T.C.
YEDITEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES
DEPARTMENT OF PHARMACOGNOSY

HIGH PERFORMANCE THIN-LAYER CHROMATOGRAPHIC METHOD APPLICATIONS FOR QUALITATIVE AND QUANTITATIVE ANALYSIS OF FLOWERING AERIAL PARTS OF *HYPERICUM PERFORATUM* L. AND THEIR IMPLEMENTATIONS FOR SOME *HYPERICUM* SPECIES AND PRODUCTS

DOCTOR OF PHILOSOPHY THESIS

ESRA SAÇICI, M.Sc. Pharm.

ISTANBUL-2017

T.C.
YEDITEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES
DEPARTMENT OF PHARMACOGNOSY

HIGH PERFORMANCE THIN-LAYER CHROMATOGRAPHIC METHOD APPLICATIONS FOR QUALITATIVE AND QUANTITATIVE ANALYSIS OF FLOWERING AERIAL PARTS OF *HYPERICUM PERFORATUM* L. AND THEIR IMPLEMENTATIONS FOR SOME *HYPERICUM* SPECIES AND PRODUCTS

DOCTOR OF PHILOSOPHY THESIS

ESRA SAÇICI, M.Sc. Pharm.

SUPERVISOR
Prof. Dr. Erdem YEŞİLADA

ISTANBUL-2017

THESIS APPROVAL

Institute	:	Yeditepe University Institute of Health Sciences
Programme	:	Pharmacognosy
Title of the Thesis	:	High Performance Thin-Layer Chromatographic Method Applications for Qualitative and Quantitative Analysis of Flowering Aerial Parts of *Hypericum perforatum* L. and their Implementations for Some *Hypericum* species and Products
Owner of the Thesis	:	Esra SAÇICI
Examination Date	:	11.12.2017

This study have approved as a Doctorate Thesis in regard to content and quality by the Jury.

		SIGNATURE
Chair of the Jury: (Supervisor)	Prof. Dr. Erdem YEŞİLADA Yeditepe University	
Member:	Prof. Dr. Hasan KIRMIZIBEKMEZ Yeditepe University	
Member:	Doç. Dr. Hayati ÇELİK Yeditepe University	
Member:	Yrd. Doç. Dr. İrem ATAY BALKAN Sağlık Bilimleri University	
Member:	Yrd. Doç. Dr. Hilal BARDAKÇI ALTAN Acıbadem University	

APPROVAL

This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated 15.12.2017 and numbered 2017/25-04

Prof. Dr. Bayram YILMAZ
Director of Institute of Health Sciences

ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to my advisor, Prof. Dr. Erdem Yeşilada for his wisdom, continuous support, patience, and motivation. His guidance helped me in all the time of research and writing of this thesis. He is really helpful for alternative ideas for each my endless question. He never hesitated to share his knowledge with me during my studies.

I am pleased to thank Prof. Dr. Hasan Kırmızıbekmez, and all academicians as well as members who supported me in Department of Pharmacognosy and Faculty of Pharmacy in Yeditepe University (Turkey) for their knowledge, help and kindness to me throughout my studies.

Last but not the least, I am grateful to my parents, who provide a care for environment for me, so that I can concentrate on my study and they are willing to support any decision I make. I am so lucky to have them as my parents. Above all, I would like to thank my mother, father and sister for their personal supports and great patience at all times.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

2D	2-dimensional
α	Selectivity factor
ABTS	2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)
ADC	Automatic developing chamber
ANOVA	Analysis of variance
CAM	Chicken embryo chorioallantoic membrane
COX	Cyclooxygenase
DAD	Diode array detector
DCF	2′,7′-dichlorofluorescein
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DS	von Zerssen depression scale
EPM	Elevated plus maze test
ESI	Electrospray ionization
EtOH	Ethanol
F	Fluorescence indicator
FST	Porsolt's forced swim test
FTIR	Fourier transform infrared (spectroscopy)
H	Height
H_2SO_4	Sulfuric acid
HAMD	Hamilton rating scale for depression
HeLa	Human cervical carcinoma cells
HETP	Height equivalent of a theoretical plate
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HPTLC	High performance thin-layer chromatography
HyF	Hyperforin
HyP	Hypericin
HyS	Hyperoside
ICH	International Conference on Harmonization
ISTE	Herbarium of Faculty of Pharmacy in İstanbul University
İTK	İnce tabaka kromatografisi

JNK	c-Jun-NH_2 terminal kinase
K	Partition coefficient (partition ratio)
k	Capacity factor
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantitation
LSD	Least significant difference
MAP	Mitogen-activated protein
MeOH	Methanol
MP	Mobile phase
MS	Mass spectrometry
N	Theoretical plate number
ndt	Not detected
NF	Nuclear factor
NMR	Nuclear magnetic resonance
NP	Normal phase
OFT	Open-field exploration test
P′	Polarity index
Ph. Eur.	European Pharmacopoeia
PMA	Phorbol-12-myristate 13-acetate
PMS	Premenstrual syndrome
PTP1B	Protein tyrosine phosphatase 1B
REM	Rapid eye movement
R_f	Retardation factor
RP	Reversed phase
RPF	Radical protection factor
R_s	Resolution factor
RSD	Relative standard deviation
SD	Standard deviation
Si	Silica gel
SJW	St. John’s wort
SMD	Standardized mean difference
SSR	Solar stimulated radiation
SSRI	Selective serotonin reuptake inhibitor

TEAA	Triethylammonium acetate
TLC	Thin layer chromatography
TNF	Tumor necrosis factor
t_R	Retention time
UPLC	Ultra performance liquid chromatography
UV	Ultraviolet
VIS/NIR	Visible/near infra-red
YPİTK	Yüksek performanslı ince tabaka kromatografisi
YPSK	Yüksek performanslı sıvı kromatografisi

ABSTRACT

Saçıcı, E. (2017). High Performance Thin-Layer Chromatographic Method Applications for Qualitative and Quantitative Analysis of Flowering Aerial Parts of *Hypericum perforatum* L. and their Implementations for Some *Hypericum* species and Products. Yeditepe University, Institute of Health Sciences, Department of Pharmacognosy, PhD Thesis, İstanbul.

Hypericum species (Hypericaceae) have been traditionally used as remedial drugs for ages across the world and in Turkey. Among *Hypericum* L. species *H. perforatum* L. is recognized as the medicinally most important member. A vast number of experimental studies have been published so far and pharmacological activities have been mostly attributed to its hyperforin (phloroglucinol derivative), hypericin (naphthodianthrone derivative) and/or hyperoside (flavonoid derivative) contents. In the European Pharmacopoeia, these three marker components in "Hyperici herbae extractum siccum quantificatum" were described for qualitative (by TLC method) and quantitative analysis (by HPLC method). On the other hand, HPTLC, as a new analytical tool, has become increasingly used as a suitable technique in analysis of herbal drugs. Within the context of this thesis, three HPTLC methods were developed and validated on HPTLC plates coated with silica gel 60 F_{254} for qualitative and quantitative analysis of hyperforin, hypericin and hyperoside in the flowering aerial parts of *H. perforatum* as an alternative to TLC and HPLC methods stated in the European Pharmacopoeia. Three different mobile phases were used in HPTLC analysis; for hyperforin analysis *n*-hexane-ethyl acetate (8:2); for hypericin analysis toluene-chloroform-ethyl acetate-formic acid (8:5:3.5:0.6); and for hyperoside analysis ethyl acetate-formic acid-acetic acid-water (15:2:2:1). Then the developed and validated HPTLC methods were applied on different *Hypericum* species and practiced to test their applicability for the quality analysis of several *Hypericum* samples and oily macerates purchased from herbal shops based on the concentrations of these three marker components.

Key words: *Hypericum perforatum* L., hyperforin, hypericin, hyperoside, High Performance Thin-Layer Chromatography (HPTLC)

ÖZET

Saçıcı, E. (2017). *Hypericum perforatum* L. Toprak Üstü Kısımlarının Kalitatif ve Kantitatif Analizi için Yüksek Performanslı İnce-Tabaka Kromatografik Yöntem Uygulamaları ve Bazı *Hypericum* Türleri ve Ürünlerinde Uygulanması. Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Farmakognozi ABD, Doktora Tezi, İstanbul.

Hypericum türleri (Hypericaceae), tüm dünyada ve Türkiye'de tedavi edici amaçla geleneksel olarak kullanılmaktadır. *H. perforatum* L., *Hypericum* L. cinsi içinde yer alan önemli tıbbi bitkilerden biridir. *H. perforatum* L. içerdiği hiperforin (floroglusinol bileşiği), hiperisin (naftodiantron bileşiği) ve/veya hiperozit (flavonoit bileşiği) ile çeşitli farmakolojik aktiviteler göstermektedir. Avrupa Farmakopesi'nde "Hyperici herbae extractum siccum quantificatum"da üç işaretleyici bileşenin kalitatif analizi İTK yöntemi ile ve kantitatif analizi YPSK yöntemi ile verilmiştir. Ancak bitkilerin analizinde HPTLC uygun bir teknik olarak giderek daha fazla kullanılmaktadır. Bu tez kapsamında, Avrupa Farmakopesi'nde belirtilen İTK ve YPSK yöntemlerine alternatif olarak, *H. perforatum* toprak üstü kısımlarında hiperforin, hiperisin ve hiperozidin kalitatif ve kantitatif analizi için silika jel 60 F254 YPİTK plakaları üzerinde üç YPİTK metodu geliştirilmiş ve valide edilmiştir. YPİTK analizinde üç farklı mobil faz kullanılmıştır; hiperforin analizi için *n*-hekzan-etil asetat (8:2); hiperisin analizi için toluen-kloroform-etil asetat-formik asit (8:5:3.5:0.6); ve hiperozit analizi için etil asetat-formik asit-asetik asit-su (15:2:2:1). Geliştirilen yeni YPİTK yöntemleri kullanılarak farklı *Hypericum* türü örneklerinde ve geleneksel yöntemle hazırlanmış yağlı özütünde üç bileşenin kalitatif ve kantitatif analizi yapılmıştır. Aynı yöntemler, piyasada satılan *Hypericum* materyali örnekleri ve yağ özütlerinin kalite değerlendirmesi amacıyla da uygulanmıştır.

Anahtar kelimeler: *Hypericum perforatum* L., hiperforin, hiperisin, hiperozit, Yüksek Performanslı İnce-Tabaka Kromatografisi (YPİTK)

***H. perforatum* L.**

***H. bithynicum* Boiss.**

***H. calycinum* L.**

***H. cerastoides* Spach**

***H. perfoliatum* L.**

***H. triquetrifolium* L.**

***H. kotschyanum* Boiss.**

***H. scabrum* L.**

***H. thymopsis* Boiss.**

1. INTRODUCTION and AIM

INTRODUCTION and AIM

Hypericaceae (Guttiferae, Clusiaceae) family has 8 genus and approximately 900 species throughout the world (1). *Hypericum* L. is the most important genus of this family. It includes more than 450 species distributed all over the world (2). Although the number of *Hypericum* species in Turkey was recorded 69 in the "*Flora of Turkey and The East Aegean Islands*" (3), recent studies have indicated that the number of distinct types in Turkey is over 90 and the half of them are endemic (4, 5). Amongst these species, *Hypericum perforatum* L. has got a wide distribution in Turkish flora and also finds frequent application in Turkish folk medicine (3).

Hypericum perforatum L. (St. John's wort) is indigenous to Asia, Europe, America, North Africa, Australia, and New Zealand (6-8). The English name "St. John's wort" comes from harvesting its flowers bloom in late June (24 June) around St. John's day (9, 10). In Greek, "*Hypericum*" derives from "*hyper* (over, above)" and "*eikon* (icon, image, picture)" as well as "*perforatum*" means "*the existence of oil glands on its leaves that resemble perforations*" (9, 11, 12). The dried flowering tops, including leaves, unopened buds and flowers are recognized as Hyperici herba. Hyperici herba contains numerous compounds, such as naphtodianthrones (hypericin), phloroglucinols (hyperforin), flavonoids (hyperoside, quercetin, quercitrin, rutin), biflavonoids, phenolic acids, proanthocyanidins, xanthones, essential oils and other components (13).

Hyperici herba, the flowering aerial parts of the plant, is a popular herbal remedy worldwide. However, amongst the various therapeutic benefits reported so far, it is a well-known remedy with its effects on anxiety and depression (9, 12). Hyperforin and hypericin were determined to be the major active constituents for antidepressant and anxiolytic activities (14). Besides, antimicrobial (11), antibacterial (15), antioxidant (16, 17), antinociceptive (18), anti-menopausal (19), topical anti-inflammatory (20) and wound-healing (21, 22) activities of *H. perforatum* have been evidenced through scientific investigations.

Hyperforin (a phloroglucinol derivative) is one of the leading constituents responsible for antidepressant effect (23). In addition, hyperforin has shown to possess

other pharmacological activities, including neurological, antibacterial, anti-inflammatory, and antitumoral (24-27). Hypericin (a naphthodianthrone derivative) is another major active constituent of *H. perforatum* that is responsible for its antidepressant effects (23). Hypericin is a photodynamic agent and has also been shown to exert antiviral, antimicrobial, anti-inflammatory and anticancer properties (28-32). The other main component of *H. perforatum*, hyperoside is a flavonoid possessing potent antioxidant activity (33). It is also reported to contribute to the antidepressant activity which was evidenced by providing a significant reduction of the motionless time in mice of forced swimming and tail suspension tests (34).

Hypericum perforatum L. is the subject of this study because of its historical importance, widespread application in traditional and modern medicine. In European Pharmacopoeia (Ph. Eur.), thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) methods are described for the quality assessment of "Hyperici herbae extractum siccum quantificatum". TLC has been utilized for the qualitative analysis that allows the characterization of main compounds, while HPLC has been employed for the quantitative analysis.

The aim of this study was to develop useful and validated methods for quality assessment of the flowering aerial parts of *H. perforatum* based on its three marker components, namely hyperforin, hypericin and hyperoside, by high performance thin-layer chromatography (HPTLC). Three separate validated HPTLC systems for qualitative and quantitative analysis of hyperforin, hypericin and hyperoside were developed as an alternative to TLC and HPLC techniques designated in Ph. Eur. These new HPTLC systems were practiced for the qualification and quantification of these main active components in various *Hypericum* species provided from different sources and also in oily macerate prepared by traditional technique. Furthermore, the methods were also employed on several *H. perforatum* samples purchased from herbal markets as well as commercially available St. John's wort (SJW) oily macerates.

2. THEORETICAL SECTION

2.1. Botanical Chapter

2.1.1. Hypericaceae (Guttiferae)

Hypericaceae comprises herbs or shrubs that generally have translucent glands comprising essential oils and occasionally black or red glands including hypericin. It has opposite, simple or rarely whorled leaves, 5 sepals that are imbricate in bud and 5 free petals that are contorted in bud. Its stamens are in fascicles or apparently indefinite. Its superior ovary has axile or parietal placentation. The seeds do not include endosperm (3).

2.1.2. The *Hypericum* L. Genus

Flowers are bisexual. It has 5 sepals and 5 petals. The colors of its petals are mostly yellow, frequently tinged red or with red veins and they rarely include nectary appendages. Its stamens have 5 fascicles that are antipetalous, free or 4 of them combined in pairs to constitute 2 antisepalous compound fascicles. The ovary may be 3-5-locular or partially or entirely 1-locular and it has 2-many ovules on each placent. 3-5 styles are free and slender. The capsular fruit dehisces septicidally, and it has generally with resin-including vittae or vesicles in the wall, or it is rarely fleshy and indehiscent (3).

There are 69 species of *Hypericum* in Turkey and they are classified into 7 groups in "Flora of Turkey and the East Aegean Islands". The key to identify these groups and species is presented as follows (3):

1. Broad-leaved shrub, or herb with leaves perfoliate, 7-12 cm; black glands absent or rarely present on sepal and petal margins ..... Group **A**

1. Low ± sclerophyllous shrub, erect subshrub or herb; leaves rarely perfoliate but then only up to 4.5 cm

2. Low ± sclerophyllous shrub up to 30 cm, rounded or spreading; black glands often completely absent..... Group **B**

2. Perennial herb (sometimes shrubby at base) or erect and suffructicose; black glands usually present

3. Leaves auriculate and gland-fringed or sepals entire and broadly imbricate .....Group **F**

3. Leaves not auriculate, entire or rarely the uppermost glandular-ciliate; sepals not entire or, if so, then not or scarcely imbricate

4. Black glands absent from anthers and leaves (or at apex only); intra-marginal leaf glands absent or pale to amber; seeds papillose-tuberculate to almost smooth

5. Petals unguiculate, usually without red tinges or veins, with superficial glands (where present) usually round or oval; stems ± erect, herbaceous, not rooting; leaves often narrow, not markedly discolorous or coriaceous ..... Group **C**

5. Petals not unguiculate, usually red-tinged or red-veined, with superficial glands (where present) mostly elongate or linear; stems often shrubby or wiry, erect to prostrate, usually rooting; leaves rarely narrow, often markedly discolorous and coriaceous ..... Group **D**

4. Black glands present on anthers and intramarginally on leaves; seeds reticulate or foveolate to ribbed or rugulose

6. Stems eglandular, usually terete ..... Group **E**

6. Stems with black or reddish or amber glands (sometimes confined to raised lines or near base only), rarely terete

7. Sepals fimbriate or long denticulate (teeth c. 0.5-3 mm); capsule longitudinally vittate ..... Group **E**

7. Sepals entire or shortly ciliate or denticulate (teeth less than 0.5 mm); capsule variously glandular ..... Group **G**

Group **A**

1. Leaves free; shrubs; black or reddish glands absent

2. Petals and stamen fascicles deciduous after flowering; sepals entire

3. Styles 5; anthers red; flowers usually solitary (Sect. *Eremanthe*)

1. *calycinum*

3. Styles 3; anthers yellow; flowers in terminal cymes (Sect. *Androsaemum*)

4. Styles longer than the ovary; sepals deciduous; fruit capsular

2. *hircinum*

4. Styles shorter than the ovary; sepals persistent; fruit baccate

3. *androsaemum*

2. Petals and stamen fascicles persistent after flowering; sepals sometimes with yellow marginal glands (Sect. *Inodorum*)

4. *xylosteifolium*

1. Leaves perfoliate; perennial herb; sepals and petals often with minute black or reddish marginal glands (Sect. *Bupleuroides*)

5. *bupleuroides*

Group **B**

1. Petals persistent after flowering, without nectariferous appendages; stamens free to near base; flowers homostyled; fasciclodes absent

2. Leaves obtuse to rounded or apiculate, without axillary, condensed, gemma-like shoots

3. Black glands completely absent or present around petal and sepal margins only; leaves densely reticulate-veined (Sect. *Arthrophyllum*)

4. Inflorescence corymbose, 9-many-flowered; leaves sessile, 15-45 mm

5. Sepals and bracts entire; leaf base truncate to cordate-amplexicaul

6. *cardiophyllum*

5. Sepals and bracts with small black marginal glands; leaf base cuneate

7. *rupestre*

4. Inflorescence 1(-2-3)-flowered; leaves subsessile, 6-16 mm

8. *vacciniifolium*

3. Black glands present on anthers and sometimes on sepals, petals and leaves; leaves not or obscurely reticulate-veined (Sect. *Triadenioides*)

6. Leaves in whorls of 3, without black glands; dense ± rounded shrub

9. *ternatum*

6. Leaves paired, often with black glands; straggling shrublet

10. *pallens*

2. Leaves acute, narrow, the lower with condensed, gemma-like shoots (Sect. *Heterophyllum*)

11. *heterophyllum*

1. Petals deciduous after flowering, with nectariferous appendages; stamens united above the middle; flowers heterostyled; fasciclodes present (Sect. *Triadenia*)

12. *russeggeri*

Group **C** (Sect. *Drosanthe*)

1. Inflorescence broadly pyramidal to subspicate
    2. Leaves on main stem perfoliate or broadly cordate-amplexicaul, triangular-ovate to semi-circular

13. *spectabile*

    2. Leaves on main stem free, cuneate at the base, relatively narrower
        3. Leaves on main stem ovate to lanceolate or oblong; petals often red-tinged or red-veined
            4. Sepals rounded, suborbicular to oblong or spathulate; leaves rounded, glabrous or undulate-papillose above

14. *amblysepalum*

            4. Sepals acute, lanceolate to oblong or spathulate; leaves acute or apiculate to obtuse, glabrous or slightly scabrid above or on the margin

15. *lysimachioides*

        3. Leaves on main stem linear or narrowly lanceolate; petals not red-tinged or red-veined (except sometimes in **14** & **16**)
            5. Leaves retuse with apical black gland; stem, leaves and petals usually black-gland-dotted; sepals usually with 2 black streaks

18. *retusum*

            5. Leaves rounded to mucronate; stem, leaves and petals without black dots; sepals without black streaks
                6. Sepals obovate-spathulate to oblong, rounded; petals 10-16 mm; capsule 7-10 mm

14. *amblysepalum*

                6. Sepals ovate to lanceolate or, if oblong and rounded, then petals and capsule smaller
                    7. Capsule ovoid, acuminate or markedly rostrate; buds acute or obtuse, usually ellipsoid

8. Sepals ± unequal, obtuse or apiculate to rounded with margin usually at least partly eglandular; stem glands absent or small, not prominent

16. *hyssopifolium*

8. Sepals equal, acute or subacute, with margin regularly glandular; stem glands usually numerous and ± prominent

17. *lydium*

7. Capsule globose, not or only shortly rostrate; buds rounded, usually globose

9. Sepals ± unequal, often imbricate and with the margin irregularly glandular

16. *hyssopifolium*

9. Sepals equal or subequal, not imbricate, with margin ± regularly glandular

10. Leaves rounded without apical gland; sepals rarely ribbed

11. Capsule shortly rostrate: leaves usually undulate-papillose; inflorescence broadly pyramidal to cylindric

19. *pseudolaeve*

11. Capsule globose; leaves usually glabrous; inflorescence cylindric or narrowly pyramidal to subspicate

20. *helianthemoides*

10. Leaves apiculate or mucronate or with apical black gland; sepals ribbed

12. Sepals acute, 3-ribbed at base; leaves with eglandular mucro; inflorescence shortly cylindric to pyramidal

21. *thymbrifolium*

12. Sepals mucronate to rounded, 5-ribbed at the base; leaves usually with glandular apex; inflorescence ± subspicate

13. Leaves and usually sepals with glandular or eglandular mucro; stem and leaves glabrous to puberulous

22. *uniglandulosum*

13. Leaves and sometimes sepals with glandular apiculus or apical gland; stems and leaves puberulous

23. *salsolifolium*

1. Inflorescence subcorymbose to corymbose

14. Inflorescence subcorymbose; stem glabrous; petals sometimes orange-red to crimson; leaves rounded

24. *capitatum*

14. Inflorescence corymbose; stem usually pubescent or glandular-scabrid; petals yellow; leaves rounded to mucronate

15. Stem puberulous to pubescent; sepals free or united only at the base

25. *scabroides*

15. Stem glandular-scabrid or rarely glabrous but then leaves apiculate or mucronate; sepals united for 1/3-2/3 of their length

16. Stem glands (when present) unbranched; inflorescence c. 15-many-flowered; stems 10-60 cm

26. *scabrum*

16. Stem glands branched; inflorescence 1-c. 22-flowered; stems 3-11 cm

27. *thymopsis*

Group **D**

1. Leaves in whorls of 3; petals and stamens deciduous after flowering; capsule with vittae and vesicles (Sect. *Coridium*)

44. *empetrifolium*

1. Leaves in pairs; petals and stamens persistent; capsule with longitudinal vittae (Sect. *Taeniocarpium*)

2. Leaves and usually stems pruinose to pubescent or hirsute

3. Leaves 30-55 mm, oblong to elliptic or lanceolate; sepals narrowly oblong

28. *hirsutum*

3. Leaves 5-14 mm, varying in shape; sepals lanceolate to oblong or ovate

4. Stems glabrous to puberulous; leaves on main stem usually pruinose to papillose, oblong or linear-oblong to elliptic or lanceolate

5. Sepals broadly oblong to broadly elliptic, margin minutely or entire glandular-denticulate

29. *pruinatum*

5. Sepals lanceolate to ovate or oblong, margin glandular-ciliate to fimbriate

32. *confertum*

4. Stems pubescent; leaves on main stem pubescent, linear to linear-oblong or linear-lanceolate

6. Sepals 3-5-ribbed, broadly oblong to ovate

30. *kotschyanum*

6. Sepals 7-9-ribbed, oblong to spathulate

31. *neurocalycinum*

2. Leaves and stems glabrous

7. Leaves thin with densely reticulate venation; sepals narrowly oblong, usually with margin glandular-ciliate

33. *venustum*

7. Leaves parchment-like to coriaceous, with or without obscure reticulate venation; sepals usually relatively broader, margin entire to glandular-denticulate

8. Sepals entire or glandular near the apex only; leaves without a submarginal row of pellucid glands below; inflorescence spicate to narrowly cylindric

34. *linarioides*

8. Sepals with ± regularly glandular margin; leaves with submarginal row of pellucid glands below; inflorescence cylindric to corymbose or 1-flowered

9. Bracts entire or with 1-2 marginal glands; leaves subsessile

35. *armenum*

9. Bracts with margin gland-fringed; leaves petiolate

10. Leaves markedly discolorous, cuneate at the base; stems usually erect

11. Stems 15-22 cm, not or little branched, woody at the base

36. *fissurale*

11. Stems 20-70 cm, usually with ascending branches, ± suffruticose and wand-like

37. *thymifolium*

10. Leaves ± concolorous, usually rounded or cordate at the base; stems erect to prostrate

12. Leaves with margins undulate

38. *crenulatum*

12. Leaves with margins plane or indurate

13. Petals 10-16 mm; leaves green with plane margin and ± conspicuous lateral venation

39. *nummularioides*

13. Petals 4-9 mm; leaves ± glaucous, usually with indurate margin, without visible lateral venation

14. Upper leaves with 1-2(-3) apical black glands

15. Leaves broadly oblong-ovate to circular, (4-)5-11 mm, with one prominent apical gland

40. *monadenum*

15. Leaves oblong to ovate, 3-7(-9) mm, with (1-)2-5 apical glands, not prominent

41. *pumilio*

14. Upper leaves without apical black glands

16. Leaves oblong-elliptic to circular; sepals obtuse to rounded; petals with 1-4 apical black glands

42. *marginatum*

16. Leaves narrowly oblong; sepals acute to obtuse; petals without apical glands

43. *saxifragum*

Group **E**

1. Sepals fimbriate or long-denticulate; stem markedly 2-lined; capsule longitudinally vittate

2. Styles 3; sepals lanceolate to ovate-lanceolate, scarcely imbricate (Sect. *Oligostema*)

55. *aucheri*

2. Styles 5; sepals oblong to ovate, broadly imbricate (Sect. *Thasia*)

56. *thasium*

1. Sepals entire to ciliate or, if fimbriate, then capsule with vesicles; stems terete or slightly 2-4-lined

3. Capsule valves longitudinally vittate; indumentum often present on leaves and sometimes stems (Sect. *Adenosepalum*)

4. Bracts with densely glandular auricles; leaves usually scabrous beneath, indumentum otherwise absent

45. *montanum*

4. Bracts not or scarcely auriculate but sometimes with long-stalked glands near the base; indumentum various or absent

5. Petals and sepals without superficial black glands

46. *lanuginosum*

5. Petals and sepals with superficial black glands

6. Sepal margin glandular-denticulate; leaves and stems pruinose to pubescent or rarely completely glabrous; leaves ovate to oblong-elliptic or lanceolate

7. Stems erect or rarely decumbent, not rooting, ± stout; petals 8-12 mm; leaves 15-50 mm; inflorescence many-flowered

47. *atomarium*

7. Stems decumbent or diffuse, often rooting, slender; petals 5-7(-8) mm; leaves 2-15 mm; inflorescence 1-7(-21)-flowered

48. *cuisinii*

6. Sepal margin glandular-ciliate to subentire; leaves and stems glabrous; leaves broadly ovate or ovate-oblong to suborbicular

8. Stems erect, not cushion-forming; sepals acute to obtuse, with only black glands; petals with only black glands

49. *huber-morathii*

8. Stems densely caespitose or straggling; sepals obtuse to rounded, with amber and sometimes black glands; petals with amber superficial glands

9. Sepals oblong, margin irregularly glandular to subentire, with numerous superficial black glands; leaves somewhat fleshy; stems 1-2 cm

50. *minutum*

9. Sepals broadly oblong to obovate-spathulate, margin regularly glandular-ciliate, without or almost without superficial black glands; leaves thin; stems 3-10 cm

51. *formosissimum*

3. Capsule with dorsal vittae and lateral vesicles, or vesicles only; indumentum completely absent (Sect. *Drosocarpium*)

10. Leaves (at least the upper) densely pellucid-punctate, with lax obscure reticulate venation; petals with black glands usually confined to near the apex

11. Capsule ovoid, with dorsal vittae and lateral vesicles; sepals ± rounded, erect in fruit, with numerous superficial black glands mostly in 2 rows

52. *perfoliatum*

11. Capsule narrowly ovoid-pyramidal with vesicles only; sepals ± acute, reflexed in fruit with few or no superficial black glands

53. *montbretii*

10. Leaves (rarely except the upper) not pellucid-punctate, with dense conspicuous reticulate venation; petals with black glands over the whole surface

54. *bithynicum*

Group **F**

1. Leaves gland-fringed, auriculate (Sect. *Crossophyllum*)

2. Plant with black glands; leaf and sepal margin glandular-fimbriate

57. *adenotrichum*

2. Plant without black glands; leaf and sepal margin glandular-denticulate

58. *orientale*

1. Leaves entire, not auriculate

3. Stem, leaves and sepals glabrous; capsule erect (Sect. *Olympia*)

4. Sepals ± long-acuminate; sepals and petals usually without black dots; leaves without or with irregular intramarginal black glands

59. *olympicum*

4. Sepals apiculate or acute to rounded; sepals and petals usually black-dotted; leaves with regular intramarginal black glands

60. *polyphyllum*

3. Stem, leaves and sepals pubescent; capsule pendulous (Sect. *Campylopus*)

61. *cerastoides*

Group **G**

1. Stems without axillary shoots; leaves usually glaucous or papillose to pubescent; sepal margin glandular (Sect. *Origanifolia*)

2. Stems pubescent or puberulous

3. Stems and leaves whitish-pubescent; sepals usually puberulous

62. *origanifolium*

3. Stems puberulous; leaves papillose; sepals glabrous

63. *aviculariifolium*

2. Stems glabrous

4. Leaves cuneate to rounded at the base, not frequently imbricate, 5-35 mm with intramarginal black glands

63. *avicularifolium*

4. Leaves (at least the lower) cordate-amplexicaul ± densely imbricate, 1-7 mm, often without intramarginal black glands

5. Pedicels absent or up to 0.5 mm; inflorescence lax; leaves markedly heteromorphic, with pellucid glands

64. *salsugineum*

5. Pedicels c. 1 mm; inflorescence capitate; leaves slightly heteromorphic, without pellucid glands

65. *imbricatum*

1. Stems with ± well-developed axillary shoots; leaves glabrous, rarely glaucous; sepal margin (except in **69**) entire (Sect. *Hypericum*)

6. Stems 4-lined or narrowly 4-winged

66. *tetrapterum*

6. Stems 2-lined

7. Sepals entire or eglandular-denticulate

8. Leaves sessile or subsessile, with margin plane; inflorescence branches ascending; capsule valves with dorsal vittae and lateral vesicles

67. *perforatum*

8. Leaves amplexicaul, margins undulate; inflorescence branches widely spreading; capsule valves usually longitudinally vittate

68. *triquetrifolium*

7. Sepals with marginal black glands

69. *elegans*

2.1.3. *Hypericum calycinum* L.

Stems are erect from creeping rhizomes, usually unbranched, and grow up to 20-60 cm. Leaves are oblong to elliptic or ovate, evergreen, and grow up to 4.5-9.5 cm. Sepals are markedly unequal, elliptic to suborbicular, entire, persistent, and grow up to 1-2 cm. Petals are markedly asymmetric, and grow up to 2.5-4(-4.5) cm. Stamens have reddish anthers. Capsule is 20 mm, ovoid, on a reflexed peduncle (3).

Flowering season: May – August (-October)
Habitat: Shady woods and banks
Altitude: 30 – 1200 m.
Distribution in Turkey: Regions of Marmara and Black Sea
Worldwide distribution: Northern America, Bulgaria

2.1.4. *Hypericum scabrum* L.

Stems are decumbent or erect at the base, glabrous, scabrid with unbranched red-gland-tipped emergences or rarely smooth, eglandular, and grow up to (10-)15-45(-60) cm. Leaves are on main stems, oblong or oblong-elliptic to lanceolate or linear, sometimes revolute, rounded to mucronate, glabrous or slightly undulate-papillose, rarely glaucous and grow up to 7-20(-25) mm. Inflorescence is corymbose, 15-many-flowered. Sepals are oblong, subacute to rounded, 1/3-2/3 united, irregularly glandular-denticulate to –ciliate or eglandular-ciliate to subentire. The length of petals are 5-7(-8) mm. Capsule is 5-8 mm, ovoid or ovoid-trigonous, not or scarcely rostrate (3).

Flowering season: May – August
Habitat: Dry rocky slopes, open woodland or steppe
Altitude: 750 – 3200 m.
Distribution in Turkey: Regions of Mediterranean, Aegean, Black Sea, Central, Eastern and Southeast Anatolia
Worldwide distribution: Asia

2.1.5. *Hypericum thymopsis* Boiss.

Stems are erect, sometimes creeping, rooting at the base, glabrous, scabrid with branched glandiferous emergences towards the base, and grow up to 3-11 cm. Leaves are on main stems, linear, revolute, rounded or subapiculate, glabrous to undulate-papillose, glaucous or not, and grow up to 6-18 mm. Inflorescence is corymbose. It has 22-flowered or flowers solitary. Sepals are lanceolate to oblong-elliptic, acute to rounded, united in the lower and they include subsessile or sessile glands. Petals grow up to 5-7 mm and do not contain black glands. Capsule is 4 mm, ovoid, not or slightly rostrate (3).

Flowering season: June – July

Habitat: Calcareous hillsides and lavas

Altitude: 980 – 1500 m.

Distribution in Turkey: Regions of Mediterranean, Central Anatolia and Eastern Anatolia

2.1.6. *Hypericum kotschyanum* Boiss.

Stems are erect or ascending from a rooting and branching base, pubescent, and grow up to 10-30 cm. Leaves are on main stem, narrowly oblong to linear-lanceolate, strigose-pubescent, and grow up to 5-15 mm. Inflorescence is narrowly pyramidal or cylindric, (1-)3-c. 20-flowered. Sepals are ± broadly oblong to ovate, united at the base, rounded, 3-5-ribbed, black-glandular-denticulate or –ciliate. Petals are 7-12 mm long, and often red-veined. Capsule is 7-12 mm, and ovoid (3).

Flowering season: April – June

Habitat: Calcareous rocks

Altitude: 1800 – 2000 m.

Distribution in Turkey: Regions of Mediterranean and Central Anatolia

2.1.7. *Hypericum perfoliatum* L.

Stems are erect or decumbent, and grow up to 15-80 cm. Leaves are ovate to triangular-lanceolate or seldom linear, they have occasionally black-glandular-ciliate, generally densely pellucid-dotted, with obscure reticulate venation, and grow up to 13-60 mm. Its sepals are oblong, subacute to rounded, densely and irregularly black-glandular-denticulate or –ciliate and they consist of numerous superficial black streaks and dots mostly in 2 vertical rows. Its petals are with or without black dots or streaks towards the apex and grow up to 9-14 mm. The capsule is 5-6 mm, broadly ovoid, with dorsal vittae and lateral vesicles (3).

Flowering season: April – May

Habitat: Damp meadows and shady places among rocks

Altitude: 20 – 880 m.

Distribution in Turkey: Regions of Marmara, Aegean and Mediterranean

Worldwide distribution: Southern Europe, Cyprus, Northwest Africa, Mediterranean

2.1.8. *Hypericum bithynicum* Boiss.

Stems are erect or ascending, rooting, and grow up to 10-60 cm. Leaves are ovate or ovate-oblong to suborbicular, entire, without pellucid dots or with a few in the uppermost pairs, sometimes with superficial black dots, with conspicuous reticulate venation, and grow up to (10-)15-55 mm. Sepals are lanceolate to narrowly oblong, acute, glandular-denticulate to –fimbriate or rarely entire, with numerous superficial black dots, erect in fruit. Petals are 10-13 mm long, with numerous black dots over the whole surface. Capsule is 6-8 mm, ± narrowly ovoid, with round and elongate vesicles (3).

Flowering season: May – October

Habitat: Damp meadows, Rhododendron scrub and deciduous woodland

Altitude: 120 – 2700 m.

Distribution in Turkey: Regions of Marmara and Black Sea

Worldwide distribution: Georgia, Western Caucasus

2.1.9. *Hypericum cerastoides* Spach

Stems are decumbent or ascending, sometimes branching and rooting at the base, and grow up to 7-27 cm. Leaves are oblong to elliptic or ovate, with or without few black glands, and grow up to 8-30 mm. Sepals are ± unequal, broadly imbricate, broadly ovate or elliptic to oblong or lanceolate, rounded or rarely obtuse to acute, entire, without black glands. Petals are 9-21 mm long, with marginal and sometimes a few intramarginal black glands. Capsule is 5-12 mm, and broadly ovoid to globose (3).

Flowering season: April – September
Habitat: Siliceous stony places or woodland
Altitude: 0 – 1500 m.
Distribution in Turkey: Regions of Marmara
Worldwide distribution: Southern Bulgaria, Greece

2.1.10. *Hypericum perforatum* L.

Stems are erect, sometimes rooting, and 2-lines, with branches ± ascending, and grow up to 10-110 cm. Leaves are narrowly ovate or lanceolate to elliptic-oblong or linear, occasionally oblanceolate, sessile or subsessile, plane, and they always contain large pellucid dots, as well as grow up to 5-35 mm. The sepals are lanceolate to oblong or elliptic, acute to acuminate or shortly aristate, entire, and they have no or a few superficial black dots. Its petals are (5-)8-15 mm long, include a few marginal black dots and sometimes superficial black streaks. Capsule is (4-)5-9 mm, ovoid to pyramidal, and contains lateral vesicles and dorsal vittae (3).

Flowering season: (April-) May – August (-September)
Habitat: Dry habitats in mesophytic regions, near water
Altitude: 0 – 2500 m.
Distribution in Turkey: Regions of Marmara, Aegean, Mediterranean, Black Sea, Eastern and Central Anatolia
Worldwide distribution: Europe, Northern Africa, Caucasia, Siberia, Central Asia, Iran, Northern Iraq, Cyprus, Western Syria

2.1.11. *Hypericum triquetrifolium* L.

Stems are erect or decumbent, 2-lined, include branches widely spreading, frequently forming a pyramid, and grow up to 15-55 cm. Its leaves are triangular-lanceolate or rarely narrowly ovate to linear-oblong, amplexicaul, undulate, and occasionally involve medium to small pellucid dots, as well as grow up to 3-20 mm. The sepals are oblong to ovate-oblong, rounded or apiculate, entire or denticulate, and do not embrace black dots. Its petals have no or occasionally one superficial black dot, and grow up to 5-7 mm. The capsule is 3-5 mm, ovoid, as well as includes longitudinal vittae and occasionally a few lateral vesicles (3).

Flowering season: May – September

Habitat: Open dry sandy and stony ground and cultivated fields

Altitude: 0 – 1250 m.

Distribution in Turkey: Regions of Marmara, Aegean, Mediterranean, Black Sea, Eastern and Central Anatolia

Worldwide distribution: Southern Europe, Northwest Africa, Cyrenaica, Cyprus, Syrian Desert, Sinai, Northern Iraq, Southern Iran, Greece, Sicily, Calabria

2.1.12. Local Names of *Hypericum* Species

In Turkish folk medicine, *Hypericum* species are mostly named as “binbirdelik otu, sarı kantaron, gatran, gatran otu, katran, katran otu, kangren otu, gangren otu, gantıran, kantıran, kantoran, kantoron, kantoron otu, mayasıl otu, yara otu, kuzukıran, koyunkıran, kılıç otu, kan otu, kepir otu, püren, and sarı püren” (35, 36).

In other languages, they are locally known as “St. John’s wort, goatweed, tipton weed, klamath weed, amber, hardhay” (English) (6); “johanniskraut, sonnenwendkraut, hartheu” (German) (12, 37); “millepertuis” (French) (11); “iperico” (Italian) (37); “prikbladet perikon” (Danish) (37).

2.2. Literature Review on *Hypericum perforatum*

2.2.1. Phytochemical Studies

The major characteristic constituents of *H. perforatum* are phloroglucinols, naphthodianthrones and flavonoids. The amount of chemical components in *H. perforatum* are varied due to genetic diversity, ecological growth conditions, harvesting time of samples, exposure to light, drying methods, and storage conditions. The secondary metabolites of *H. perforatum* are classified in Figure 2.1 (13, 38).

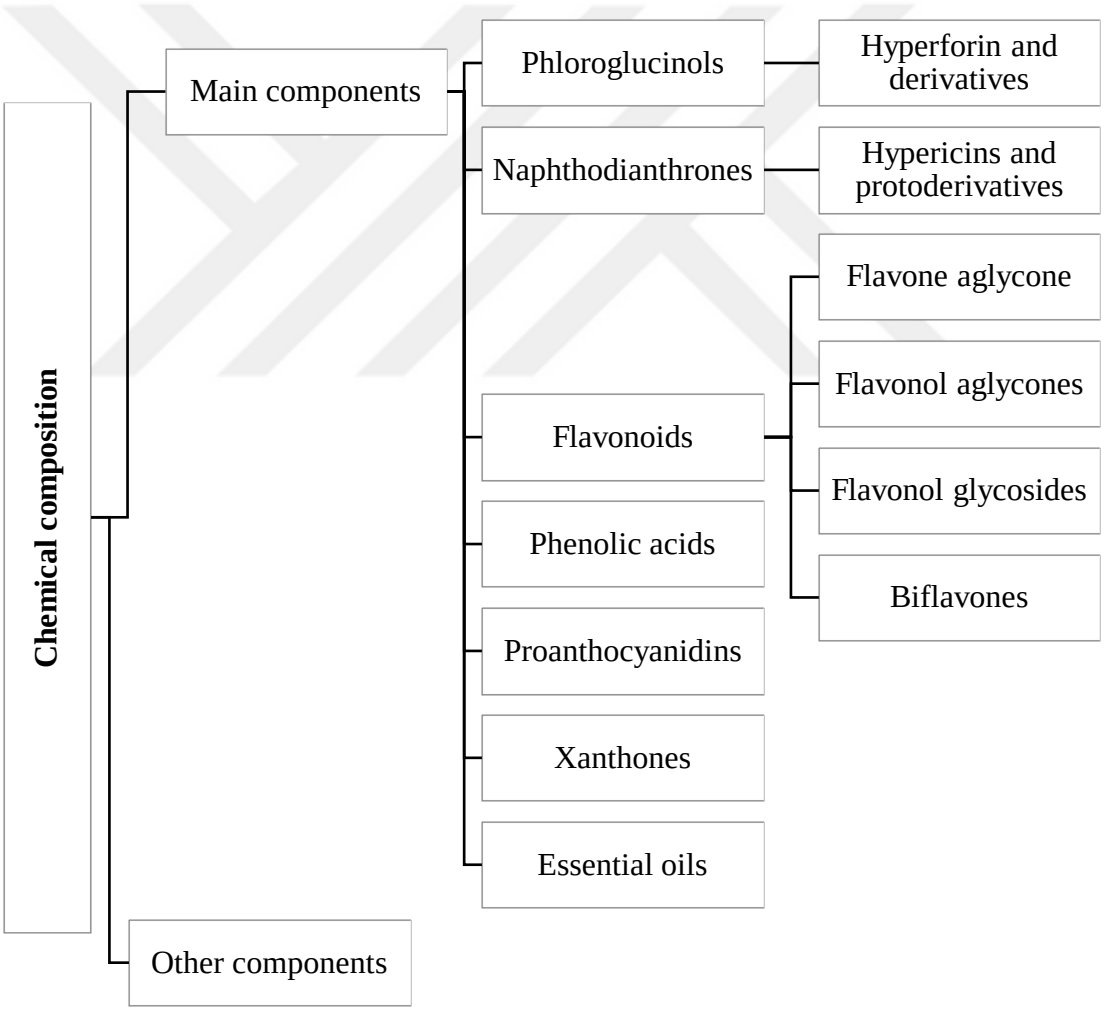


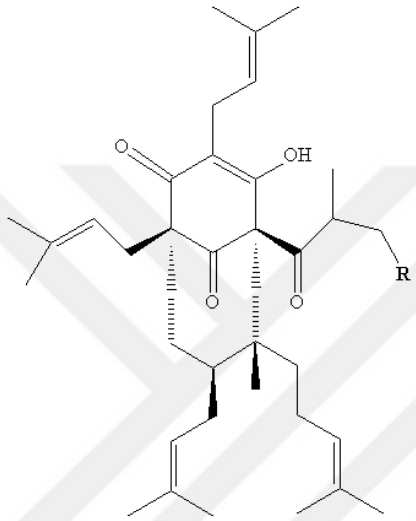
Figure 2.1. Categorization of *H. perforatum* secondary metabolites

2.2.1.1. Main components

2.2.1.1.1. Phloroglucinols

Phloroglucinols are found in the flower and fruit (13). The major phloroglucinol derivative constituents of *H. perforatum* are showed in Table 2.1.

(1)



(2)

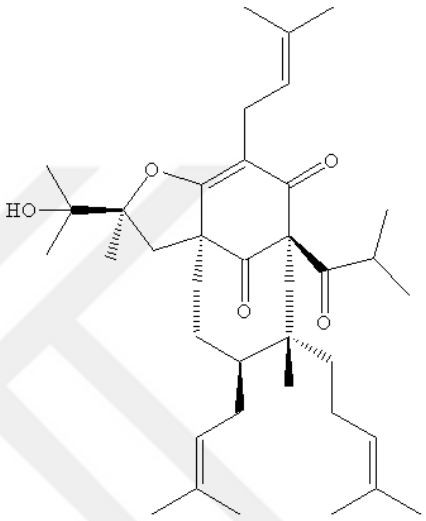


Table 2.1. Phloroglucinols isolated from *H. perforatum*

Compound	**R**	**Reference**
Hyperforin (1)	H	(13, 39-41)
Adhyperforin (1)	CH_3	(7, 13, 39, 42)
Furohyperforin (2)		(42-44)

2.2.1.1.2. Naphthodianthrones

Naphthodianthrones are existed in buds and flowers of *H. perforatum*. Hypericin and pseudohypericin, which are responsible for the color of juice contained in the black dots on the leaves and flowers, and biosynthetic precursors of these components,

protohypericin and protopseudohypericin, and the trace amount of cyclopseudohypericin are naphthodianthrones derivative compounds (13, 45). The chemical structures of naphthodianthrones in *H. perforatum* are given in Table 2.2.

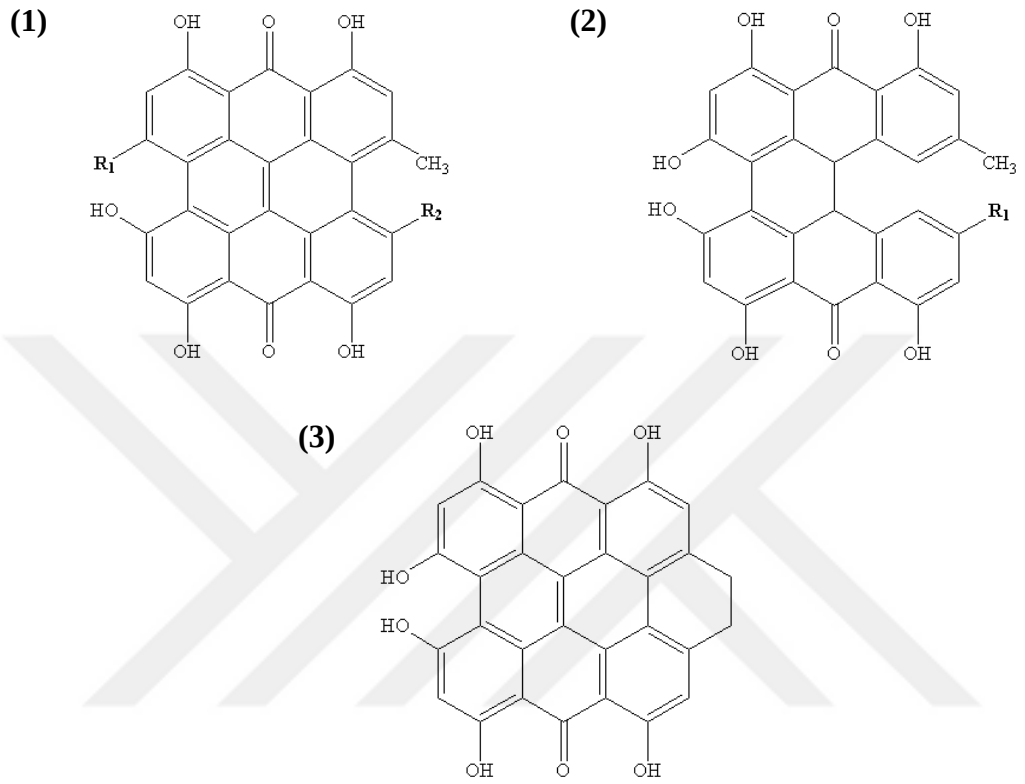


Table 2.2. Naphthodianthrones isolated from *H. perforatum*

Compound	**R_1**	**R_2**	**Reference**
Hypericin (1)	OH	CH_3	(13, 40, 41, 46)
Pseudohypericin (1)	OH	CH_2OH	(13, 40, 41, 46)
Isohypericin (1)	CH_3	OH	(47, 48)
Protohypericin (2)	CH_3		(46, 47, 49)
Protopseudohypericin (2)	CH_2OH		(46-48)
Cyclopseudohypericin (3)			(46, 47, 50)

2.2.1.1.3. Flavonoids

Flavone, flavonol aglycones and glycosides are located in buds, flowers and leaves. There are also dimeric flavones found in buds and flowers (50). The chemical structures of flavonoid constituents in *H. perforatum* are presented in Table 2.3 .

A. Flavone aglycone
B. Flavonol aglycones
C. Flavonol glycosides
D. Biflavones

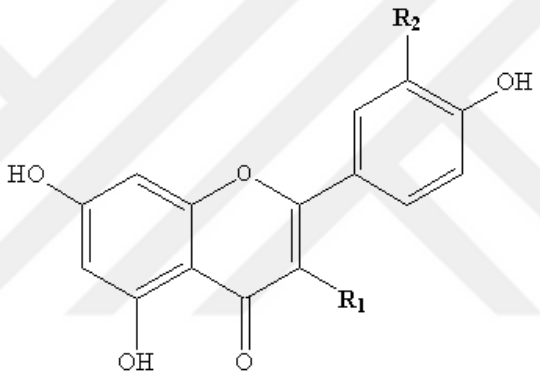
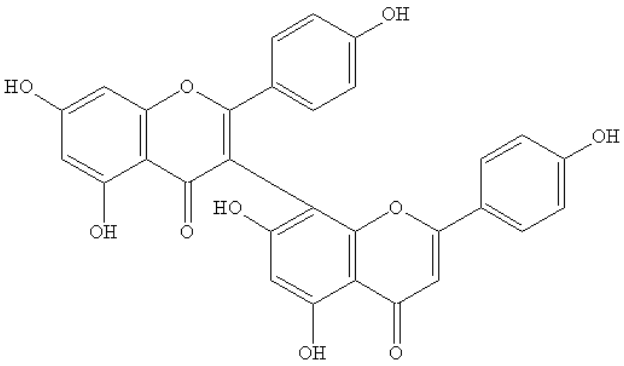


Table 2.3. Flavonoids isolated from *H. perforatum*

Compound	**Group**	**R_1**	**R_2**	**Reference**
Luteolin	A	H	OH	(11, 46, 48, 51)
Kaempferol	B	OH	H	(11, 45, 48, 52)
Quercetin	B	OH	OH	(7, 17, 40, 46)
Hyperoside (Hyperin)	C	OGal	OH	(7, 23, 40, 46)
Quercitrin	C	ORha	OH	(7, 23, 40, 46)
Isoquercitrin	C	OGlc	OH	(7, 17, 23, 46)
Quercetin 3-*O*-rutinoside (Rutin)	C	ORut	OH	(7, 23, 40, 53)

Gal: Galactose; Glc: Glucose; Rha: Rhamnose; Rut: Rutinose (Rhamnoglucoside)

(1)



(2)

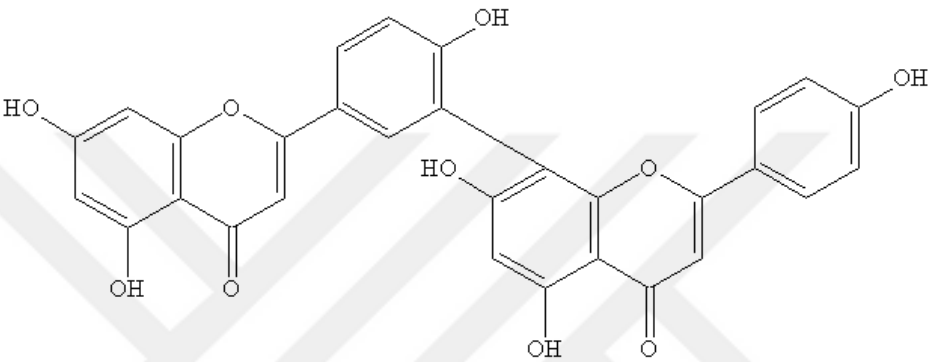
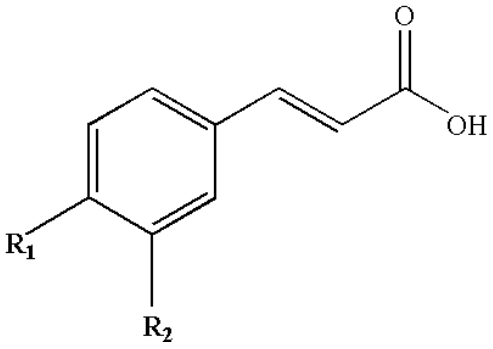


Table 2.3. Flavonoids isolated from *H. perforatum* (continued)

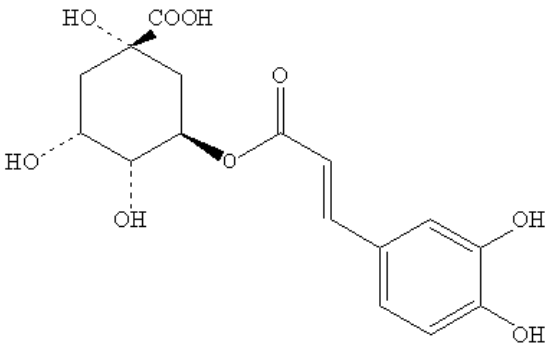
Compound	Group	Reference
I3, II8 – biapigenin (1)	D	(23, 47, 48)
Amentoflavone (I3', II8 – biapigenin) (2)	D	(21, 46, 54)

2.2.1.1.4. Phenolic acids

The chemical composition of *H. perforatum* includes common phenolic acids that are derivated from hydroxycinnamic acids, i.e., caffeic acid, ferulic acid, isoferulic acid, *p*-coumaric acid, and chlorogenic acid (45). The chemical structures of phenolic acids in *H. perforatum* are given in Table 2.4.



(1)



(2)

Table 2.4. Phenylpropanes isolated from *H. perforatum*

Compound	**R_1**	**R_2**	**Reference**
p-coumaric acid (1)	OH	H	(47, 48)
Caffeic acid (1)	OH	OH	(47, 48)
Ferulic acid (1)	OH	OCH_3	(47, 48)
Isoferulic acid (1)	OCH_3	OH	(47)
Chlorogenic acid (2)			(46, 47)

2.2.1.1.5. Proanthocyanidins

Catechin and epicatechin are the basic patterns of condensed tannins (45). The chemical structures of proanthocyanidins in *H. perforatum* are presented in Table 2.5.

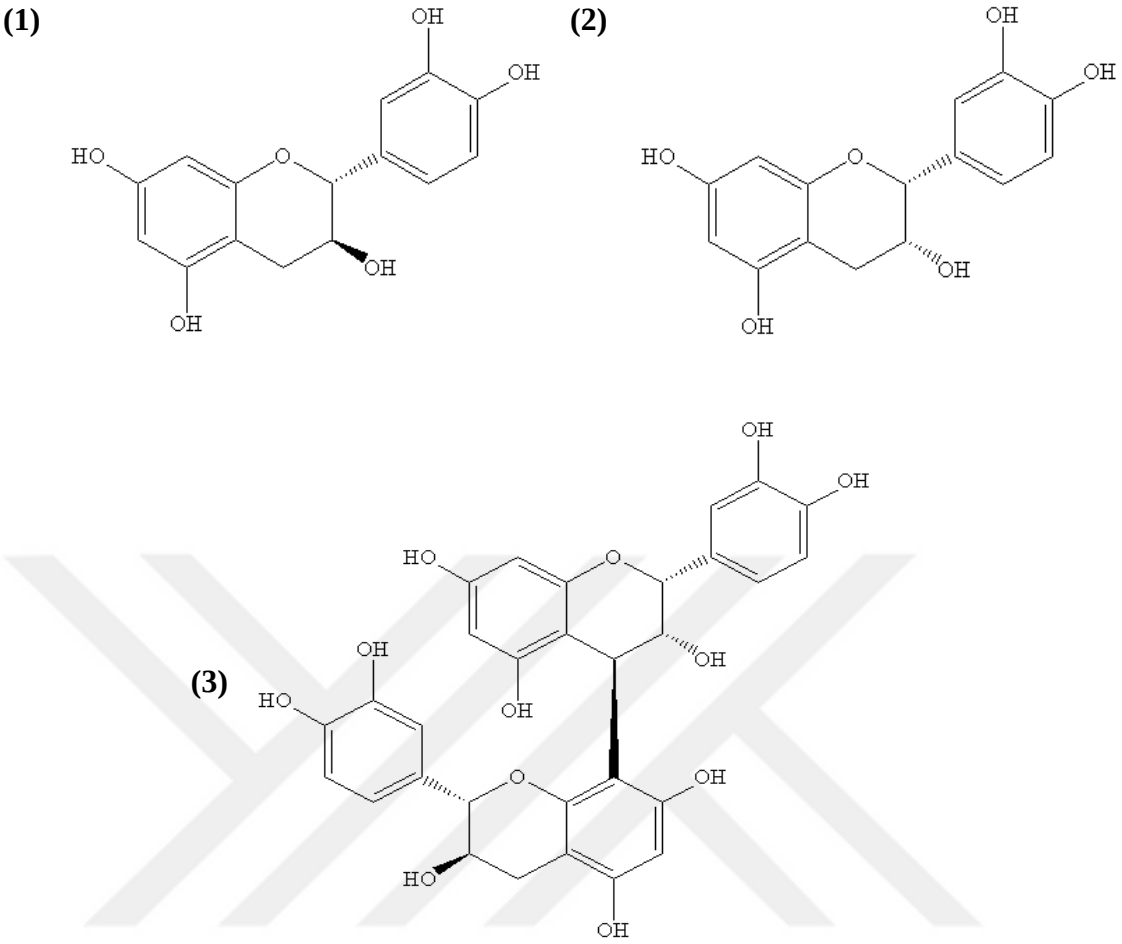


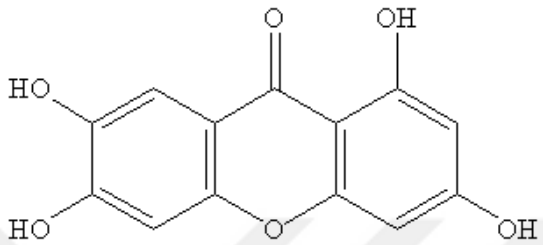
Table 2.5. Proanthocyanidins isolated from *H. perforatum*

Compound	**Reference**
Catechin (1)	(21, 50)
Epicatechin (2)	(11, 45, 50)
Procyanidin B2 (3)	(21, 46, 50)

2.2.1.1.6. Xanthones

Trace amounts of xanthones are found in the flowering aerial parts and roots of *H. perforatum* (6, 13, 46, 50, 55). The chemical structures of xanthones in *H. perforatum* are showed in Table 2.6.

(1)



(2)

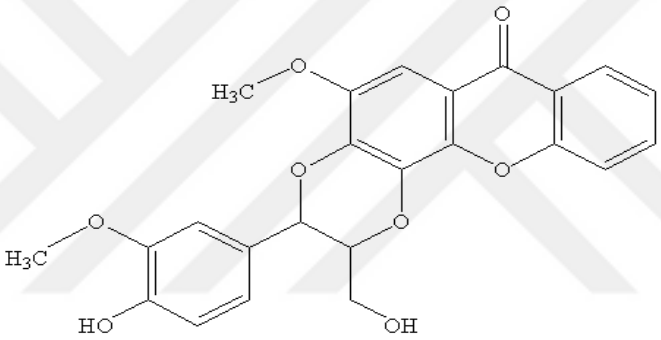


Table 2.6. Xanthones isolated from *H. perforatum*

Compound	**Reference**
1,3,6,7-tetrahydroxyxanthone (1)	(6, 46, 50)
Kielcorin (2)	(13, 46, 50)

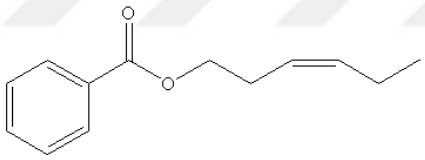
2.2.1.1.7. Essential oils

The volatile oil of *H. perforatum* comprises aliphatic, monoterpene and sesquiterpene compounds (15, 45, 56), and the detailed composition is shown in Table 2.7.

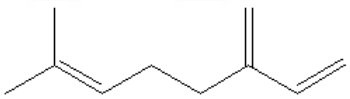
Table 2.7. Volatile components isolated from *H. perforatum*

Compound	Chemical group	Reference
n-nonane	Hydrocarbon (aliphatic)	(56-58)
n-undecane	Hydrocarbon (aliphatic)	(56, 59, 60)
n-tridecane	Hydrocarbon (aliphatic)	(56, 60, 61)
nonadecane	Hydrocarbon (aliphatic)	(56, 62)
n-heneicosane	Hydrocarbon (aliphatic)	(56, 63)
n-tricosane	Hydrocarbon (aliphatic)	(56)
n-pentacosane	Hydrocarbon (aliphatic)	(56)
n-heptacosane	Hydrocarbon (aliphatic)	(56)
n-nonacosane	Hydrocarbon (aliphatic)	(56)
2-methyloctane	Hydrocarbon (aliphatic)	(56-58, 62)
3-methylnonane	Hydrocarbon (aliphatic)	(56, 57, 59)
2-methyldecane	Hydrocarbon (aliphatic)	(56, 57, 59, 60)
2-methyldodecane	Hydrocarbon (aliphatic)	(56, 60, 64)
dodecanol	Hydrocarbon (alcohol)	(58, 60, 63)
tetradecanol	Hydrocarbon (alcohol)	(56, 58-60)

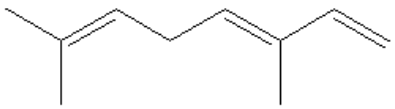
(1)



(2)



(3)



(4)

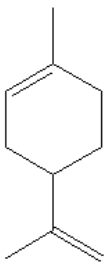


Table 2.7. Volatile components isolated from *H. perforatum* (continued)

Compound	Chemical group	Reference
3-hexenylbenzoate (1)	Hydrocarbon (ester)	(56, 60, 65)
myrcene (2)	Monoterpene (acyclic hydrocarbon)	(56, 57, 66, 67)
β-ocimene (3)	Monoterpene (acyclic hydrocarbon)	(56, 57, 60)
limonene (4)	Monoterpene (monocyclic hydrocarbon)	(56, 57, 60, 66)

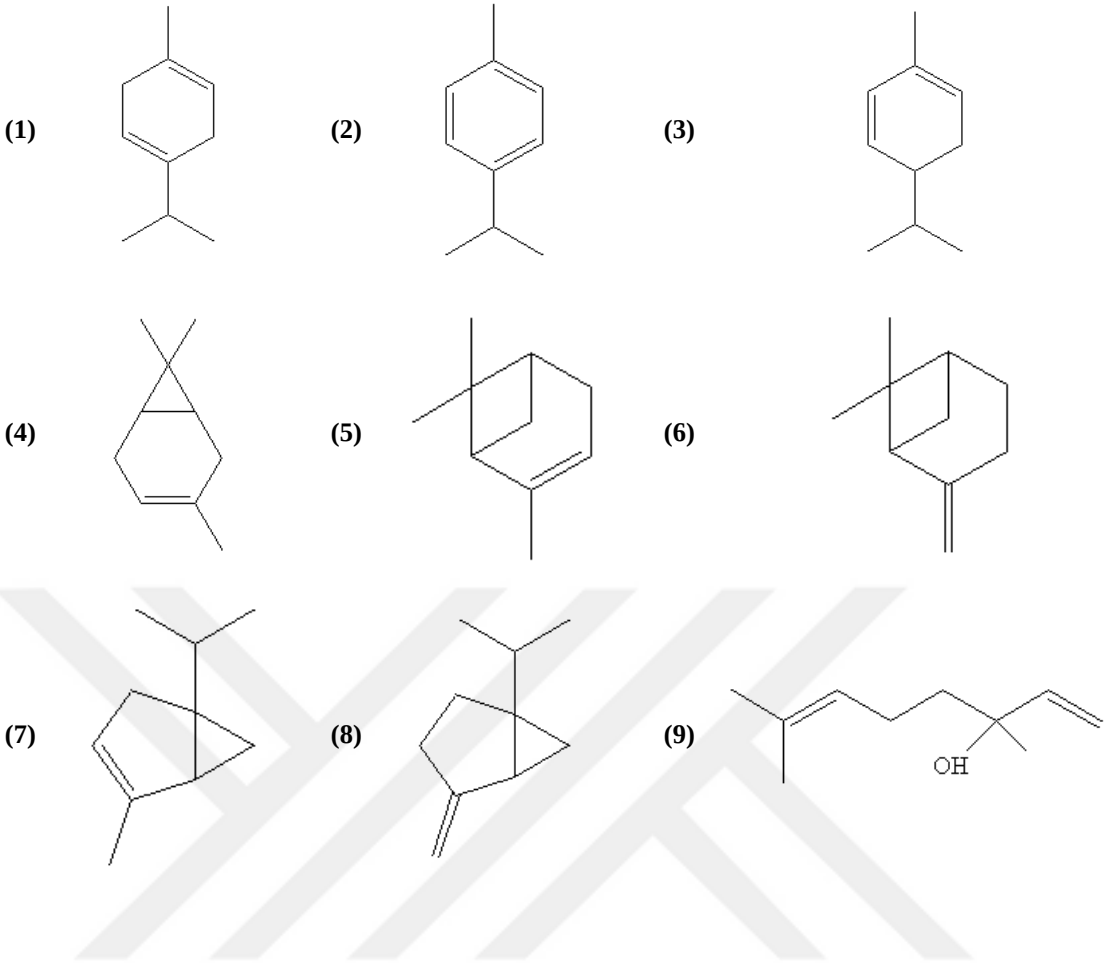
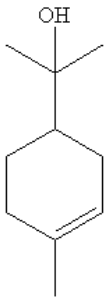


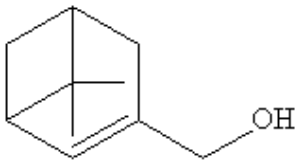
Table 2.7. Volatile components isolated from *H. perforatum* (continued)

Compound	Chemical group	Reference
γ-terpinene (1)	Monoterpene (monocyclic hydrocarbon)	(57, 66)
p-cymene (2)	Monoterpene (monocyclic hydrocarbon)	(56, 63, 66, 68)
α-phellandrene (3)	Monoterpene (monocyclic hydrocarbon)	(63, 66, 69)
3-carene (4)	Monoterpene (bicyclic hydrocarbon)	(66)
α-pinene (5)	Monoterpene (bicyclic hydrocarbon)	(56-59, 66)
β-pinene (6)	Monoterpene (bicyclic hydrocarbon)	(56, 57, 59, 66)
thujene (7)	Monoterpene (bicyclic hydrocarbon)	(57)
sabinene (8)	Monoterpene (bicyclic hydrocarbon)	(63, 66)
linalool (9)	Monoterpene (acyclic alcohol)	(56, 57)

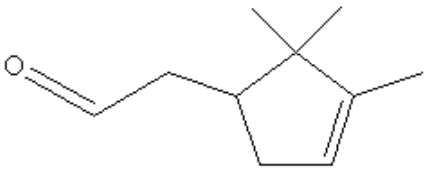
(1)



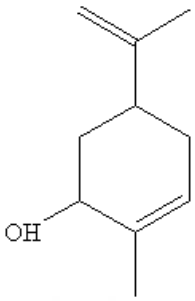
(2)



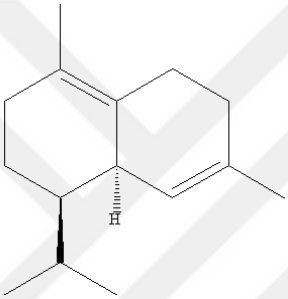
(3)



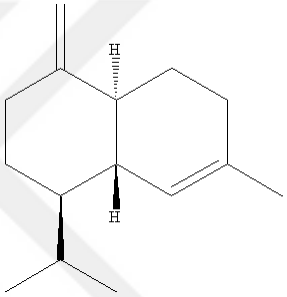
(4)



(5)



(6)



(7)

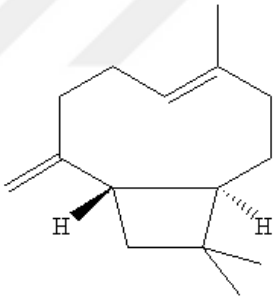


Table 2.7. Volatile components isolated from *H. perforatum* (continued)

Compound	Chemical group	Reference
terpineol (1)	Monoterpene (monocyclic alcohol)	(56, 60, 63)
myrtenol (2)	Monoterpene (monocyclic alcohol)	(60, 62, 66)
α-campholenal (3)	Monoterpene (acyclic aldehyde)	(56, 60)
carveol (4)	Monoterpene (phenol)	(60)
δ-cadinene (5)	Sesquiterpene (hydrocarbon)	(56, 58, 60, 63)
γ-cadinene (6)	Sesquiterpene (hydrocarbon)	(56, 57, 60, 66)
β-caryophyllene (7)	Sesquiterpene (hydrocarbon)	(56, 58, 60, 66)

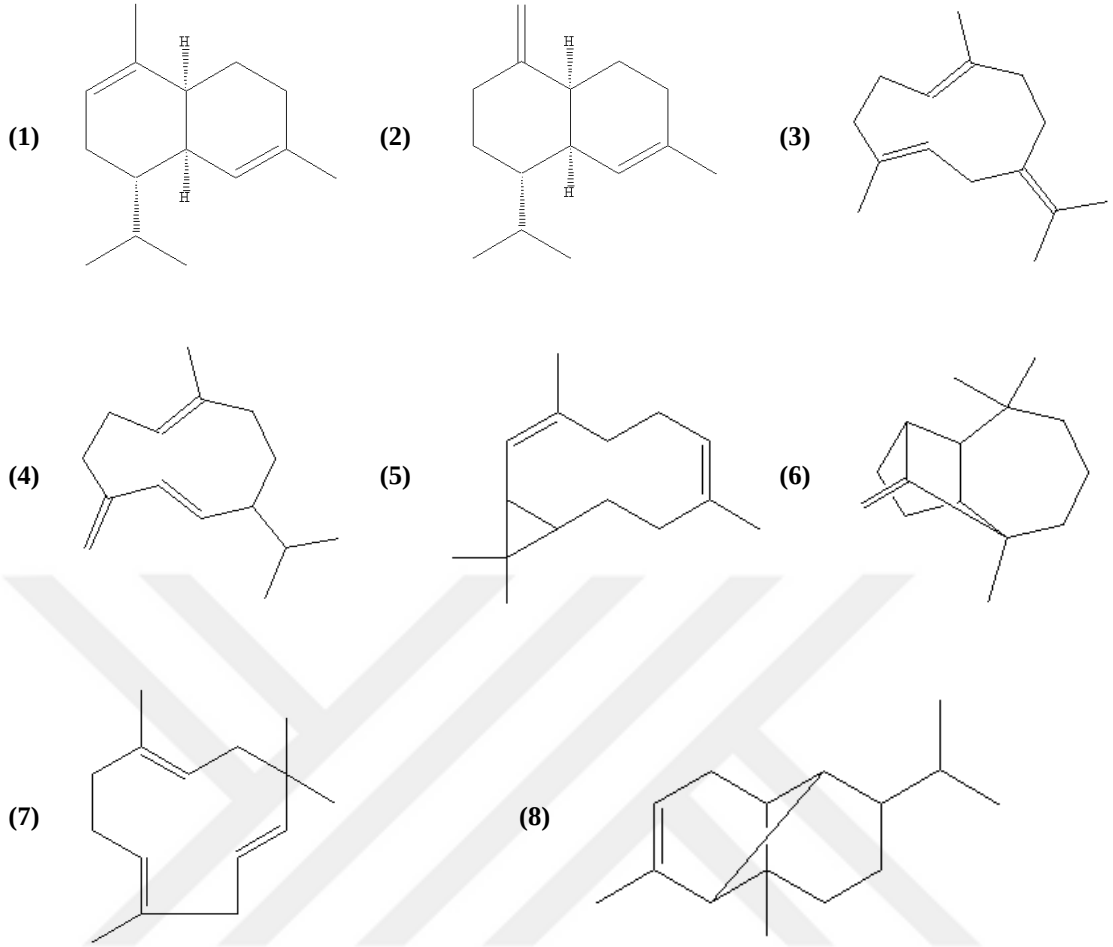


Table 2.7. Volatile components isolated from *H. perforatum* (continued)

Compound	Chemical group	Reference
α-muurolene (1)	Sesquiterpene (hydrocarbon)	(56, 60, 66)
γ-muurolene (2)	Sesquiterpene (hydrocarbon)	(56, 57, 60)
germacrene B (3)	Sesquiterpene (hydrocarbon)	(70)
germacrene D (4)	Sesquiterpene (hydrocarbon)	(56-58, 60, 66)
bicyclogermacrene (5)	Sesquiterpene (hydrocarbon)	(60, 62, 65)
longifolene (6)	Sesquiterpene (hydrocarbon)	(66, 70)
humulene (7)	Sesquiterpene (hydrocarbon)	(58, 60, 68)
α-copaene (8)	Sesquiterpene (hydrocarbon)	(56, 57, 60, 66)

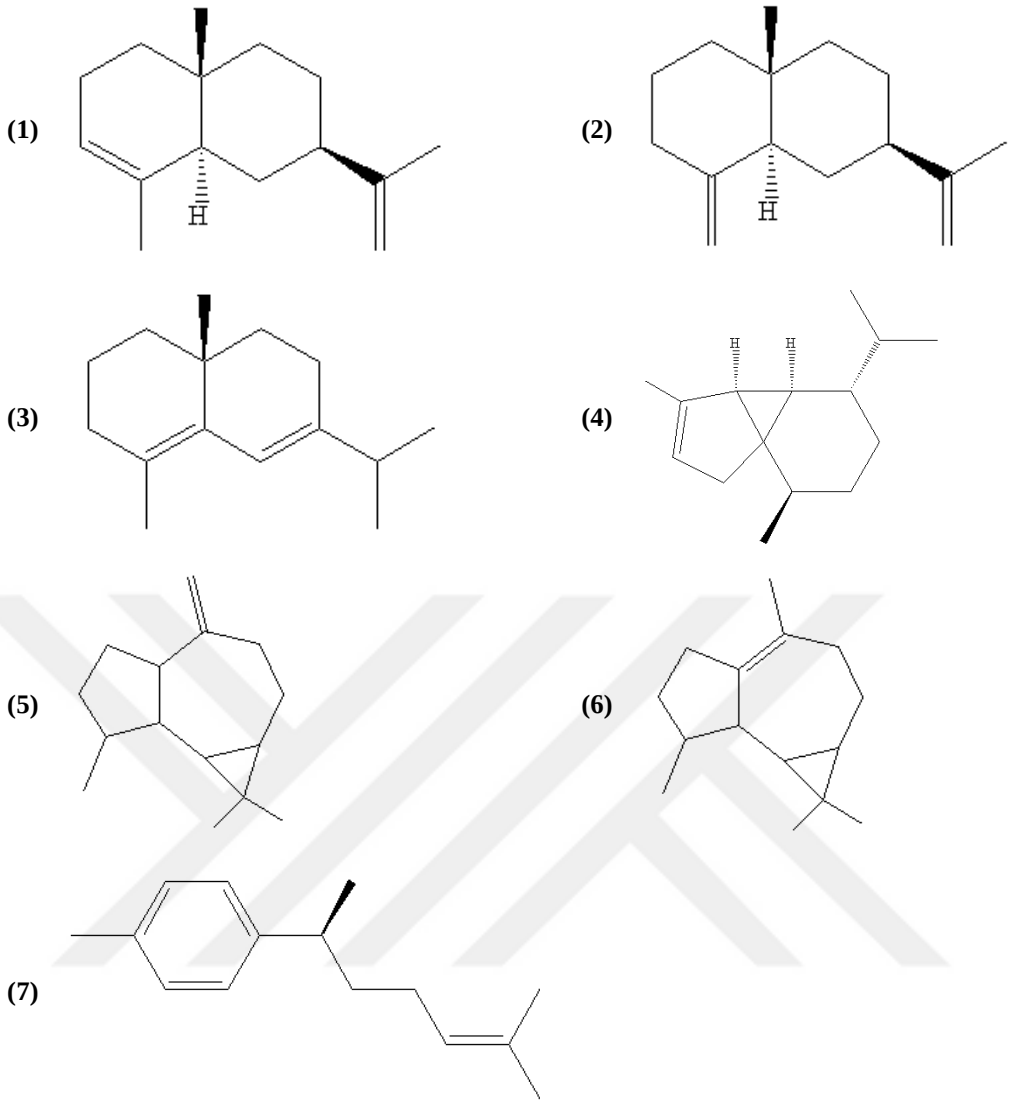


Table 2.7. Volatile components isolated from *H. perforatum* (continued)

Compound	Chemical group	Reference
α-selinene (1)	Sesquiterpene (hydrocarbon)	(59-61, 69)
β-selinene (2)	Sesquiterpene (hydrocarbon)	(57, 59-61)
δ-selinene (3)	Sesquiterpene (hydrocarbon)	(61)
α-cubebene (4)	Sesquiterpene (hydrocarbon)	(60, 64, 65, 69)
aromadendrene (5)	Sesquiterpene (hydrocarbon)	(57, 60, 66, 69)
viridiflorene (6)	Sesquiterpene (hydrocarbon)	(66)
curcumene (7)	Sesquiterpene (hydrocarbon)	(57, 60, 68)

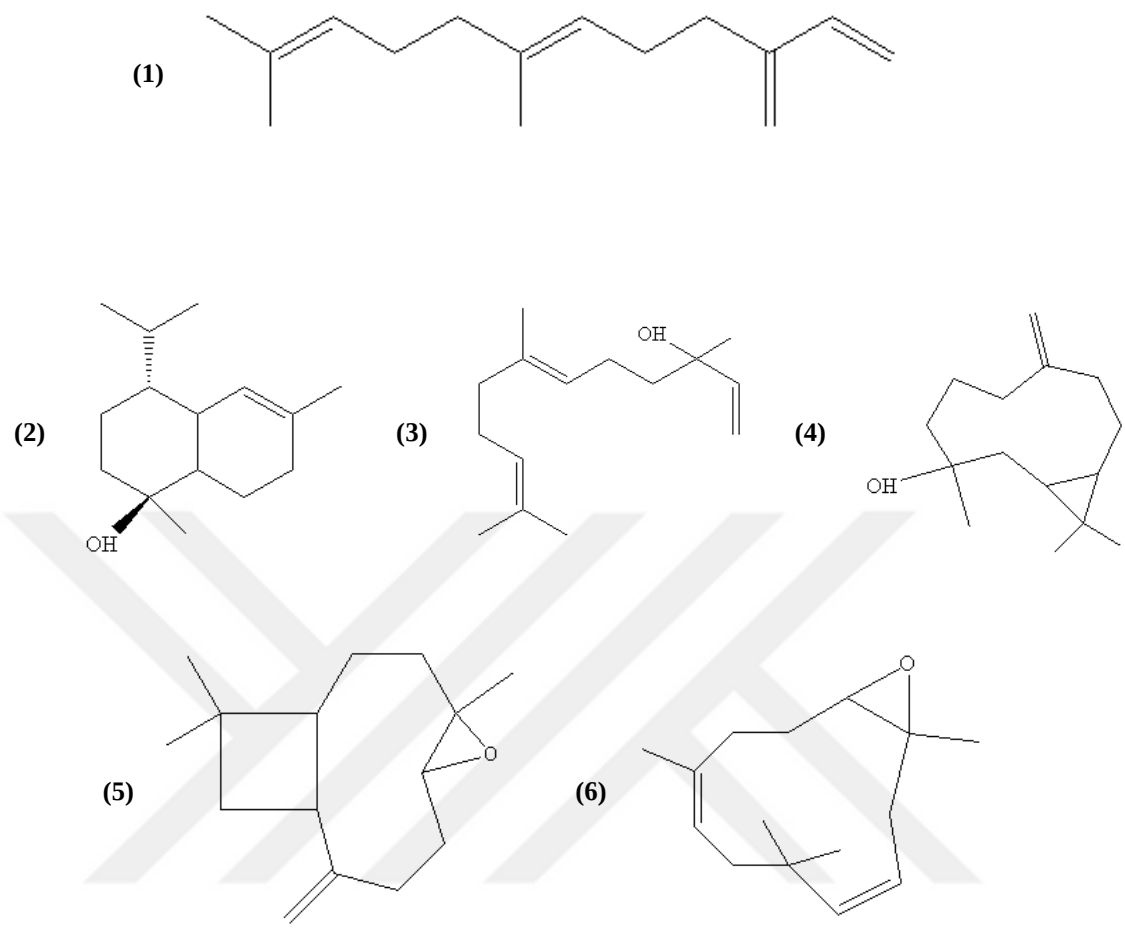


Table 2.7. Volatile components isolated from *H. perforatum* (continued)

Compound	Chemical group	Reference
β-farnesene (1)	Sesquiterpene (hydrocarbon)	(56-60, 69)
α-cadinol (2)	Sesquiterpene (alcohol)	(56, 58, 60)
nerolidol (3)	Sesquiterpene (alcohol)	(56-58, 60, 61)
spathulenol (4)	Sesquiterpene (alcohol)	(56-58, 60, 69)
caryophyllene oxide (5)	Sesquiterpene (epoxide)	(56-58, 60, 69)
humulene epoxide II (6)	Sesquiterpene (epoxide)	(56, 58, 60, 62)

2.2.1.2. Other components

Other compounds of *H. perforatum* include acids, i.e., palmitic, palmitoleic, myristic, lauric, oleic, linoleic, arachidic and stearic; pectin, carotenoids, choline, nicotinamide, β-sitosterol, and amino acids (γ-aminobutyric acid) (45, 48, 50, 71).

2.2.2. Analytical Studies

Numerous techniques have been developed for qualification and/or quantification of the components in the plant parts of *H. perforatum* or SJW preparations such as hyperforin, hypericin, hyperoside, rutin, quercetin, quercitrin, and chlorogenic acid, in alcoholic or hydroalcoholic or aqueous extracts. Qualitative and quantitative studies on *H. perforatum* are summarized in Table 2.8 and Table 2.9.

Table 2.8. LC studies on *H. perforatum*

Extract	Method	Column	Mobile phase	Compound	Reference
Methanol:acetone extract from blossom of *H. perforatum* L.	HPLC λ = 590 nm HPLC-MS	LiChrosorb RP 18 (5 µm, 4 x 125 mm)	**A:** methanol-acetonitrile (5:4, v/v) **B:** 0.1 M aqueous triethylammonium acetate **Gradient system:** initial 70% A, 0-8 min 90% A, 8-13 min 70% A **Flow rate:** 0.6 mL/min	**Qualitative analysis** hypericin, pseudohypericin	(72)
Methanolic extract from flowering top of *H. perforatum*	HPLC-DAD λ = 270 nm HPLC-ESI-MS	201 TP 54 RP-18 (5 µm, 4.6 x 250 mm)	**A:** water-phosphoric acid (85%) (99.7:0.3, v/v) **B:** acetonitrile **C:** methanol **Gradient system:** initial 100% A & 0% B, 10 min 85% A & 15% B, 30 min 70% A & 20% B, 40 min 10% A & 75% B, 56 min 100% A & 0% B, 65 min 100% A & 0% B **Flow rate:** 1.0 mL/min	**Qualitative and quantitative analysis** RSD values of reproducibility & repeatability of adhyperforin (0.66% & 1.95%), chlorogenic acid (1.26% & 1.59%), hyperforin (1.27% & 2.00%), hypericin (0.84% & 3.96%), hyperoside (0.18% & 1.92%), isoquercitrin (0.22% & 1.90%), I3,II8-biapigenin (0.17% & 1.93%), quercetin (0.40% & 2.15%), quercitrin (0.38% & 2.70%), pseudohypericin (2.32% & 2.62%), rutin (0.17% & 1.92%)	(73)
Hydroalcoholic extract of herba and flower of *H. perforatum* L.	HPLC-DAD λ = 590 nm	LiChrosorb RP18 column (5 µm, 4.6 x 250 mm)	**A:** water (pH 3.2 with phosphoric acid) **B:** methanol **C:** acetonitrile **Gradient system:** 0.1 min 88% A & 12% C, 10-15 min 82% A & 18% C, 30 min 55% A & 45% C, 35-42 min 55% B & 45% C **Flow rate:** 1.0 mL/min	**Qualitative and quantitative analysis** hypericin, pseudohypericin Total amount in Hyperici herba samples (1.72-2.22-2.48-2.52 mg/g), in dried flowers (1.82-2.84 mg/g)	(74)

Table 2.8. LC studies on *H. perforatum* (continued)

Extract	Method	Column	Mobile phase	Compound	Reference
Hexane extract from blossom of *H. perforatum* L.	HPLC-DAD-UV λ = 272 nm HPLC-NMR, HPLC-MS, HPLC-FTIR	ET 100 C18 (5 µm, 4 x 250 mm)	**A:** acetonitrile **B:** twice-distilled water **Isocratic system:** A-B (89.5:10.5, v/v) **Flow rate:** 1.2 mL/min	**Qualitative and quantitative analysis** hyperforin (3%)	(75)
SJW pharmaceutical preparations (capsule, tablet & tincture)	HPLC-DAD-UV λ = 236 & 592 nm HPLC-MS	Ultrasphere ODS C18 (5 µm, 4.6 x 250 mm)	**A:** methanol-acetonitrile (5:4, v/v) **B:** triethylammonium acetate buffer **Gradient system:** initial 30% B, 0-2 min 30% B, 2-10 min 30-10% B, 10-14 min 10% B, 14-16 min 10-0% B, 16-21 min 0% B, 21-22 min 0-30% B, 22-26 min 30% B **Flow rate:** 1.0 mL/min	**Qualitative and quantitative analysis** hypericin [1.10-467.78 µg/capsule, 25.74-292.83 µg/tablet, 28.20-54.24 µg/mL(tincture)]; pseudohypericin [8.12-1023.33 µg/capsule, 39.13-520.63 µg/tablet, 48.67-67.43 µg-mL(tincture)]	(76)
SJW dietary supplement (capsule)	HPLC-DAD λ = 284-590 nm HPLC-ESI-MS	LiChrospher RP-C18 (3 µm, 4 x 250 mm)	**A:** 0.5% trifluoroacetic acid in water **B:** 0.5% trifluoroacetic acid in mixture of methanol-acetonitrile (13:7, v/v) **Gradient system:** initial 10% B, 0-20 min 50% B, 20-40 min 60% B, 40-50 min 100% B, 50-60 min 100% B, 60-70 min 10% B, 70-95 min equilibrated **Flow rate:** 0.6 mL/min	**Qualitative and quantitative analysis** hyperforin (1.9-10.0 mg/g), hypericin (0.2-0.9 mg/g), hyperoside (3.5-15.8 mg/g), rutin (7.0-23.1 mg/g), isoquercitrin (1.3-9.1 mg/g), pseudohypericin (0.3-1.7 mg/g), quercetin (0.9-2.6 mg/g), quercitrin (0.6-2.9 mg/g)	(77)

Table 2.8. LC studies on *H. perforatum* (continued)

Extract	Method	Column	Mobile phase	Compound	Reference
Methanolic extract from flowering and fruiting top of *H. perforatum* L. and commercial dried extract of *H. perforatum* L.	HPLC-DAD λ = 200-590 nm HPLC-MS	Protein C4 (5 µm, 0.5 x 250 mm)	**A:** water-phosphoric acid (85%) (99.7:0.3, v/v) **B:** acetonitrile **C:** methanol **Gradient system:** 0 min 100% A, 10 min 85% A & 15% B, 30 min 70% A & 20% B, 40 min 25% A & 65% B, 55 min 20% A & 70% B, 57 min 5% A & 80% B, 60 min 100% A **Flow rate:** 1.0 mL/min	**Qualitative and quantitative analysis** flavonoids in flowering tops (5.64-15.60%), in fruiting tops (4.58-15.90%), in commercial extracts (10.64-15.01%); naphthodianthrones in flowering tops (0.05-0.11%), in fruiting tops (0.08-011%), in commercial extracts (0.03-0.20%); furohyperforin in flowering tops (0.30-3.66%), in fruiting tops (0.83-3.95%), in commercial extracts (0.24-0.57%); hyperforin in flowering tops (0.63-10.95%), in fruiting tops (5.20-10.14%), in commercial extracts (0.52-4.62%); adhyperforin in flowering tops (0.25-2.58%), in fruiting tops (2.90-4.22), in commercial extracts (0.08-1.36%)	(78)
Commercial dried extract and capsule of SJW	HPLC-DAD λ = 200-590 nm HPLC-MS	Protein C4 (5 µm, 0.5 x 250 mm)	**A:** acetonitrile **B:** methanol **C:** water (pH 3.2 phosphoric acid) **Gradient system:** 0 min 15% A & 0% B, 12 min 15% A, & 5% B, 20 min 75% A & 15% B, 27 min 80% A & 15% B, 30 min 15% A & 0% B **Flow rate:** 1.0 mL/min	**Qualitative and quantitative analysis** hyperoside (6.35 mg/100 mg), isoquercitrin (0.61 mg/100 mg), quercetin (0.83 mg/100 mg), quercitrin (0.65 mg/100 mg), rutin (4.28 mg/100 mg), total flavonols (12.67-12.72 mg/100 mg), total hyperforins (4.13-4.23 mg/100 mg), total hypericins (0.29-0.32 mg/100 mg)	(79)

Table 2.8. LC studies on *H. perforatum* (continued)

Extract	Method	Column	Mobile phase	Compound	Reference
Methanolic extract of SJW dietary supplement	HPLC-DAD λ = 270 & 590 nm	YMC ODS-AQ™ RP-18 (5 µm, 4.6 x 250 mm)	**A:** water containing 20% methanol & 0.5% trifluoroacetic acid **B:** acetonitrile containing 10% methanol & 0.5% trifluoroacetic acid **Gradient system:** initial 90% A, 0-20 min 30% A, 20-25 min 10% A, 25-30 min 0% A, 30-60 min 0% A, 60-65 min 90% A **Flow rate:** 1.0 mL/min	**Qualitative and quantitative analysis** rutin (2.52%), hyperoside (2.19%), isoquercitrin (1.44%), quercitrin (0.31%), quercetin (0.71%), pseudohypericin (0.11%), hyperforin (1.62%), hypericin (0.09%)	(80)
Methanolic extract from flower and fruit of SJW	HPLC λ = 280 & 590 nm HPLC-MS	Symmetry C18 (5 µm, 4.6 x 250 mm)	**A:** 30 mM sodium dihydrogen phosphate (pH 3) **B:** acetonitrile **Gradient system:** 0-40 min 90-60% A, 40-50 min 60-10% A, 50-70 min 10% A **Flow rate:** 1.8 mL/min	**Qualitative and quantitative analysis** hyperforin in flowers (1.24-2.11%), in fruits (3.00-4.15%); hypericin in flowers (0.28-0.66%), in fruits (0.06-0.29%); pseudohypericin in flowers (0.52-1.08%), in fruits (0.21-0.62%); quercetin & derivatives [quercitrin, isoquercitrin, hyperoside] in flowers (0.92-1.45%), in fruits (0.33-0.87%); rutin in flowers (1.56-2.11%), in fruits (0.45-1.27%)	(81)

Table 2.8. LC studies on *H. perforatum* (continued)

Extract	Method	Column	Mobile phase	Compound	Reference
Methanolic extract from flowering top of *H. perforatum*	HPLC-DAD λ = 270 & 590 nm	Nucleosil-10 RP18 (10 µm, 4.6 x 125 mm)	**A:** acetonitrile **B:** water-phosphoric acid (85%) (99.7:0.3, v/v) **C:** methanol **Gradient system:** 0 min 30% A & 60% B, 5 min 55% A & 20% B, 7 min 70% A & 5%B, 10 min 70% A & 5% B, 16 min 30% A & 60% B, 17 min 30% A & 60% B **Flow rate:** 1.2 mL/min	**Qualitative and quantitative analysis** hypericins (1.2-8.0%), hyperforins (1.15-5.77%)	(82)
Hydroalcoholic extract from SJW capsule	HPLC-UV λ = 273 & 590 nm	Discovery C18 (5 µm, 4.6 x 150 mm)	**A:** acetonitrile **B:** *ortho*phosphoric acid (0.3%, v/v) **Isocratic system:** A-B (90:10, v/v) **Flow rate:** 1.5 mL/min	**Qualitative and quantitative analysis** Concentration range of hyperforin (5-50 µg/mL), hypericin (0.5-2.5 µg/mL), pseudohypericin (0.35-1.6 µg/mL)	(83)
Methanolic extract of Hyperici herba	HPLC-UV-DAD the whole UV range	C18 column (5 µm, 4.6 x 250 mm)	**A:** 5% glacial acetic acid **B:** acetonitrile **Gradient system:** 0-10 min 70% A, 20-30 min 40% A **Flow rate:** 1.0 mL/min	**Qualitative and quantitative analysis** quercetin (0.276 mg/mL, 0.552%)	(84)
Chloroform-acetone-methanolic floral, leaf, and stem extract	HPLC-DAD λ = 254 & 590 nm	Diazem-phenyl™ guard column	**A:** acetonitrate **B:** methanol **C:** water **D:** phosphoric acid **Isocratic system:** A-B-C-D (48:40:10:2, v/v/v/v) **Flow rate:** 1.0 mL/min	**Qualitative and quantitative analysis** hypericin in floral (0.018-0.044%), in leaf (0.012-0.032%), in stem (0.001-0.003%); pseudohypericin in floral (0.107-0.427%), in leaf (0.054-0.228%), in stem (0.007-0.033%)	(85)

Table 2.8. LC studies on *H. perforatum* (continued)

Extract	Method	Column	Mobile phase	Compound	Reference
Methanolic extract of commercial SJW sample	HPLC-UV λ = 270 nm HPLC-MS	Synergi MAX-RP (4 µm, 4.6 x 150 mm)	**A:** 10 mM ammonium acetate buffer (pH 5.0 with glacial acetic acid) **B:** acetonitrile-methanol (9:1, v/v) **Gradient system:** initial 13% B, 0-10 min 17% B, 10-35 min 100% B **Flow rate:** 1.0mL/min	**Qualitative and quantitative analysis** hyperoside (0.16-1.41 mg/100 mg), isoquercitrin (0.09-0.65 mg/100 mg), quercitrin (0.03-0.31 mg/100 mg), quercetin (0.10-0.37 mg/100 mg), pseudohypericin (0.02-0.07 mg/100 mg), hypericin (0.01-0.09 mg/100 mg), hyperforin (0.00-1.30 mg/100 mg), I3,II8 biapigenin (0.11-0.56 mg/100 mg)	(86)
Commercial SJW preparations (powder, caplet, capsule)	HPLC-UV λ = 273 nm	Discovery C18 (5 µm, 4.6 x 150 mm)	**A:** acetonitrile **B:** *ortho*phosphoric acid (0.3%, v/v) **Isocratic system:** A-B (90:10, v/v) **Flow rate:** 1.5 mL/min	**Qualitative and quantitative analysis** hypericins (0.03-0.29%), hyperforin (0.01-0.29%)	(87)
Methanolic extract from the whole plant of *H. perforatum* (flower, bud, leaf & stem)	HPLC-DAD λ = 254 & 590 nm	Diazem-phenyl™ guard column	**A:** acetonitrile **B:** methanol **C:** water **D:** phosphoric acid **Isocratic system:** A-B-C-D (48:40:10:2, v/v/v/v) **Flow rate:** 1.0 mL/min	**Qualitative and quantitative analysis** hypericin (0.004-0.037%), pseudohypericin (0.043-0.277%)	(88)
Acetone:ethanol:methanol solution extract from callus of *H. perforatum*	HPLC-MS	XTerra RP18 (3.5 µm, 2.1 x 50 mm)	**A:** 20 mM ammonium acetate **B:** acetonitrile **Gradient system:** initial 50% A, 0-5 min 0% A, 5-9 min 0% A, 9-14 min 50% A **Flow rate:** 0.5 mL/min	**Qualitative and quantitative analysis** pseudohypericin, hyperforin, adhyperforin, hypericin LOQ of hypericin (2 ng/mL), hyperforin (0.5 ng/mL)	(89)

Table 2.8. LC studies on *H. perforatum* (continued)

Extract	Method	Column	Mobile phase	Compound	Reference
Methanolic extract of *Hypericum* stem	HPLC-DAD λ = 270 nm λ = 590 nm	Vydac 201TPS4 (4.6 x 280 mm) Phenomex Luna 00F-4252-E0 (4.6 x 150 mm)	**Hyperforin:** **A:** acetonitrile **B:** methanol **C:** 0.3% phosphoric acid-99.7% water **Isocratic system:** A-B-C (75:10:10, v/v/v) **Flow rate:** 1.5 mL/min **Hypericin & Pseudohypericin:** **A:** acetonitrile **B:** methanol **C:** 0.77% w/v ammonium acetate in water **Isocratic system:** A-B-C (50:30:20, v/v/v) **Flow rate:** 1.5 mL/min	**Qualitative analysis** hyperforin, hypericin, pseudohypericin	(90)

Table 2.8. LC studies on *H. perforatum* (continued)

Extract	Method	Column	Mobile phase	Compound	Reference
Methanolic (M) and aqueous (A) extract of SJW tea product & SJW dietary supplement extract with methanol (M), acetonitrile (Ac), dichloromethane (D), hexane (Hx), water (W)	HPLC-UV λ = 290 nm HPLC-MS	Luna C18 (5 µm, 4.6 x 250 mm)	**A:** acetonitrile **B:** triethylammonium acetate buffer **Gradient system:** 0-2 min 20% B, 2-9 min 10% B, 9-10 min 0% B, 10-16 min 0% B **Flow rate:** 1.0 mL/min	**Qualitative and quantitative analysis** SJW herbal teas hypericin (M: 3.0-36.9 µg/mL, A: 0.4-10.7 µg/mL); pseudohypericin (M: 7.9-61.1 µg/mL, A: 2.6-37.9 µg/mL); hyperforin (M: 26.0-36.5 µg/mL, A: 1.3-2.5 µg/mL); adhyperforin (M: 5.8-79.9 µg/mL, A: 0.9-1.5 µg/mL) SJW dietary supplement hypericin (M: 52 µg/mL, Ac: 3.0 µg/mL, D & Hx: ndt, W: 36 µg/mL); pseudohypericin (M: 13 µg/mL, Ac: 0.062 µg/mL, D & Hx: ndt, W: 11 µg/mL); hyperforin (M: 6.2 µg/mL, Ac: 1.2 µg/mL, D: ndt, Hx: 0.80 µg/mL, W: 0.86 µg/mL); adhyperforin (M: 3.4 µg/mL, Ac: 0.81 µg/mL, D: ndt, Hx: 0.18 µg/mL, W: 0.59 µg/mL)	(91)
Acetone:methanol extract from top of flower of *H. perforatum* L.	HPLC-DAD λ = 270 & 590 nm	Jupiter C18 (5 µm, 4.6 x 250 mm)	**A:** water (0.02% phosphoric acid pH 2.7) **B:** acetonitrile-methanol (9:1, v/v) **Gradient system:** 0-10 min 85% A, 10-30 min 85-65% A **Flow rate:** 1.0 mL/min	**Qualitative and quantitative analysis** chlorogenic acid (1.114 µg/g), hyperforin (27.884 µg/g), hypericin (8.339 µg/g), hyperoside (2.068 µg/g), isoquercitrin (7.767 µg/g), quercetin (0.900 µg/g), quercitrin (1.619 µg/g), rutin (17.656 µg/g)	(92)

ndt: not detected

Table 2.8. LC studies on *H. perforatum* (continued)

Extract	Method	Column	Mobile phase	Compound	Reference
Commercial SJW tablet	HPLC λ = 470 & 600 nm	Wakosil-II 5C18 (4.6 x 150 mm)	**A:** 0.1% ammonium phosphate (pH 7.0)- acetonitrile (7:3, v/v) **B:** acetonitrile-deionized water (7:3, v/v) **Gradient system:** initial 0% B, 0-10 min 100% B, 10-15 min 0% B, 20-25 min 0% B **Flow rate:** 1.2 mL/min	**Qualitative and quantitative analysis** hypericin (15-198 µg/tablet), pseudohypericin (11-465 µg/tablet)	(93)
Commercial preparations of SJW (tablet & capsule)	HPLC-UV λ = 590 nm	Phenomenex C18 Synergi hydro-RP (4 µm, 4.6 x 150 mm)	**A:** acetonitrile **B:** methanol **C:** 100 mM ammonium acetate **D:** glacial acetic acid **Isocratic system:** A-B-C-D (50:30:20:0.15, v/ v/v/v) **Flow rate:** 1.4 mL/min	**Qualitative and quantitative analysis** hypericin (7.72-38.57%), pseudohypericin (0.05-0.19%)	(94)
Methanolic extract from commercial chopped, dried *H. perforatum* L. plant material and SJW finished products (tablet & capsule)	HPCL-UV λ = 340 & 590 nm HPLC-MS	Luna C18 (5 µm, 4.6 x 250 mm)	**Flavonoids:** **A:** 1.0% trimethylamine in water (pH 4.5 with acetic acid) **B:** acetonitrile **Gradient system:** 0-15 min 95% A, 15-55 min 75% A, 55-75 min 0% A, 75-95 min 0% A **Flow rate:** 1.0 mL/min **Hypericin & Pseudohypericin:** **A:** TEAA buffer [1.0% trimethylamine in water (pH 4.5 with acetic acid)] **B:** acetonitrile **Isocratic system:** A-B (16:84, v/v) **Flow rate:** 1.0 mL/min	**Qualitative and quantitative analysis** In SJW: amentoflavone (0.32-0.60 mg/g), chlorogenic acid (6.21-6.80 mg/g), hypericin (0.28-0.65 mg/g), hyperoside (14.75-16.30 mg/g), isoquercitrin (5.19-5.62 mg/g), pseudohypericin (0.79-1.04 mg/g), quercetin (1.90-2.28 mg/g), quercitrin (2.66-3.58 mg/g), rutin (16.66-21.38 mg/g) In brands of SJW: hypericin (0.082-0.322 mg/g), pseudohypericin (0.044-0.546 mg/g)	(95)

Table 2.8. LC studies on *H. perforatum* (continued)

Extract	Method	Column	Mobile phase	Compound	Reference
Hydroalcoholic extract from leaf and flower of *H. perforatum*	HPLC-DAD λ = 280 & 350 nm HPLC-ESI-MS	Luna C18(2) (4.6 x 250 mm)	**A:** 25 mM phosphate buffer at pH 2.5 **B:** methanol **Gradient system:** 0 min 95% A, 35 min 90% A, 50 min 20% A, 52 min 0% A **Flow rate:** 1.0 mL/min	**Qualitative and quantitative analysis** caffeic acid in leaves (0.15 mg/g), in flowers (0.17 mg/g); chlorogenic acid in leaves (1.19 mg/g), in flowers (3.28 mg/g); hyperoside in leaves (1.78 mg/g), in flowers (4.70 mg/g); quercetin in leaves (0.31 mg/g), in flowers (7.52 mg/g); quercitrin in leaves (0.38 mg/g), in flowers (1.84 mg/g); rutin in leaves (3.45 mg/g), in flowers (1.49 mg/g)	(96)
Ethanolic extract from aerial part of *H. perforatum* L.	HPLC-UV λ = 360 nm λ = 590 nm	XTerra RP18 (3.5 µm, 3.9 x 150 mm) Hypersil ODS C18 (5 µm, 4.6 x 150 mm)	**Phenolic acid & Flavonoids:** **A:** 5% 0.1% trifluoroacetic acid in water **B:** 95% 0.1% trifluoroacetic acid in acetonitrile **Gradient system:** 0-45 min 5-45% B, 45-50 min 45% B, 50-55 min 45-5% B **Flow rate:** 0.4 mL/min **Hypericins:** **A:** ethyl acetate **B:** 15.6g/L sodiumhydrogen phosphate-phosphoric acid **C:** methanol **Isocratic system:** A-B-C (16:17:67, v/v/v) **Flow rate:** 1.0 mL/min	**Qualitative and quantitative analysis** apigenin-7-*O*-glucoside (0.21 mg/g), chlorogenic acid (0.66 mg/g), hypericin (2.82 mg/g), hyperoside (16.58 mg/g), pseudohypericin (1.86 mg/g), quercetin (1.40 mg/g), quercitrin (2.85 mg/g), rutin (0.66 mg/g)	(97)

Table 2.8. LC studies on *H. perforatum* (continued)

Extract	Method	Column	Mobile phase	Compound	Reference
Ethanolic extract from aerial part of different *H. perforatum* plants	HPLC-UV λ = 360 nm λ = 590 nm	XTerra RP 18 (3.5 µm, 3.9 x 150 mm) Hypersil ODS c18 (5 µm, 4.6 x 150 mm)	**Phenolic acid & Flavonoids:** **A:** 5% 0.1% trifluoroacetic acid in water **B:** 95% 0.1% trifluoroacetic acid in acetonitrile **Gradient system:** 0-45 min 5-45% B, 45-50 min 45% B, 50-55 min 45-5% B **Flow rate:** 0.4 mL/min **Hypericin:** **A:** ethyl acetate **B:** 15.6 g/L sodium dihydrogen phosphate-phosphoric acid **C:** methanol **Isocratic system:** A-B-C (16:17:67, v/v/v) **Flow rate:** 1.0 mL/min	**Qualitative and quantitative analysis** apigenin-7-*O*-glucoside (0.10-0.43 mg/g), chlorogenic acid (0.00-1.86 mg/g), hypericin (0.44-2.82 mg/g), hyperoside (5.41-22.28 mg/g), quercetin (1.01-1.76 mg/g), quercitrin (1.22-3.98 mg/g), rutin (0.00-8.77 mg/g)	(98)
Hydroalcoholic extract from herbal part of *H. perforatum*	HPLC-UV λ = 280 & 320 & 360 nm	Purospher RP-18 (5 µm, 3 x 125 mm)	**A:** formic acid (4.5%) **B:** acetonitrile-solvent A (80:20, v/v) **Gradient system:** 0-1 min 5% B, 1-17 min 5-80% B, 17-24 min 80-100% B **Flow rate:** 1.0 mL/min	**Qualitative and quantitative analysis** caffeic acid (229 mg/100 g), ferulic acid (9.38 mg/100 g), kaempferol (5.89 mg/100 g), *p*-coumaric acid (32.3 mg/100 g), quercetin (49.7 mg/100 g)	(99)
Acetone:methanol extract from stem, leaf and flower of *H. perforatum*	HPLC-DAD λ = 588 nm	Phenomenex Hypersil C18 (3.0 µm, 4.6 x 100 mm)	**A:** 0.1 M triethylammonium acetate **B:** acetonitrile **Isocratic system:** A-B (33:67, v/v) **Flow rate:** 1.0 mL/min	**Qualitative and quantitative analysis** hypericin in stem (0.05 mg/g), in leaf (2.03 mg/g), in flower (0.83 mg/g); pseudohypericin in stem (0.05 mg/g), in leaf (1.24 mg/g), in flower (0.51 mg/g)	(100)

Table 2.8. LC studies on *H. perforatum* (continued)

Extract	Method	Column	Mobile phase	Compound	Reference
Methanolic extracts from two commercial extracts of SJW	HPLC λ = 270 nm	Kromasil 100 C18 (3.5 µm, 4.6 x 150 mm)	**A:** 0.2% formic acid in water **B:** 0.2% formic acid in methanol **Gradient system:** initial 90% B, 0-30 min 100% B, 30-35 min 100% B, 35-36 min 90% B **Flow rate:** 1.0 mL/min	**Qualitative and quantitative analysis** hyperforin (2.0-27.0% w/w)	(101)
Methanolic extract from aerial part of *H. perforatum* L.	HPLC-UV HPLC-MS	**I:** Luna C18 (3 µm, 2 x 50 mm) **II:** Luna C18 (3 µm, 2 x 150 mm)	**A:** 10 mM ammonium acetate buffer (pH 5.0 with glacial acetic acid) **B:** methanol-acetonitrile (1:9, v/v) **Gradient system:** initial 13% B, 17% B in 10 min, 90% B in 25 min, 100% B in 5 min **Flow rate:** 0.25 mL/min	**Qualitative and quantitative analysis** **I:** hyperforin (3.84-24.58 mg/g), hypericin (1.19-2.68 mg/g), pseudohypericin (1.66-4.62 mg/g) **II:** hyperoside (13.74-41.64 mg/g), quercetin (0.19-1.54 mg/g), quercitrin (1.33-12.85 mg/g), rutin (5.63-23.77 mg/g)	(40)
Extract from Hyperici herba and commercial products of SJW	HPLC-UV λ = 590 nm	Hypersil Gold RP18 (5 µm, 4.6 x 150 mm)	**A:** ethyl acetate **B:** 15.6 g/L solution of sodium dihydrogen phosphate (pH 2 with phosphoric acid) **C:** methanol **Isocratic system:** A-B-C (39:41:160, v/v/v) **Flow rate:** 1.0 mL/min	**Qualitative and quantitative analysis** total hypericins of capsules (0.327 mg/capsule), tablets (0.048-0.106 mg/tablet), tincture (0.942 mg/100mL), infusion (0.023-0.029%), succus (0.057 mg/100mL)	(102)

Table 2.8. LC studies on *H. perforatum* (continued)

Extract	Method	Column	Mobile phase	Compound	Reference
Methanolic extract from aerial part of *H. perforatum*	HPLC-UV λ = 590 nm	Separon SGX C18 (7 µm, 4 x 150 mm)	**A:** water-acetonitrile-phosphoric acid (80:19:1, v/v/v) **B:** acetonitrile **Gradient system:** 0-3 min 95% A, 3-10 min 45% A, 10-20 min 0% A, 20-25 min 95% A **Flow rate:** 0.5 mL/min	**Qualitative and quantitative analysis** hypericin (1.17-2.59 mg/g), pseudohypericin (3.45-6.82 mg/g)	(103)
Methanolic extract from leaf and flower of *H. perforatum*	HPLC-UV λ = 270 & 590 nm λ = 270 & 590 nm	Lichrosorb RP-18 (7 µm, 4.1 x 250 mm) Nova-Pak RP-18 (4 µm, 3.9 x 150 mm)	**Hypericin** **A:** phosphoric acid (0.01%, v/v) **B:** methanol **C:** acetonitrile **Gradient system:** 0-5 min 95-70% A & 5-20% C, 5-10 min 70% A & 20% C, 10-25 min 10% A & 70% C, 25-40 min 100% B, 40-50 min 100% B **Flow rate:** 1.0 mL/min **Other compounds** **A:** phosphoric acid (0.01%, v/v) **B:** methanol **C:** acetonitrile **Gradient system:** 0-10 min 95-85% A & 5-15% C, 10-30 min 70% A & 20% C, 30-40 min 10% A & 75% C, 40-55 min 5% A & 80% C **Flow rate:** 1.0 mL/min	**Qualitative and quantitative analysis** biapigenin (0.060-0.549%), chlorogenic acid (0.083-0.612%), hyperforin (0.236-2.163%), hypericin (0.070-0.242%), hyperoside (0.466-1.550%), isoquercitrin (0.133-0.366%), quercetin (0.252-0.480%), quercitrin (0.080-0.610%), rutin (0.343-0.695%)	(104)

Table 2.8. LC studies on *H. perforatum* (continued)

Extract	Method	Column	Mobile phase	Compound	Reference
Methanolic extract of aerial part of *H. perforatum*	HPLC-DAD λ = 270 & 590 nm	Hypersil ODS C18 (5 µm, 4.6 x 250 mm)	**A:** phosphoric acid in water (0.3%) **B:** acetonitrile **C:** methanol **Gradient system:** 0-12 min 100-80% A & 0-15% B & 0-5% C, 12-20 min 10% A & 75% B & 15% C, 20-27 min 5% A & 80% B & 15% C, 27-30 min 85% A & 15% B **Flow rate:** 1.0 mL/min	**Qualitative and quantitative analysis** adhyperforin (0.9 mg/g), chlorogenic acid (0.9 mg/g), ferulic acid (0.7 mg/g), hyperforin (5.5 mg/g), hypericin (0.4 mg/g), hyperoside (21.8 mg/g), isoquercitrin (1.92 mg/g), I3,II8-biapigenin (1 mg/g), pseudohypericin (0.3 mg/g), quercetin (1.4 mg/g), quercitrin (2.9 mg/g), rutin (0.8 mg/g)	(105)
Methanolic (M), hydroalcoholic (H) and aqueous (A) extracts from aerial part of *H. perforatum* L.	HPLC-UV λ = 270 nm	Polaris C-18 (5 µm, 4.6 x 150 mm)	**A:** water-phosphoric acid (85%) (99.7:0.3, v/v) **B:** acetonitrile **C:** methanol **Gradient system:** initial 100% A & 0% C, 10 min 85% A & 0% C, 30 min 70% A & 10% C, 40 min 10% A & 15% C, 56 min 100% A & 0% C, 65 min 100% A & 0% C **Flow rate:** 1.0 mL/min	**Qualitative and quantitative analysis** chlorogenic acid (M: 0.83-0.99%, H: 1.29%, A: 1.15-1.28%); hyperforin (M: 0.26-1.39%, H&A: ndt); hypericin (M: 0.99-1.40%, H&A: ndt); hyperoside (M: 2.62-3.18%, H: 1.42%, A: 0.43-1.33%); isoquercitrin (M: 1.34-1.63%, H: 1.26%, A: 0.44-0.96%); I3, II8-biapigenin (M: 0.11-0.71%, H: 0.31%, A: 0.04%); quercetin (M: 0.65-1.05%, H: 2.13%, A: 0.14-0.22%); quercitrin (M: 1.32-1.52%, H: 1.37%, A: 0.41-0.75%); rutin (M: 3.49-3.64%, H: 1.69%, A: 0.17-2.15%)	(106)

ndt: not detected

Table 2.8. LC studies on *H. perforatum* (continued)

Extract	Method	Column	Mobile phase	Compound	Reference
Methanolic extract from aerial part of *H. perforatum* L.	HPLC-UV λ = 254 nm	Separon SGX C 18 (7 µm, 4 x 150 mm)	**A:** water-acetonitrile-phosphoric acid (80:19:1, v/v/v) **B:** acetonitrile **Gradient system:** 0-3 min 95% A, 3-10 min 45% A, 10-20 min 0% A, 20-25 min 95% A **Flow rate:** 0.5 mL/min	**Qualitative and quantitative analysis** biapigenin (0.03-4.63 mg/g), hyperoside (12.84-24.49 mg/g), quercetin (0.14-1.50 mg/g), quercitrin (0.00-16.18 mg/g), rutin (0.00-32.61 mg/g)	(107)
Methanolic extract from aerial part of *H. perforatum* L.	HPLC-DAD-MS λ = 270 nm	Eclipse XDB-C18 (5 µm, 4.6 x 150 mm)	**A:** acetonitrile **B:** 40 mM formic acid in water **Gradient system:** initial 100% A, 10 min 15% B, 30 min 30% B, 40 min 90% B, 55 min 95% B, 56 min 100% A, 65 min 100% A **Flow rate:** 1.0 mL/min	**Qualitative and quantitative analysis** adhyperforin (156 µg/g), biapigenin (189 µg/g), chlorogenic acid (374 µg/g), hyperforin (1164 µg/g), hypericin (16 µg/g), hyperoside (805 µg/g), pseudohypericin (14 µg/g), quercetin (39 µg/g), quercitrin (144 µg/g), rutin (1124 µg/g)	(108)
Hydroalcoholic extract from aerial part of *H. perforatum* L.	HPLC-DAD HPLC-MS	Zorbax Plus C18 (1.8 µm, 2.1 x 100 mm)	**A:** 0.1 % formic acid in deionized water **B:** 0.1 % formic acid in acetonitrile **Gradient system:** initial 95% A, 0-30 min 5% A **Flow rate:** 0.4 mL/min	**Qualitative analysis** caffeic acid, chlorogenic acid, quercetin, quercetin-3-*O*-galactoside (hyperoside), quercetin-3-*O*-rutinoside (rutin)	(109)
Methanolic extract from flower of *H. perforatum*	UPLC-MS NMR	Water HSS T3 (1.8 µm, 1.0 x 100 mm)	**A:** 50 mM ammonium acetate in water **B:** acetonitrile **Gradient system:** 0-1 min 95% A, 1-16 min 95-5% A, 16-18 min 5% A, 18-20 min 95% A **Flow rate:** 150 µL/min	**Qualitative analysis** adhyperforin, amentoflavone, chlorogenic acid, furohyperforin, hyperforin, hypericin, hyperoside, isoquercitrin, pseudohypericin, quercetin, rutin	(110)

Table 2.8. LC studies on *H. perforatum* (continued)

Extract	Method	Column	Mobile phase	Compound	Reference
Commercial extract of *H. perforatum*	UPLC λ = 590 nm	Waters Acquity HSS T3 (1.8 µm, 2.1 x 100 mm)	**A:** ethyl acetate **B:** sodium dihydrogen phosphate buffer (pH 2) **C:** methanol **Isocratic system:** A-B-C (39:41:160, v/v/v) **Flow rate:** 0.4 mL/min	**Qualitative and quantitative analysis** pseudohypericin (0.21-0.81 mg/g), hypericin (1.27-5.86 mg/g)	(111)

Table 2.9. HPTLC studies on *Hypericum* species

Extract	Stationary phase	Developing solvent system	Derivatization	Detection	Compound	Reference
Hydroalcoholic extract of herba and flower of *H. perforatum* L.	Silica gel TLC plates	toluene-formic acid-ethyl acetate (5:1:4, v/v/v)	-	Scanner: λ = 590 nm	**Qualitative and quantitative analysis** hypericin, pseudohypericin Total amount in Hyperici herba samples (1.67-2.14-2.36-2.44 mg/g), in dried flowers (1.92-2.79 mg/g)	(74)
Hexane extract from blossom of *H. perforatum* L.	HPTLC silica gel 60 F_{254}	**A:** acetone-*n*-heptane- t-butyl methyl ether-96% acetic acid (35:33:30:2, v/v/v/v) **B:** toluene- formic acid-formic acid ethyl ester (5:1:4, v/v/v)	Fast blue salt B 0.5% in water	Visualizer: 254 nm	**Qualitative analysis** hyperforin (R_f =0.45, 0.8, respectively)	(75)
Methanol: acetone extract from leaf, flower and flower bud of *H. richeri*	HPTLC silica gel 60 F_{254}	ethyl acetate-water-formic acid (30:2:3, v/v/v)	-	Scanner: λ = 400 nm, 590 nm	**Qualitative analysis** hyperoside, quercitrin, isoquercitrin, quercetin, hypericin, pseudohypericin	(112)
Ethanolic extract from flower of *H. hirsutum*, *H. empetrifolium* and *H. formosissimum*	Silica gel plates	toluene-formic acid-ethyl acetate (5:1:4, v/v/v) ethyl acetate-formic acid (25:3, v/v)	0.5 N potassium hydroxide in ethanol	Visualizer: 366 nm	**Qualitative analysis** hypericin, pseudohypericin	(113)
Methanol: acetone extract from flower of *H. perforatum* from different localities in Italy	HPTLC silica gel 60 F_{254}	ethyl acetate-water-formic acid (30:2:3, v/v/v)	-	Scanner: λ = 400 nm, 590 nm	**Qualitative and quantitative analysis** hypericin (0.03-0.40%), hyperoside (0.14-0.86%), isoquercitrin (0.05-0.36%), pseudohypericin (0.10-0.34%), quercetin (0.10-0.66%), quercitrin (0.20-1.04%), rutin (0.00-0.54%)	(114)

Table 2.9. HPTLC studies on *Hypericum* species (continued)

Extract	Stationary phase	Developing solvent system	Derivatization	Detection	Compound	Reference
Methanolic extract and commercial trade sample of Hyperici herba	HPTLC silica gel 60 F_{254}	**A:** ethyl acetate- glacial acetic acid-formic acid- water (100:11:11:26, v/v/v/v) **B:** toluene-ethyl formate-formic acid (5:4:1, v/v/v)	**A:** Natural products-polyethylene glycol **B:** 10% pyridine in ethanol	**A:** vis & λ = 365 nm **B:** visible	**Qualitative analysis** hypericin, rutin, chlorogenic acid, hyperoside, isoquercitrin, quercitrin	(115)
Methanolic extract from Hyperici herba	HPTLC silica gel 60 F_{254}	**A:** ethyl acetate-water-formic acid (20:1:2, v/v/v) **B:** ethyl acetate-dichloromethane-acetic acid-formic acid-water (100:25:10:10:11, v/v/v/v/v) **C:** ethyl acetate-formic acid-acetic acid-water (100:11:11:27, v/v/v/v) **D:** formic acid-water-ethyl acetate (6:8:86, v/v/v) **E:** ethyl acetate-formic acid-methanol (9:0.2:0.8, v/v/v) **F:** formic acid-water-ethyl acetate (2:1:17, v/v/v) **G:** chloroform-water-methanol (18:2:8, v/v/v) **H:** water-formic acid-ethyl methyl ketone-ethyl acetate (1:1:3:5, v/v/v/v)	Natural product reagent	Visualizer: 366 nm	**Qualitative analysis** chlorogenic acid, hypericin, hyperoside, isoquercitrin, pseudohypericin, rutin	(116)

Table 2.9. HPTLC studies on *Hypericum* species (continued)

Extract	Stationary phase	Developing solvent system	Derivatization	Detection	Compound	Reference
Methanolic extract from Hyperici herba	HPTLC silica gel 60 F_{254}	toluene-dichloromethane (4:1, v/v)	Godin reagent	Visualizer: 366 nm	**Qualitative analysis** hyperforin	(116)
Methanolic extract from flowering aerial part of *H. perforatum*	HPTLC silica gel 60 F_{254}	**A:** ethyl acetate-glacial acetic acid-formic acid-water (100:11:11:27, v/v/v/v) **B:** toluene-ethyl formate-formic acid (5:4:1, v/v/v) **C:** ethyl acetate-dichloromethane-acetic acid-formic acid-water (100:25:10:10:11, v/v/v/v/v)	-	Scanner: λ = 590 nm Visualizer: 366 nm	**Qualitative analysis** hypericin (R_f = 0.50, 0.95, 0.48 respectively)	(117)
Hydroalcoholic extract from Hyperici herba	HPTLC silica gel 60 F_{254}	ethyl acetate-hexane (1:9, v/v)	Acetic acid-sulfuric acid-anisaldehyde (100:2:1, v/v/v)	Visualizer: 366 nm	**Qualitative analysis** hyperforin	(52)
Ethanolic extract from Hyperici herba	Kieselgel 60 F_{254} aluminum plates	ethyl acetate-water-methanol (100:16.5:13.5, v/v/v) chloroform-water-methanol (61:7:32, v/v/v) chloroform-water-methanol (7:0.1:3, v/v/v) chloroform-methanol (8:2, v/v) chloroform-methanol (9:1, v/v)	5% sulfuric acid, 10% pyridine in ethanol	Visualizer: 254 nm, 366 nm	**Qualitative analysis** rutin, quercitrin, hyperoside	(118)
Methanolic extract from commercial extracts of *H. perforatum* L.	Silica gel 60 F_{254}	petroleum ether-ethyl acetate (9:1, v/v)	10% methanolic-sulfuric acid reagent	Scanner: λ = 290 nm	**Qualitative and quantitative analysis** hyperforin (2.06% & 27.04% in two *H. perforatum* commercial extracts)	(119)

Table 2.9. HPTLC studies on *Hypericum* species (continued)

Extract	Stationary phase	Developing solvent system	Derivatization	Detection	Compound	Reference
Hydroalcoholic extract from flowering aerial part of *H. perforatum*	HPTLC G60 F_{254} silica gel plates	**A:** ethyl acetate-glacial acetic acid-formic acid-water (10:1.1:1.1:2.7, v/v/v/v) **B:** ethyl acetate-glacial acetic acid-formic acid-ethyl methyl ketone-water (5:0.3:0.7:3:1, v/v/v/v/v) **C:** ethyl acetate-methanol-water-formic acid (25:1:1.5:3, v/v/v/v)	Natural product and Polyethylene glycol 400 reagent	Visualizer: 254 nm, 366 nm	**Qualitative analysis** **A:** flavonoids & polyphenols (rutin, chlorogenic acid, hyperoside, caffeic acid) **B:** rutin **C:** hyperoside	(120)
H. perforatum extracts and commercial extract	HPTLC 60 silica gel glass plates	formic acid-ethyl acetate-water (2:17:1, v/v/v)	Natural product and Macrogol reagent	Scanner: λ = 350 nm	**Qualitative analysis** rutin, quercetin, isoquercetin, luteolin, apigenin, hypericin, chlorogenic acid	(121)
Methanolic extract from flowering plant of *H. perforatum*	HPTLC silica gel 60 F_{254}	formic acid-ethyl acetate-water (2:17:1, v/v/v)	Natural product and Macrogol reagent	Visualizer: 254 nm, 366 nm	**Qualitative analysis** hypericin, hyperoside, rutin, quercetin, isoquercetin, chlorogenic acid, luteolin, apigenin	(51)
Marketed product of hydroalcoholic extract of *H. perforatum* and hydroalcoholic extract from flowering aerial part of *H. perforatum*	HPTLC silica gel 60 F_{254}	ethyl acetate-dichloromethane-formic acid-acetic acid-water (20:5:2:2:2.2, v/v/v/v/v)	Natural product and Macrogol reagent	Visualizer: 254 nm, 366 nm, white light	**Qualitative analysis** marketed product compared with raw materials	(122)

Table 2.9. HPTLC studies on *Hypericum* species (continued)

Extract	Stationary phase	Developing solvent system	Derivatization	Detection	Compound	Reference
Methanolic extract from flowering aerial part of *H. perforatum*	HPTLC silica gel 60 F_{254}	**A:** formic acid-ethyl acetate-water (2:17:1, v/v/v) **B:** toluene-dichloromethane (4:1, v/v)	**A:** Natural product reagent **B:** Godin reagent	Visualizer: 254 nm, 366 nm	**Qualitative analysis** **A:** hypericin, hyperoside, rutin, quercitrin, isoquercetin, chlorogenic acid, luteolin, apigenin **B:** hyperforin	(123)
Hydroalcoholic extract from flowering aerial part of *H. perforatum* L.	HPTLC silica gel 60 F_{254}	**A:** ethyl acetate-dichloromethane-formic acid-acetic acid-water (20:5:2:2:2.2, v/v/v/v/v) **B:** toluene-dichloromethane (4:2, v/v)	**A:** Natural product reagent **B:** Godin reagent	Visualizer: 254 nm, 366 nm, white light	**Qualitative analysis** **A:** hypericin, quercetin, quercitrin, rutin, biapigenin, chlorogenic acid **B:** hyperforin	(124)
Methanolic extract from flowering aerial part of *H. perforatum* and marketed product	Silica gel 60 glass plates	**A:** ethyl acetate-dichloromethane-formic acid-acetic acid-water (20:5:2:2:2.2, v/v/v/v/v) **B:** toluene-dichloromethane (4:1, v/v)	**A:** Anisaldehyde reagent **B:** Godin reagent	Visualizer: 366 nm	**Qualitative analysis** **A:** hypericin, pseudohypericin, quercetin, isoquercitrin, rutin, chlorogenic acid **B:** hyperforin	(125)
Commercial extract of *H. perforatum*	HPTLC silica gel 60 F_{254}	ethyl acetate-water-formic acid (90:9:6, v/v/v)	Natural product and Polyethylene glycol 400 reagent	Visualizer: 366 nm	**Qualitative analysis** hyperoside, rutin, chlorogenic acid, hypericin, pseudohypericin	(111)
Hydroalcoholic extract from aerial part of *H. perforatum* L.	Silica gel TLC plates Si60 F_{254}	**A:** toluene-diethyl ether-acetic acid (6:4:1, v/v/v) **B:** ethyl acetate-formic acid-acetic acid-water (100:11:11:26, v/v/v/v) **C:** chloroform-ethyl acetate-acetone-formic acid (4:3:2:1, v/v/v/v)	Natural product and Polyethylene glycol 400 reagents	Visualizer: 366 nm	**Qualitative analysis** **A:** flavonoid aglycone: quercetin **B:** flavonoid glycosides: quercetin-3-*O*-galactoside (hyperoside), quercetin-3-*O*-rutinoside (rutin) **C:** phenolic acid: chlorogenic acid	(109)

2.2.3. Activity Studies

2.2.3.1. Antidepressant activity

In a double-blind, placebo-controlled and cross-over study, the effect of commercial *H. perforatum* (two doses: 0.9 mg and 1.8 mg) was evaluated on the sleep polysomnogram of healthy participants. The REM latency (rapid eye movement) was significantly increased by both doses of *H. perforatum*. No effect on REM sleep duration or any other effect on sleep architecture was produced by *H. perforatum*. The results evidenced that *H. perforatum* to possess antidepressant efficacy in a way possibly similar to that of conventional antidepressant drugs (126).

In a randomized, controlled and double-blind study, a *H. perforatum* preparation [containing 150 mg of *H. perforatum* herba extract (0.450-0.495 mg of total hypericin)] was compared to fluoxetine hydrochloride in patients with mild to moderate depression. One tablet of a commercial *H. perforatum* extract (150 mg) or fluoxetine hydrochloride (selective serotonin reuptake inhibitor) (20 mg) was taken two times in a day during 6 weeks. For the Hamilton Rating Scale for depression (HAMD), significant reductions were observed by 50% of *H. perforatum* group and 58% of the fluoxetine group. For von Zerssen Depression Scale (DS), the groups treated with the *H. perforatum* and fluoxetine exhibited highly significant reductions by 42% and 52%, respectively. 83% of the effectiveness of fluoxetine on HAMD was achieved by *H. perforatum* preparation while 78% of the effectiveness of fluoxetine on the DS was achieved by *H. perforatum* preparation. Besides, *H. perforatum* preparation did not demonstrate any relevant changes in clinical or laboratory values as well as it did not cause any serious undesirable effects. Accordingly, *H. perforatum* was recommended as a well-tolerated therapeutic alternative to synthetic antidepressants (127).

In an experiment, commercial *H. perforatum* hydroalcoholic extract was examined to treat the comorbid conditions of mood disturbances and anxiety in type 2 diabetic rats induced by streptozotocin (i.p.) and then nicotinamide (i.p.). The *H. perforatum* extract containing hyperforin (3.00 %) and hypericins was administrated orally for 14 days. Anxiolytic effect was determined using elevated plus maze (EPM) and open-field exploration tests (OFT), while antidepressant effect was assessed using

Porsolt's forced swim test (FST). According to EPM and OFT results, significant increase in anxiety was observed in diabetic rats comparing to non-diabetic rats. Regarding to FST findings, immobility period increased in vehicle treated diabetic rats, while the period decreased dose-dependently in diabetic rats treated with *H. perforatum* extract. Moreover, the level of fasting blood glucose in diabetic rats was significantly reduced by treatment with *H. perforatum* extract. Therefore, it was suggested that *H. perforatum* extract could be a therapeutic candidate to prevent and treat comorbid disorders related to diabetes, anxiety and depression (128).

In an *in vivo* study, antidepressant efficacy of a commercial *H. perforatum* extract [standardized on 0.3% hypericin] in animal models [rats, mice] of depression was compared with clinically used standard antidepressants. In force swim test, immobility time in rats was dose-dependently reduced with maximal effect by *H. perforatum* extract that was almost similar to fluoxetine as well as greater than venlafaxine. Weak antidepressant activity was presented by venlafaxine. Besides, yohimbine induced lethality was potentiated by the following order: venlafaxine > dothiepin > fluoxetine > *H. perforatum* extract. Additionally, pnetylenetetrazole induced toxicity was enhanced by the following order: venlafaxine > dothiepin > fluoxetine > *H. perforatum* extract. In the locomotor activity test, the general locomotor behavior was decreased by *H. perforatum* extract and standard antidepressants. Based on the findings, *H. perforatum* showed antidepressant activity, resembling to standard antidepressants (129).

Peron et al. assessed the potential cytotoxicity, mutagenicity and antimutagenicity of *H. perforatum* extract prepared from 300 mg of SJW capsules (dried leaves and ground) using meristematic cells obtained from the bulbs of *Allium cepa* and bone marrow cells obtained from Wistar rats. The antimutagenic activity of *H. perforatum* extract was compared to that of a mutagenic chemotherapeutic drug, cyclophosamide. In the vegetal and animal systems, no cytotoxic and mutagenic effects based on time, concentration and type of treatments were observed by *H. perforatum* extract. On the other hand, antimutagenic action was detected by *H. perforatum* extract against cyclophosphamide in pre-treatment, simultaneous treatment and post-treatment. The outcomes indicated that *H. perforatum* provided a protective efficacy on DNA within bone marrow cells of rats cured with *H. perforatum* extract. Therefore, *H.*

perforatum could be used as an antidepressant as well as the administration of *H. perforatum* might be protective for human continuously treated with cyclophosphamide (130).

A meta-analysis of 27 clinical trials (involving 3808 participants) was performed for comparing the clinical efficacy of *H. perforatum* with SSRIs (selective serotonin reuptake inhibitors) in mild-to-moderate depressions. In patients with depression, the comparable therapeutic benefit and remission rates were determined by *H. perforatum*. In addition, lower discontinuation / dropout complaints were observed comparing to standard SSRIs treatment. Additionally, the remarkable clinical effectiveness for the improvement of depressive symptoms was confirmed by the pooled standardized mean difference (SMD) from baseline Hamilton Rating Scale for depression scores. *H. perforatum* was concluded to possess comparable antidepressant effectiveness and safety to SSRIs treatment (131).

2.2.3.2. Antimicrobial activity

The crude extracts of Hyperici herba were found to be more effective on gram-positive bacteria than gram-negative bacteria. The aqueous extracts were found to be less active than the alcoholic extracts. The isolated constituents from *H. perforatum* extracts, containing essential oils, phloroglucinols, flavonoids and tannins, showed the antimicrobial, antibacterial and antifungal activities. Hyperforin and hypericin were the major active compounds for antimicrobial effect (46).

The antimicrobial effects of the methanol, petroleum ether, chloroform and ethyl acetate extracts prepared from the aerial parts of *H. perforatum* were examined against gram-positive bacteria (*Bacillus subtilis*, *B. cereus*, *Enterococcus faecalis*, *Staphylococcus aureus*), gram-negative bacteria (*Acinetobacter calcoaceticus*, *A. baumanii*, *Pseudomonas aeruginosa*) and yeasts (*Candida albicans*, *C. tropicalis*, *Saccharomyces cerevisiae*, *Cryptococcus laurentii*, *Aspergillus niger*). All extracts exerted antimicrobial activity only against gram-positive bacteria, while the most sensitive bacteria were *B. cereus* and *B. subtilis*. The chloroform and ethyl acetate extracts showed the highest activity against these bacteria. In addition, other gram-positive bacteria were also inhibited by the ethyl acetate extract which was composed of

flavonoids (1.75%; biapigenin, quercetin, quercitrin, isoquercitrin, hyperoside and rutin), hyperforins (0.77%; hyperforin and adhyperforin) and hypericins (0.13%; hypericin and pseudohypericin). The activities of pure constituents were further tested. The growth of the tested microorganisms was significantly inhibited by hypericin, hyperforin and its dicyclohexylammonium salt, while no activity was observed by the flavonoid mixture (rutin 23%, isoquercitrin 22%, quercetin 18% and hyperoside) (132).

Milosevic et al. studied the antibacterial activity of an ethanolic extract from the *H. perforatum* flowers towards gram-negative bacteria, including *Agrobacterium tumefaciens*, *Azotobacter chrococcum*, *Enterobacter cloacae*, *Erwinia carotovora*, *Klebsiella pneumoniae*, *Pseudomonas fluorescens*, *P. glycinea* and *P. phaseolicola* as well as gram-positive bacteria, *Bacillus mycoides* and *B. subtilis*. The highest antibacterial activity was observed against *P. glycinea*, while the least effect on *K. pneumoniae*. The ethanolic extract also reduced the number of spores of *Fusarium oxysporum* (62%) and *Penicillium canescens* (72%) (133).

Süntar et al. explored the antimicrobial properties of ethanol extract prepared from Hyperici herba and its sub-extracts, including *n*-hexane, chloroform, ethyl acetate, *n*-butanol and the remaining water, against *Enterococcus faecalis*, *Lactobacillus plantarum*, *Streptococcus mutans* and *S. sobrinus*. The water sub-extract showed strong antibacterial effect against *L. plantarum* and *S. sobrinus*, whereas moderate effect against *E. faecalis* and *S. mutans*. Among the other sub-extracts, all sub-extracts showed antimicrobial effect against *S. sobrinus*, the *n*-butanol and ethyl acetate sub-extracts against *L. plantarum*, the ethyl acetate and water sub-extracts against *E. faecalis*. Moreover, a similar activity pattern was observed against the biofilm formation by all sub-extracts. The water sub-extract had the strongest antibacterial activity against all oral pathogens and therefore concluded that *H. perforatum* could be used as a natural antibacterial agent in oral care products (134).

Antimicrobial and antiprotozoal potential of twenty-one Turkish St. John's wort (SJW) oil macerates, including traditionally-prepared and commercial oil samples, were comparatively investigated. For antifungal activity *Candida albicans*, *Trichophyton rubrum* and *Aspergillus fumigatus*, for antibacterial activity *Staphylococcus aureus* and *Escherichia coli*, and for antiprotozoal activity *Leishmania infantum*, *Plasmodium*

falciparum, *Trypanosoma rhodesiense* and *T. cruzi* were used as test microorganisms. A mild antitrypanosomal activity against *T. rhodesiense* was exhibited by ten oil macerate samples [four home-made types obtained from Konya, Adana, and Manisa; as well as six ready-made types, namely Bosphorus Flora from Aydın, Kardelen, Karden, Talya and Sepe from Konya, Kirinti from Aksaray]. On the other hand, the antimicrobial effect against *S. aureus* was observed by only one commercial oil sample [Bosphorus Flora from Aydın]. The remaining oil materials exerted no activity against the tested fungi, bacteria and protozoa. No cytotoxic potential on MRC-5 fibroblasts was observed by all oil macerates. Besides, all macerates were examined for their naphthodianthrones (hypericin and pseudohypericin), chlorogenic acid, phloroglucinols (hyperforin and adhyperforin) as well as flavonoids (quercetin and biapigenin) via LC-DAD-MS. Hypericin and pseudohypericin were determined in all samples, whereas chlorogenic acid was detected in only one sample [Mecitefendi SJW oil from Konya]. In addition, hyperforin was determined in three home-made SJW oil samples [from Adana and Aydın] and one ready-made SJW oil sample [Defne/Doga from Adana]. Adhyperforin was found in five traditionally-prepared oil samples [Adana and Aydın] and a commercial oil sample [Defne/Doga from Adana]. Furthermore, quercetin was determined in four home-made SJW oil materials [Konya, Adana, Manisa and Aydın] and two ready-made SJW oil samples [Mecitefendi from Konya and Ege Lokman from Hatay]. Biapigenin was determined in five traditionally-prepared SJW oil macerates [Konya, Adana, Manisa and Aydın] and two commercial SJW oil macerates [Mecitefendi from Konya and Nurs Lokman Hekim from Adana] (135).

2.2.3.3. Antinociceptive activity

In an *in vivo* study, antinociceptive activity of a commercial *H. perforatum* extract was investigated in rats using hot plate and tail electric stimulation, capsaicin-induced hind paw licking as well as acetic acid-induced writhing tests. The effects of commercial *Hypericum* extract was compared with that of fluoxetine (selective serotonin reuptake inhibitor) or etodolac (COX-2 inhibitor). *H. perforatum* extract showed significant antinociceptive effect in the hot plate and tail electric stimulation tests on oral administration, while no effect was observed in nociception caused by capsaicin and the acetic acid-induced writhing. Then it was concluded that *H. perforatum* might be useful to manage inflammatory painful conditions (136).

Hatanaka et al. developed a new formulation of *H. perforatum* extract for improving its pharmacokinetics as well as antinociceptive activity. Four novel formulations of *H. perforatum* extract [nano-emulsion, dry-emulsion, solid dispersion and cyclodextrin inclusion] were prepared and tested. Nano-sized particles, a high negative charge and the highest physicochemical stability in water were showed by the nano-emulsion preparation among the analyzed formulations. According to the results obtained from the formalin test to evaluate the pain response in mice, the nociceptive response was remarkably reduced by the single oral administration of nano-emulsion formulation of *H. perforatum* extract because of the active component, hyperforin. Therefore, the nano-emulsion solubilization technique might be an effective option to improve the oral bioavailability and to increase antinociceptive response of *H. perforatum* extract in the treatment of chronic pain (137).

In an animal study, the effect of commercial sample of *H. perforatum* dried extract for the relief of meningeal nociception on mice induced by nitric oxide donor glyceryl trinitrate and sodium nitroprusside was investigated using cold plate, hot plate and hole-board tests. Pain hypersensitivity and meningeal activation were counteracted by a single oral administration of *H. perforatum* dried extract and hypericin. Therefore, it was suggested that *H. perforatum* might be a significant and safe alternative to treat migraine pain (138).

In an animal study, the efficacy of commercial sample of *H. perforatum* dried extract (containing total hypericins, 0.32%) for the relief of nociceptive hypersensitivity induced by nitric oxide donor nitroglycerin and sodium nitroprusside were searched using cold and hot plate test and Western blotting. Additionally, the identification of the effective compound was determined. The nitric oxide-induced nociceptive hypersensitivity was reversed by a single oral administration of *H. perforatum* extract because of the existence of hypericin. Hence, the results revealed that *H. perforatum* or hypericin might be used as a therapeutic option to treat pain due to efficiency and safety profile (139).

Galeotti et al. explored the effectiveness of commercial *H. perforatum* dried extract (containing total hypericins, 0.32%) for the determination of a safe and tolerable adjuvant to opioid analgesia to treat migraine. Mouse was induced by nitric oxide donor

sodium nitroprusside in meningeal nociception model. Cold plate test was employed for the presence of thermal allodynia; inspection activity (hole-board test), spontaneous mobility and locomotor activity (rotarod test) were evaluated for the presence of behavioral side effects. The opioid analgesia was importantly increased by co-injection of a single low dose of *H. perforatum* extract with morphine. While administrating *H. perforatum* extract alone, it did not counteract sodium nitroprusside-induced allodynia. Besides, a potentiating efficacy was produced by hypericin whereas that effect was not showed by flavonoids and hyperforin. According to the findings, *H. perforatum* extract exhibited the potentiating effect on morphine antinociception in an animal study of migraine. It was revealed that *H. perforatum* extract might be an adjuvant against opioid antagonists to produce analgesic efficacy using lower and safer doses of opioids. Hence, the combination of *H. perforatum* extract and morphine might be used as a therapeutic choice to treat migraine pain (140).

In an *in vivo* study, the chemical compounds and the antihyperalgesic effects of *H. perforatum* seed extract and feverfew flower and leaf extracts were examined in diabetic rats induced by streptozotocin. Mechanical hyperalgesia was reversed by acute administration of *H. perforatum* seed extract with a prolonged efficacy. Additionally, neuropathic pain was relieved by the extracts prepared from aerial parts of *H. perforatum* and flowers of feverfew, but the leaf extract of feverfew was inactive. Besides, the phytochemical compositions of the plants indicated that the responsible compounds for antihyperalgesic property were hyperforin and hypericin for *H. perforatum* and parthenolide for feverfew. Moreover, no behavioral side effect or sign of altered locomotor activity was observed in rats cured with *H. perforatum* and feverfew. Thus, it was suggested that *H. perforatum* and feverfew might be novel therapeutic options to treat painful diabetic peripheral neuropathy (141).

2.2.3.4. Anticancer activity

The properties of aqueous extracts prepared from *H. perforatum, H. androsaemum* and *H. undulatum* as well as some of their phenolic contents, namely chlorogenic acid, rutin and quercetin were investigated for the prevention and repairing of oxidative and alkylating DNA damage induced by hydrogen peroxide and methyl-methanesulfonate in human colon adenocarcinoma cells. The protective activity was

demonstrated against oxidative DNA damage induced by hydrogen peroxide in colon cells by *H. perforatum*, *H. androsaemum*, *H. undulatum*, rutin and quercetin whereas chlorogenic acid was inactive. Additionally, the protective activity was exhibited against alkylating DNA damage induced by methyl-methanesulfonate by *H. perforatum*, *H. undulatum*, rutin and quercetin. The base excision repair activity of alkylating DNA damage was increased by *H. perforatum*, *H. androsaemum* and chlorogenic acid. However, all tested extracts and all analyzed compounds did not show any effect on nucleotide excision repair pathway. According to the results, colon cells were protected from oxidative and alkylating DNA damage and alkylating DNA damage was increased as well as base excision repair activity was induced by *Hypericum* water extracts. Thus, *Hypericum* water extracts had antigenotoxic properties. In addition, the existence of high level of phenolic compounds was found to be protective and could be contributed for the protection of DNA. Therefore, it was suggested that herbal teas containing *H. perforatum*, *H. androsaemum* and *H. undulatum* might be consumed for the prevention of colon cancer (142).

Menichini et al. studied the antioxidant, anti-inflammatory and phototoxic properties of hydroalcoholic extract (70% ethanol) prepared from the flowering aerial parts of *H. perforatum*. Hydroalcoholic extract of *H. perforatum* presented better antioxidant effect in the β-carotene bleaching test compared to 2,2-diphenyl-1-pcrylhydrazyl (DPPH) assay. Furthermore, hydroalcoholic extract of Hyperici herba displayed a significant anti-proliferative effect on human tumour cell line (melanoma A375 cells) in concentration- and time-dependent manner. Besides, nitric oxide production in murine monocytic macrophage cell line RAW 264.7 was inhibited significantly in a dose-dependent manner by hydroalcoholic extract of *H. perforatum*. Based on the results, the proliferation of human malignant melanoma A375 cells was suppressed by the hydroalcoholic extract of *H. perforatum*. Additionally, high phototoxicity was enhanced by the hydroalcoholic extract with UVA irradiation. Therefore, the hydroalcoholic extract of *H. perforatum* might be used as an anti-cancer agent (143).

Kıyan et al. searched the components and the anti-angiogenic effects of essential oils obtained from *H. perforatum*, *H. hyssopifolium*, *H. confertum*, and *H.* hircinum, on the chicken embryo chorioallantoic membrane (CAM) from the fertilized hen eggs. The

anti-angiogenic activity was exerted by the CAM cured with the essential oil of *H. perforatum*. However, no effect was demonstrated by other *Hypericum* essential oils. Besides, membrane toxicity and irritation were not observed by *Hypericum* oils. In addition, the major constituent was found to be germacrene D in the essential oil of *H. confertum* and α-pinene in the essential oils of *H.* hircinum, *H. hyssopifolium* and *H. perforatum*. Regarding to findings, it was indicated that essential oil of *H. perforatum* displayed anti-angiogenic activity that contributed to wound healing, anti-inflammatory activities and anticancer activities (144).

2.2.3.5. Neuroprotective activity

Zerrouki et al. explored the protective, antioxidant, cytotoxic and anti-Alzheimer properties of ethanolic extract obtained from the aerial parts of *H. perforatum* in Alzheimer's model. A significant antioxidant effect was showed by *Hypericum* extract because of the rich hyperforin and quercitrin contents. The consequences showed that the efficacy of *H. perforatum* lead to a decrease in neurotoxicity and Alzheimer's disease appeared as shrunken reduced in pyramidal cells of mice brains. Hence, it was suggested that the oxidative stress was modulated by *H. perforatum* as well as it had protective effect on oxidative damage and neurodegenerative diseases in mice (145).

In a study, inhibitory effects of three extracts prepared from the aerial parts of *H. perforatum*, including ethyl acetate, methanol and water, were examined against acetylcholinesterase and butyrylcholinesterase associated with Alzheimer's disease as well as tyrosinase linked to Parkinson's disease. Total phenol and flavonoid constituents of extracts were detected. The highest inhibition towards acetylcholinesterase was presented by the methanol extract whereas the best butyrylcholinesterase inhibition was obtained from the ethyl acetate extract. In addition, the lowest tyrosinase inhibition was presented by only the methanol extract while a greater antioxidant activity was exerted by the methanol extract. Therefore, it was observed that the methanol extract had significant anticholinesterase and antioxidant activities related to the high proportion of total phenol constituents contained in aerial parts of *H. perforatum* (146).

2.2.3.6. Antigastritis and gastric ulcer activity

Yeşilada et al. investigated the anti-ulcerogenic folk remedies used in Turkey. *H. perforatum* was one of these Turkish plants. Anti-*Helicobacter pylori* activity of water extract as well as ethanol extract and its sub-extracts, including chloroform, *n*-butanol and aqueous fractions, were studied and chloroform and butanol fractions exerted the highest activity (147).

Tanideh et al. searched the healing activity of *H. perforatum* administrated as two dietary and two gel forms as well as their effects were compared with asacol on histopathological alterations and malondialdehyde level on ulcerative colitis induced by acetic acid in rat colon. Accordingly, experimentally induced colitis was relieved by both type of products and the authors concluded that *H. perforatum* might be a suitable drug for ulcerative colitis treatment (148).

Kurt et al. assessed the healing and protective properties of commercial *H. perforatum* oil on gastric mucosal damage induced by indomethacin in rats. The evaluation of gastric pH, analysis of gastric mucus and ulcer index revealed that gastric mucosal injury and lipid peroxidation were prevented by *H. perforatum* oil. Hence, *H. perforatum* oil suggested as a protective remedy against the gastric damage (149).

2.2.3.7. Anti-inflammatory activity

In an in vivo study, anti-inflammatory property of an acute administration of a commercial *H. perforatum* extract was examined in rats using carrageenan-induced paw edema test. The effectiveness of *H. perforatum* extract was compared with that of fluoxetine (selective serotonin-reuptake inhibitor) or etodolac (COX-2 inhibitor). When *H. perforatum* extract was administered systemically, the carrageenan-induced inflammatory edema was inhibited dose-dependently and significantly. Then, *H. perforatum* was suggested as an antiedematogenic herbal drug to relieve inflammatory pain (136).

Sosa et al. studied on topical anti-inflammatory effects of three extracts obtained from the flowering tops of *H. perforatum*, including a purified hydroalcoholic extract

(ethylacetic fraction), a lipophilic extract and a hydroalcoholic extract, as well as some pure constituents which were dicyclohexylamine, hyperforin dicyclohexylammonium (DHCA) salt, isoquercitrin, hyperoside, amentoflavone, adhyperforin and hypericin. Additionally, the ability of preparations and pure compounds were evaluated for the inhibition of croton-oil-induced ear oedema in mice. Three extracts reduced the oedema in a dose-dependent manner. The lipophilic extract containing hyperforin (27.02%) and adhyperforin (5.23%) had a significant anti-inflammatory activity. The activity of ethyl acetate fraction containing hyperforin (10.27%), hyperoside (9.29%), hypericin (0.06%) and pseudohypericin (0.21%) was lower than the lipophilic preparation. The hydroalcoholic extract containing hyperforin (4.50%), adhyperforin (0.38%), hyperoside (4.88%), hypericin (0.09%) and pseudohypericin (0.18%) was the least active. Besides, adhyperforin, hyperforin DHCA salt, hypericin and amentoflavone showed anti-inflammatory activity and they were more potent than indomethacin which was a non-steroidal anti-inflammatory drug as the reference. However, isoquercitrin and hyperoside exhibited a low activity, while dicyclohexylamine was found to be inactive. It was concluded that different components in the extract contributed to the topical antiphlogistic activity of *H. perforatum* (20).

In a study, the preventive effects of topical and oral formulations prepared from the flowering aerial parts of *H. perforatum* were searched on experimentally induced myringosclerosis after myringotomy in rats. It was presented that the inflammation was inhibited, fibrosis was reduced and the development of myringosclerosis was prevented by both applications of HP extract because of its antifibrotic and anti-inflammatory activities (150).

In another study, the effect of a combined formulation of *Hippophae rhamnoides* (sea buckthorn) and *H. perforatum* (St. John's wort) oils on a surgically induced endometriosis rat model was investigated. The efficacy of oily preparation was found to be close to that of reference drug buserelin acetate which is prescribed in endometriosis treatment due to its anti-inflammatory properties (151).

2.2.3.8. Antioxidant activity

Gioti et al. explored the bioactive constituents in organic (ethanol-water, 60:40, v/v) and water extracts prepared either from flowers, floral buds, flower-bearing branches, non-flower-bearing branches and shoots with leaves of *H. perforatum* and their antioxidant properties. The highest concentrations of hypericin and pseudohypericin were determined in the organic extract of flowers whereas the highest proportion of phenolic content was determined in the organic extract of shoots. On the other hand, the highest level of hyperforin was determined in the organic extract of floral buds throughout the development period followed by the flowers of *H. perforatum*. Besides, the important antioxidant efficacy was presented by branches and shoots due to the rich phenolic compounds (152).

Meinke et al. assessed the free radical scavenging property of a verum cream [containing 1.5 % of a *H. perforatum* extract with hyperforin (44.3%)] on skin cells irradiated with solar stimulated radiation (SSR). *H. perforatum* extract did not include hypericin. The formation of SSR-induced DCF (2′,7′-dichlorofluorescein) formation in human keratinocyte cells was reduced by hyperforin. When comparing to Trolox and N-acetylcysteine, hyperforin was more effective without presenting phototoxic effect. Furthermore, the radical protection factor (RPF) method was used as well as 0.5%, 1.5%, 3.0% and 5.0% of *H. perforatum* concentrations in the cream were used in order to detect the radical scavenging property. The RPF of the verum cream was increased by the increment in concentration of *H. perforatum* extract in cream. Moreover, when verum and vehicle creams were applied on porcine ear skin after infrared irradiation, the radical formation was reduced by 54% and 31%, respectively. Besides, in a randomized, double-blind and vehicle-controlled experiment, UVB-induced skin erythema on human skin was significantly reduced by the verum cream compared to the vehicle cream and untreated skin. Any skin irradiation was not observed by the application of the verum cream on the non-irradiated area. Accordingly, all results indicated that the cream containing hyperforin-rich HP extract showed an antioxidative activity and hyperforin could be a powerful free radical scavenger (153).

In a placebo-controlled and double-blind study, radical skin protection of topically treatment with a verum cream [containing *H. perforatum* extract (1.5%), rich

in hyperforin] during visible/near infra-red (VIS/NIR) irradiation and the effectiveness of the verum cream on the skin barrier function were evaluated. When a single application of basic cream without *H. perforatum* extract used as placebo and the verum cream was performed, the radical formation was almost completely inhibited by both placebo and verum creams as well as an immediate protection was presented by both creams. Moreover, while applying basic and verum creams for 4 weeks, the radical formation was reduced by 45% and 78%, respectively. A long-term protection was observed by the hyperforin-rich cream. Besides, when examining the effects of placebo and verum creams on skin lipid profile, the skin lipids, especially squalene, ceramide [AP] and [NP1] were directly increased by a single application of both basic and verum creams. After a treatment of 4 weeks, concentration of ceramides [AP, NP1 and NP2] and cholesterol were increased by both creams. With regard to the findings, a positive short- as well as long-terms influence was presented on the radical protection and the skin lipids by verum cream. VIS/NIR-induced radical formation was reduced by the regular application (twice daily) of the hyperforin-rich cream and skin lipids could be stabilized. Hence, the radical formation in the skin over 1 day could be decreased by topically applied antioxidants (154).

The dynamic antioxidant properties and the free radical scavenging activities of three ethanolic extracts obtained from the entire herbs of *H. perforatum*, the aerial parts of *Verbena officinalis* and the roots of *Valeriana officinalis* were examined. The total antioxidant effects of three ethanolic extracts and Nilestriol, a positive control agent in the experiment, were detected by the ferric reducing ability of plasma assay. The measurement of absorbance as a function of time of reactions of each extract with radicals, 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) and 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) was employed for the determination of the free radical scavenging ability. The highest concentration of total flavonoid was found to be in *H. perforatum* ethanolic extract, followed by *Verbena officinalis* ethanolic extract and *Valeriana officinalis* ethanolic extract. In addition, *Verbena officinalis* ethanolic extract contained a higher concentration of total phenolic acid content whereas *Valeriana officinalis* ethanolic extract included at least. Besides, a higher antioxidant effect was displayed by *H. perforatum* ethanolic extract, followed by *Verbena officinalis* ethanolic extract and *Valeriana officinalis* ethanolic extract. Furthermore, the scavenging abilities of three ethanolic extracts were time-dependent. A

significant free radical scavenging activity against $ABTS^{\cdot+}$ was presented by *H. perforatum* ethanolic extract. On the other hand, the most potent against $DPPH^{\cdot}$ was found to be *Verbena officinalis* ethanolic extract. With regard to the findings, *H. perforatum*, *Verbena officinalis* and *Valeriana officinalis* ethanolic extracts possessed a slow but stable level of dynamic $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ scavenging ability. The content of total flavonoid or phenolic acid might be an important role to the antioxidant effect and the free radical scavenging activity (155).

2.2.3.9. Anti-menopausal activity

In a double-blind, randomized and placebo-controlled study, effectiveness of an herbal combination consisting of *Cimicifuga racemosa* [black cohosh, 3.75 mg of Cimicifugae rhizome extract siccus, equivalent to 1.0 mg of triterpene glycosides] and *H. perforatum* [St. John's wort, 70 mg of Hyperici herb extract siccus, equivalent to 0.25 mg of total hypericin] extracts was investigated in women experiencing climacteric complaints with pronounced psychological symptoms. 2 tablets were administrated orally twice per day for the first 8 weeks and after, 1 tablet was taken orally twice per day for the second 8 weeks, respectively, in the morning and in the evening. After 8-week treatment phase, Menopause Rating Scale scores were declined by 34.8% in the treatment group compared with 21.7% in the placebo group. Furthermore, groups treated with herbal combination and placebo tablet exhibited a reduction of Hamilton Depression Rating Scale scores by 30.0% and 13.7%, respectively. After 16-week treatment period, Menopause Rating Scale scores were decreased by 50.0% in the treatment group and by 19.6% in the placebo group. Moreover, groups treated with the herbal combination and placebo tablet displayed a reduction of Hamilton Depression Rating Scale scores by 41.8% and 12.7%, respectively. Therefore, the treatment therapy was found to be superior to placebo with regard to both measures over a treatment period of 16 weeks. Besides, the herbal medication was well-tolerated (156).

In a pilot, double-blind and randomized clinical trial, effectiveness of *H. perforatum* extract prepared from St. John's wort tablet [comprising 300 mg dry *H. perforatum* extract standardized to 0.3% hypericin] was searched to treat signs and quality of life of symptomatic perimenopausal women. After 3-month cure with *H. perforatum* and placebo tablet, no significant differences were found in the daily hot

flash frequency and hot flash score (frequency x severity). Besides, after 3-month treatment phase, better menopause-specific quality life was reported significantly by women treated with *H. perforatum* compared to women in the placebo group. Additionally, sleep problems were significantly declined in the group cured with *H. perforatum* as opposed to the placebo group. Besides, adverse events were recorded during 3 months of treatment. Constipation was observed frequently in both groups. However, abnormal sweating, dry mouth and fatigue were detected more frequent in the placebo group than in the *H. perforatum* group. According to the findings, it was indicated that the administration of dry *H. perforatum* extract [900 mg daily] showed a beneficial efficiency. Therefore, it was suggested that menopause-specific quality of life in perimenopausal women might be improved by *H. perforatum* (157).

In a double-blind, randomized, placebo-controlled and parallel trial, efficacy of a herbal combination of *H. perforatum* tablet [containing 300 mg extract equal to 1800 mg dry herb flowering top standardized to 990 µg of hypericins, 9 mg of hyperforin and 18 mg of flavonoid glycosides] and *Vitex agnus-castus* tablet [containing extract equal to 50 mg of dry fruit] was evaluated for managing the premenstrual syndrome (PMS)-like symptoms in late-perimenopausal women. The phytotherapeutic combination of *H. perforatum* tablet (1 tablet in the morning and 2 later in the day) and *Vitex agnus-castus* tablet (2 in the morning) and placebo tablet were administered twice times in a day for 16 weeks. After 16-week treatment period, the herbal combination was found to be superior for total PMS-like symptoms, depression and cravings. Furthermore, important improvements were presented on the anxiety and hydration by the group treated with the herbal combination even though this influence was not found to be superior over placebo. Therefore, it was suggested that the phytotherapeutic combination of *H. perforatum* and *Vitex agnus-castus* possessed a potential for the clinical application to manage PMS-like symptoms among perimenopausal women (158).

In a double-blind, randomized, placebo-controlled and parallel trial, the efficacy of an herbal combination of *H. perforatum* tablet [containing 300 mg extract equal to 1800 mg dry herb flowering top standardized to 990 µg of hypericins, 9 mg of hyperforin and 18 mg of flavonoid glycosides] and *Vitex agnus-castus* tablet [containing extract equal to 50 mg of dry fruit] was examined on physiological and psychological signs of menopause in late perimenopausal and postmenopausal women. The

phytotherapeutic combination of *H. perforatum* tablet (1 tablet in the morning and 2 later in the day) and *Vitex agnus-castus* tablet (2 in the morning) and placebo tablet were administered twice times in a day. After 16 weeks of treatment, no significant differences were determined between two groups in the reduction of frequency and severity of daily hot flushes, menopausal symptoms and depression. Besides, important improvements across the treatment stage were found in both active treatment and placebo groups. However, no important variation was showed on quality of life by both two groups. Based on the results, the herbal combination of *H. perforatum* and *Vitex agnus-castus* possessed a significant effect to manage the menopausal symptoms even though it was not found to be superior to placebo. Additionally, the phytotherapeutic combination was well-tolerated without important adverse events reported in the short term (159).

A systematic review and meta-analysis included randomized and controlled trials that evaluated the activity and adverse events of *H. perforatum* (monotherapy) or its preparations (combination therapy), placebo and standard therapy for menopause treatment. *H. perforatum* extracts and its combinations with other botanical herbs were found to be significantly superior over placebo. *H. perforatum* extracts showed more effective activity compared with placebo in the treatment of menopause. Besides, adverse events of *H. perforatum* preparations and place group were occurred by 17.4% and 15.4%, respectively. Based on the results, *H. perforatum* presented more improvement and fewer side effects against placebo in the treatment of menopausal women. Therefore, *H. perforatum* monotherapy or combination therapy might relieve menopausal primary symptoms and hot flushes (160).

Kotsiou et al. assessed the effect of *H. perforatum* extract [LI 160, 80% methanolic dry extract prepared from flowers and buds of *H. perforatum* involving hypericin (0.17%) and hyperforin (4.3%)] on menopausal symptoms in ovariectomized rats. The body weight and abdominal fat was decreased by the administration of *H. perforatum* extract similar to estradiol administration. Additionally, femur bone mass density was improved by *H. perforatum* extract. Therefore, the findings indicated that *H. perforatum* extract might be helpful to treat the menopausal signs and prevent obesity (161).

You et al. explored the therapeutic effectiveness of ethanolic extracts prepared from the whole plant of *H. perforatum* as well as the flower and leaves of *H. perforatum* on obesity, lipid metabolism and uterine epithelial proliferation in an ovariectomized rat model. The body weight, adiposity, the serum cholesterol and insulin resistance were increased in untreated ovariectomized rats whereas body weight gains, adipose tissue weight and fasting insulin level were significantly decreased in groups treated with both two extracts of *H. perforatum* and β-estradiol-3-benzoate. In addition, total cholesterol was significantly reduced by the treatment of both two extracts of *H. perforatum*. In contrast, no effect was showed on total serum cholesterol by β-estradiol-3-benzoate treatment. Besides, uterus weight and uterine epithelial proliferation rate were lowered in untreated ovariectomized rats whereas the relative uterus weight and epithelial proliferation score were increased by β-estradiol-3-benzoate treatment. Conversely, there was no difference observed in uterus weight between the rats treated with both two extracts of *H. perforatum* and untreated ovariectomized rats. Additionally, two *H. perforatum* extracts showed the significant reduction in the proliferation rates and especially, the proliferation score of rats treated with flower and leaves extract of *H. perforatum* was not significantly different compared to that of untreated ovariectomized rats. Based on the results, *H. perforatum* extracts could be used as an estrogen agonist on body weight gain, body adiposity and insulin resistance as well as an effective remedy for the prevention of ovariectomized-induced obesity without affecting uterine tissue. Hence, it was suggested that *H. perforatum* might be employed for the attenuating of metabolic syndrome in postmenopausal women without stimulatory effects on uterus (162).

2.2.3.10. Wound healing activity

Öztürk et al. examined the wound healing activity of an extract prepared from the aerial parts of *H. perforatum* [including hypericin and pseudohypericin (0.10%)] against dexpanthenol and titrated extract of *Centella asiatica* on cultured chicken embryonic fibroblasts from fertilized eggs. Collagen production, morphologic changes, mitotic ability and staining cells were assessed microscopically. Regarding to the results, fibroblast collagen production was stimulated and fibroblast cells were activated by *H. perforatum* extract. Hence, *H. perforatum* extract possessed a wound healing efficacy (163).

Süntar et al. searched the active compounds and activities of the flowering aerial parts of *H. perforatum* olive oil extract, ethanol extract and its sub-extracts, including *n*-hexane, chloroform, ethyl acetate, *n*-butanol and water fractions. *In vivo* excision, linear and circular incision wound models were used to evaluate the wound healing effect while *in vivo* model based on the inhibition of acetic acid-induced increase in capillary permeability was applied to detect the anti-inflammatory effect. The study was also applied on ethanolic extracts prepared from the flowering aerial parts of *H. scabrum*. An important healing effect on excision and circular incision was presented by *H. perforatum* oily extract. In addition, the effective inhibition of wounds in excision and incision models was exhibited the ethyl acetate sub-extract compared to other sub-extracts while all sub-fractions obtained from the ethyl acetate sub-extract demonstrated healing effect that was less effective than the whole ethyl acetate sub-extract. Besides, ethanol extract and ethyl acetate sub-extract exhibited the anti-inflammatory effect in a dose-dependent manner. Relying on all results, active constituents found in *H. perforatum* were flavonoids, namely hyperoside, rutin, isoquercitrin and epicatechin, as well as naphthoquinones, namely hypericins. Additionally, the oily macerate prepared from the aerial parts of *H. perforatum* have effective wound healing and anti-inflammatory properties whereas *H. scabrum* ethanol extract did not present wound healing or anti-inflammatory activity (164).

In a study, the probable wound healing efficacy of a novel ointment formulation (HPP crème mit Rotöl ointment) containing olive oil extract prepared from the flowering aerial parts of *H. perforatum*, olive oil, essential oils of *Origanum majorana*, *O. minutiflorum*, and *Salvia triloba*, was assessed using *in vitro* and *in vivo* models on rats and mice. The effectiveness of new preparation was compared with that of Madecassol (reference ointment). Tissue sections were histopathologically assessed. In addition, the wound healing property of each component was determined. According to comparison with the reference ointment, the highest effects on both wound models were demonstrated by HPP crème mit Rotöl ointment and *H. perforatum* ointment whereas the effect of the new preparation was superior to that of *H. perforatum* ointment. Besides, *in vitro* elastase activity was not reduced by the new preparation while the collagenase activity was inhibited by the formulation. Additionally, bactericidal and candicidal effects were presented by the new formulation. Based on the results, remarkable wound healing efficacy was demonstrated by HPP crème mit Rotöl

ointment. It was suggested that the novel formulation was effective on acute and chronic wounds; the injury area was protected from infections as well as the inflammatory cells were inhibited (165).

In an experiment, a novel phytotherapy ointment containing a total extract obtained from the aerial parts of *H. perforatum* was formulated and its healing effects was tested on three *in vivo* experimental rat models, including linear incision, circular excision and thermal burn. The new preparation was topically administrated daily for 21 days. The clinical, macroscopical and histopathological results as well as wound concentration rate and period of epithelialization indicated that a significant wound healing activity in skin injuries was shown by the new *H. perforatum* ointment and it was found to be safe for usage (166).

In a study, the fatty acid composition and the possible mechanism of wound healing activity of oil macerates of *H. perforatum*, involving 10 traditionally and 13 commercially prepared SJW oil samples towards tumor necrosis factor-alpha (TNFα)-induced NF-κB activation were evaluated. Oleic acid was the predominant constituent in the traditionally prepared oil samples. On the other hand, the majority of the commercial samples were adulterated with some other oils. Some of the oil macerates showed moderate NF-κB inhibitory effect. According to the results, it was revealed that bioactive compounds were contained in the traditionally prepared olive oil macerates from *H. perforatum* and the potential mechanism for cutaneous wound healing effect of SJW oil macerates was found to inhibit the TNFα-induced NF-κB activation to reduce the inflammation in the early phases of acute skin inflammation (167).

Tanideh et al. searched the therapeutic activities of topical and systemic administration of *H. perforatum* extract on oral mucositis induced by 5-fluorouracil in hamsters. *H. perforatum* extract was administrated as topical gel and oral form. Histopathological changes in buccal lesions and tissue malondialdehyde level were examined. An important relief in oral mucositis was shown by all *H. perforatum* extract forms. The systemic form was superior over topical formulation. Regarding to the findings of weight, blood sample, tissue malondialdehyde assessments and histopathological observations, it was revealed that the inflammation was reduced and healing of oral mucositis induced by chemotherapy in the cheek pouch mucosa of

hamsters was accelerated by both of oral and topical administrations of *H. perforatum* extract (168).

Peksen et al. examined the curative effect of *H. perforatum* olive-oil extract compared to that of silver sulfadiazine cream (1%) on scald burn wound in rats. Tissue samples were histologically examined for 17 days. The healing efficacy of silver sulfadiazine plus *H. perforatum* oil extract was more significant than the effects of other treatments containing only silver sulfadiazine and only *H. perforatum* extract on days 10 and 17. Based on the findings, scald burn wounds in rats were positively affected by *H. perforatum* olive-oil extract. Additionally, amount of collagen and an important anti-inflammatory activity was provided in the wound area by the extract. A synergistic healing effect was presented on burn wound by *H. perforatum* olive-oil extract with silver sulfadiazine cream (169).

Kıyan et al. studied the acute effects of gel formulation containing *H. perforatum* methanol extract on experimental thermal second degree burn modality in rats and its effect was compared with other burn treatment, silver sulfadiazine. Weight, epidermal thickness, number of vessels, number of degenerated hair follicles and total number of hair follicles were examined as well as skin was histopathologically assessed. Based on the findings, within the first 24 hours, the wound healing efficacy on the experimental thermal (scalding) type burns was observed by the administration of *H. perforatum* four times per day and its activity was superior to that of silver sulfadiazine treatment (170).

In an experiment, the healing property of topical *H. perforatum* oil extract on tympanic membrane perforation in ears of rats was investigated histopathologically for 21 days. Based on the histomorphological evaluation, *H. perforatum* oil extract was effectively showed the wound healing activity on the tympanic membrane. Thus, it was suggested that *H. perforatum* might be a curative option for use in otology (171).

In a study, the effectiveness of *H. perforatum* oily extract on pressure sores (decubitus ulcer) was evaluated. The ointment was daily applied to patients in an intensive care unit to treat wounds. Healing status was macroscopically screened and tissue sections were histopathologically assessed. Regarding to the evaluations, an

important healing effect on pressure sore wounds was exerted by *H. perforatum* oily extract. Therefore, it was suggested that *H. perforatum* oily extract might be a cost-effective alternative to prevent or treat the pressure sores (172).

2.2.3.11. Other activities

Taher et al. investigated the efficacy of herbal extracts prepared from capsule forms of *H. perforatum* and *Ginkgo biloba* on the ultraviolet (UV)-induced activation of human immunodeficiency virus (HIV) gene expression in the HIVcat/HeLa cells. The UV-induced HIV gene expression was inhibited dose-dependently by *H. perforatum* extract, but not inhibited by *Ginkgo biloba* extract. In contrast to *Ginkgo biloba* extract, PMA- (phorbol-12-myristate 13-acetate) and UV-induced NF-κB activation in a HeLa cell clone was completely blocked by *H. perforatum* extract. On the other hand, *H. perforatum* extract stimulated JNK1, JNK2 (c-Jun-NH_2 terminal kinase 1&2) and p38 MAP (mitogen-activated protein) kinase, while did not activate p44/42 MAP kinase in HeLa cells. Additionally, UV-induced activation of HIV gene expression in HeLa cells was inhibited by hypericin. According to these findings, *H. perforatum* was suggested as a potent therapeutic approach for the inhibition of UV activation of HIV gene expression (173).

Mitsopoulou et al. conducted a study to explore the larvicidal activity of hyperforin isolated from *H. perforatum* and deoxycohumulone synthesized from commercial phloroglucinol towards *Culex pipiens* (Cullicidae). The potent larvicidal efficacy was displayed by hyperforin and deoxycohumulone against mosquito larvae (174).

Khan et al. assessed the therapeutic potential of hydro-ethanolic extract obtained from the aerial parts of *H. perforatum* to manage opium-induced withdrawal syndrome in an animal model of dependence. For the examination of naloxone-induced jumping behavior and body shakes of rats (wet-dog shakes), the number of stereotype jumps and wet-dog shakes was significantly declined in the group chronically cured with *H. perforatum* extract against the saline control group. Furthermore, naloxone-induced diarrhea was reduced by *H. perforatum* extract in the acutely cured group. Conversely, the group acutely treated with *H. perforatum* extract did not exhibit any important effect

on naloxone-induced withdrawal jumps and wet-dog shakes. Besides, naloxone-induced hypothermia was potentiated by *H. perforatum* extract, so rectal temperature was decreased by *H. perforatum* extract in the chronic treatment group, but was not effective in the acute treatment. On the other hand, any notable effect was not demonstrated on the squeal on touch, salivation, lying painting and ptosis behaviors by *H. perforatum* extract in the acute and chronic treatments. With regard to results, some physical symptoms of opium withdrawal syndrome were attenuated by *H. perforatum* extract. Hence, *H. perforatum* might be a natural medication for the management of the withdrawal signs of opium dependence (175).

Tian et al. examined the chemical content of an extract prepared from the aerial parts of *H. perforatum* and the effect of extract on metabolic syndrome. Naphthodianthrones, including hypericin and pseudohypericin were determined in the *H. perforatum* extract. After feeding with high-fat-diet [50% of fat, 36% of carbohydrate and 14% of protein in calorie] for 14 weeks, a significant insulin resistance and hypercholesterolemia were developed in the diet induced obese mice. The improvement of glucose and lipid metabolism was presented in the diet induced obese mice treated with the extract. The levels of triglyceride content in the skeletal muscles were reduced dose-dependently by 8.9% and 25.3% in the groups treated with low dose of *H. perforatum* extract [50 mg/kg/day] and high dose of *H. perforatum* extract [200 mg/kg/day], respectively. Besides, *in vitro,* catalytic activity of recombinant human protein tyrosine phosphatase 1B (PTP1B) was inhibited by *H. perforatum* extract. Furthermore, the reduction of mRNA levels of PTP1B in skeletal muscle was exhibited by 32.8% and 50.7% in the treatment of low dose and high dose of extracts, respectively. Additionally, the protein levels of PTP1B were diminished by 21.8% and 48.8% in low dose and high dose of extract groups, respectively. Moreover, the changes in the expressions of genes associated with fatty acid uptake and oxidation were observed in skeletal muscle by *H. perforatum* extract. According to the results, insulin resistance and lipid metabolism were improved in diet induced obese mice by *H. perforatum* extract. Therefore, it was suggested that *H. perforatum* extract might be used as a new PTP1B inhibitor and a novel drug to treat metabolic syndrome (176).

2.2.4. Traditional Usage

Table 2.10. Traditional usage of *H. perforatum* in Turkey

Locality	Local name	Part(s) used	Administration, Preparation, Dosage	Usage	Reference
Andırın, Darıovası (Kahramanmaraş)	Tentürotu	Flower, herb	Decoction, tea	Stomach ache	(177)
Şile in İstanbul	Kantaron, kantarod, tentürdiyot çiçeği	Flower Aerial part	Externally, oleat Internally, infusion	Burn, wound, cut Cold	(178)
Ulukışla, Aktoprak (Niğde)	Kantoron	Flowering herb	Kept inside in olive oil for 1-2 months and applied on wounds Decoction used as tea after condensed up to 1:3 of its volume	Wound healing Stomach ache, colitis, intestinal disorders	(179)
Halkapınar, Büyükdoğan (Konya)	Koramanotu	Herb	Decoction, as tea for 6-12 months	Hemorrhoids	(179)
Gönen (Balıkesir)	Sarı kantoron, yakı otu	Flower Flowering branch	Externally, oleat Externally, decoction Externally, decoction Internally, decoction Internally, decoction, 1x1, used cold, before breakfast Internally, decoction	Burns, wounds Inflamed wounds Stomach ache Appetizer Hemorrhoids Rheumatism	(180)
Kofçaz, Demirköy, Centre, Pınarhisar, Vize (Kırklareli)	Kantaron, kantaron çayı, sarı kantaron, kantaryon, sarıcayüz, mide otu, kesik otu, kantül, kalp otu	Aerial part	Decoction	Stomach ache, enteritis, urinary diseases, kidney stones , diabetes, cold, antifungal, eczema, antihypertensive, arteriosclerosis, cardiac diseases	(181)

Table 2.10. Traditional usage of *H. perforatum* in Turkey (continued)

Locality	Local name	Part(s) used	Administration, Preparation, Dosage	Usage	Reference
Sivrice (Elazığ)	Kantaron, binbirdelik otu	Flower, leaf	Decoction, drink one cup in the evening	Anthelmintic, demulcent, sedative	(182)
East Anatolia	Binbirdelik otu	Herb	Internally, decoction	Stomach pains, ulcer, antiseptic, antihermorrhoidal, vulnerary, sedative, kidney disorders	(183)
Maden (Elazığ)	Kantaron	Flower	Infusion, drink one cup in the evening	Sedative	(184)
Edremit (Balıkesir)	Kantaron, sarı kantaron	Aerial part	Orally, waiting in olive oil for 40 days, 2 times a day 2-3 spoonful before breakfast for 1 week	Stomach ache, enteritis	(185)
			Externally, waiting in olive oil for 40 days, applied once a day for 2-3 day	Abdominal pain, local pains	
			Externally, waiting in olive oil for 40 days , applied once a day until recovery	Wounds, burns	
			Orally, waiting in olive oil for 40 days, injected into breast once a day for 1-2 week (for animal)	Mastitis (for animal)	
			Externally, waiting in olive oil for 40 days	Antibacterial	
		Flowering branch	Orally, infusion, drink one teacup two times a day for a week	Stomach diseases, enteritis, ulcers	
Turgutlu (Manisa)	Kantaron, kantiran otu, yara otu	Flowering branch	Externally, oleat	Wound	(186)

Table 2.10. Traditional usage of *H. perforatum* in Turkey (continued)

Locality	Local name	Part(s) used	Administration, Preparation, Dosage	Usage	Reference
Solhan (Bingöl)	Waş zerık	Aerial part	Infusion, drink 1 tea glass twice a day	Abdominal ache, digestive, wound healing	(187)
Alaşehir (Manisa)	Sarıkantaron, kantoron, kantoron otu, kantoran, kantıran, gantıran, kangren otu, gangren otu, gatran, gatran otu, katran, katran otu	Aerial part, flowering branch	Infusion, medical oil	Burn wound care, gastrointestinal diseases, diaphoretic, antipyretic, cancer	(36)
Hatay	Dibbeysi, kantaron, binbirdelik otu	Aerial part	Orally, decoction Externally, crushed and stored in olive oil for 20 days, filtered, applied to wounds	Stomach pains, gastritis ulcer, appetizer Inflamed or suppurating wounds, antiseptic	(188)
Aladağlar (Niğde)	Sancı otu	Aerial part	Internally, infusion Externally, crushed and mixed with olive oil	Colic, diarrhea Hemorrhoids	(189)
Espiye (Giresun)	Dişice, kantaron	Whole plant	Decoction, mash, pomade; drink one tea glass 3 times a day, compress	Ulcer, burns, wound healing	(190)
Aydıncık (Mersin)	Katran otu, kantaron otu	Aerial part	Infusion, decoction, lotion, medicinal oil	Diabetes, ulcer, reflux, pyrosis, burn and wound treatment	(191)

Table 2.10. Traditional usage of *H. perforatum* in Turkey (continued)

Locality	Local name	Part(s) used	Administration, Preparation, Dosage	Usage	Reference
Sarıgöl (Manisa)	Kantoron, kantoran, kantıran, gantıran, kangren otu, gangren otu, gatran, gatran otu, katran, katran otu	Flowering branch, aerial part	Mash, infusion, medicinal oil, gargle	Burn, boil and wound care, cancer, ulcers, rheumatism, cold and flu, antipyretic and diaphoretic, mouth sores	(192)
Marmara Island (Balıkesir)	Kantaron	Flowering branch	Internally, infusion	Stomach ailments	(193)

Table 2.11. Traditional usage of *H. perforatum* in other countries

Locality	Local name	Part(s) used	Administration, Preparation, Dosage	Usage	Reference
Samarqand, Urgut, Charchinar (Uzbekistan)	Chayeq	Flowering herb	Tea	Headache	(194)
Djizzax, Bexmel, Vadigan (Uzbekistan)	Sarichayoti	Herb	Decoction, as tea	Jaundice	(194)
Cyprus	St. John's wort, soummakin, t'agiannitou	Flowering herb, flower	Externally	Cuts, catarrh and common cold	(195)
Granada (Spain)	-	Flowering plant	Orally and topically, infusion, medicinal oil	Burns	(196)
		Flowering plant	Topically, medicinal oil	Skin problems, insect bites	
Kopaonik Mountain (central Serbia)	Kantarion	Aerial part	Internally, tea	Moderate depression, insomnia, gastrointestinal ailment (stomach ulcer, liver and bile ailments, jaundice)	(197)
			Externally, oil	Hemorrhoids	
			Externally, creams and infused oils (fresh or dried flowering tops put into olive oil and left in the sun for 30-40 days to obtain a red oil)	Burns, wounds, cuts, muscular pain, sciatica, neuralgia	
			Putting oil on the baby's abdomen and by dressing warmly	Newborn infant's gastric spasms	

Table 2.11. Traditional usage of *H. perforatum* in other countries (continued)

Locality	Local name	Part(s) used	Administration, Preparation, Dosage	Usage	Reference
Arribes de Duero (Spain)	Pericón, hipérico, corazoncillo, hierba de San Juan	Aerial part (flowered)	Externally, maceration or fried in olive oil ("aceite de pericón")	Injuries, burns, chaps	(198)
Bosnia and Herzegovina	Kantarion, gospina trava	Flower, aerial part	Decoction	Urinary tract inflammations, expectorant	(199)
Prokletije Mountains (Montenegro)	-	Aerial part	Internally Externally	Anxiety, depressive moods, gastritis Inflammation of the skin, blunt injuries, wounds, burns	(200)
Zlatibor (south-western Serbia)	Kantarion	Herb	Externally, infusion, oily extract	Dermatologic diseases, skin complaints, wounds, burns	(201)
Navarra (Spain)		Flowered aerial part	Internally (fresh, dry), macerate in oil	Earache, otitis	(202)
Sicily (Italy)	Uagliu di pricò, pricò, pericò	Flower	Oil	Burns, bruises, contusions, wounds	(203)
Navarra (Spain)	-	Flowered aerial part	Externally (fresh), olive oil macerate	Bruises	(204)
Suva planina mountain (south-eastern Serbia)	Kantarion	Aerial part	Internally, tea Externally, oil	Diseases of the internal organs, sedative, toothache, colds, wounds of internal origin, kidney sand Burns, skin complaints, wounds, hemorrhoids, varicose veins	(205)

Table 2.11. Traditional usage of *H. perforatum* in other countries (continued)

Locality	Local name	Part(s) used	Administration, Preparation, Dosage	Usage	Reference
South Kosovo	Kantarioni (among Albenians) Kantarion (among Bosniaks/Gorani)	Aerial part	Infusion	Anti-anemic, wound healing, anticoagulant, neuro-relaxant, antacid	(206)
Ripollès in eastern Pyrenees (northeast Iberian Peninsula)	Herba de cop, herba de Sant Joan, pericó, trescamp	Leaf Flowering aerial part	Embrocation Liniment, lotion, ointment, medicinal vinegar Embrocation Liniment	Vulnerary Antiecchymotic Antipyretic, vulnerary, antitoxic Antitoxic, for irritation	(207)

2.3. High Performance Thin-Layer Chromatography

Thin-layer chromatography (TLC) is a chromatographic technique that is used to conduct the analysis of herbal drugs and preparations. TLC is a simple and cheap technique due to the requirement of little instrumentation. Besides, qualitative detection via TLC requires short time to present the major compounds of the plants. Additionally, semi-quantitative TLC analysis of the characteristic components of the herbs or herbal preparations enables a quality evaluation of drugs. Besides, chromatographic fingerprints of plants are provided by TLC, so the identification and purification are monitored as well as adulterations are determined (115).

In recent years, TLC has developed rapidly and the technique has instrumentalized and automated. Therefore, the effectiveness of method has increased. Consequently, high performance thin-layer chromatography (HPTLC) is an improved and modern adaptation of TLC which has been developed to conduct accurate quantification analysis. In addition, high resolution separations as well as accurate and precise quantitative results are received quickly by HPTLC (208).

The process of HPTLC analysis is controlled by means of computer and images of chromatograms are stored electronically. A large number of samples and references are analyzed simultaneously on a single plate. Calibration curves are composed from standards chromatographed under the same experimental situations (208). Therefore, HPTLC method may find application in a wider area for the chemical identification of raw materials and finished products, the quantitation of active components in herbal drugs, the quality evaluation of botanicals, the stability assessment of herbal drug preparations and marketed products (116).

HPTLC is a simple and effective technique to evaluate visible results and chromatograms. Additionally, HPTLC based method is a flexible system that provides many parameters for a better separation, so the results obtained from HPTLC analysis can be compared with those obtained from high pressure liquid chromatography (HPLC). Principle distinguishing and common specifications between HPTLC and HPLC are summarized in Table 2.12 (208-210).

Table 2.12. Comparative evaluation of HPTLC with HPLC

HPTLC	**HPLC**
Liquid chromatography	Liquid chromatography
Planar chromatography	Column chromatography
Open chromatography system	Closed chromatography system
Off-line analysis	On-line analysis
Semi-automated	Fully automated
Computer controlled system	Computer controlled system
Easy to learn and operate	Requirement of trained personnel
Flexible	Limited flexibility
Economical	Expensive
Low maintenance	High maintenance
Multiple sample process	Only one sample can be examined at a time.
Time-saving	Taking more time
Easy preparation of samples	Laborious and time-consuming preparation of samples
Solvents do not need any filtration and degassing.	Solvents have to be pre-treated.
Small amount of mobile phase	Large amount of mobile phase
Applicability of visual detection	Not applicable
Possibility of multiple scanning of several samples at a time	Not possible
Moderate to high resolution	Very high resolution
No pressure	High pressure
High to very high data obtained from chromatography	Limited to very high data obtained from chromatography
Chromatogram image documented at 254 nm, 366 nm and visible light	Not possible
Limited chromatographic fingerprint	Comprehensive chromatographic fingerprint

2.3.1. Theoretical Notions

2.3.1.1. General principles

2.3.1.1.1. Retardation factor

Each constituent is identified by its retardation factor R_f which relates to its migration distance compared with that of the solvent. R_f values vary from 0 to 1. The retention/retardation factor is expressed as the following equation (211).

$$R_f = \frac{Z_s}{Z_f}$$

R_f : Retardation factor (unitless)

Z_s : Distance run by analyte

Z_f : Distance run by solvent

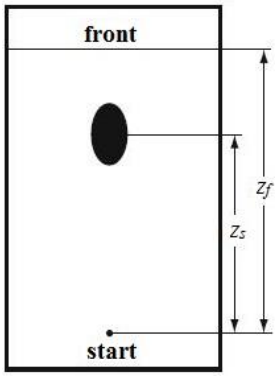


Figure 2.2. Parameters used in calculation of retardation factor

2.3.1.1.2. Partition coefficient

The important physico-chemical factor of chromatography is the equilibrium constant *K*, as named partition ratio or partition coefficient. The proportion of concentrations of each constituent within the two phases, namely stationary phase (c_s)

and mobile phase (c_m) is quantified. The partition ratio is described as follows (116, 208).

$$K = \frac{c_s}{c_m}$$

K : Partition coefficient (partition ratio)

c_s : Concentration of substance in stationary phase

c_m : Concentration of substance in mobile phase

2.3.1.1.3. Capacity factor

The ratio of the retention time of a compound in the stationary phase to that in mobile phase is described by capacity factor (k). Retention time of a constituent represents the time elapsed from the sample introduction to the detection of the peak maximum on the chromatogram. The capacity factor is related to R_f by the equation stated as the following equation (212).

$$\text{Capacity factor} = k = \frac{1}{R_f} - 1$$

2.3.1.1.4. Selectivity factor

Selectivity factor (α) enables a chromatographic technique to separate compounds that are retained to a different extent. The selectivity factor is defined as follows (213).

$$\alpha = \frac{k_B}{k_A}$$

α : Selectivity factor

k_b : Capacity factor of B

k_a : Capacity factor of A

2.3.1.1.5. Plate number and height

Plate number and height are terms that express the efficiency of a chromatographic method. Theoretical plate number and height equivalent of a theoretical plate are expressed as follows (211).

$$N = 16 \times \left(\frac{Z_s}{W_s}\right)$$

N : Theoretical plate number

Z_s : Migration length of analyte

W_s : Chromatographic spot width in the direction of mobile phase migration

$$H = \frac{Z_f}{N}$$

H : Height (HETP, height equivalent of a theoretical plate)

Z_f : Migration length of mobile phase

N : Theoretical plate number

2.3.1.1.6. Resolution factor

Resolution factor (R_s) is provided to evaluate the separation of two compounds based on the corresponding peaks in the chromatogram. The resolution factor is determined as the following equation (211).

$$R_s = \frac{R_{f(2)} - R_{f(1)}}{0.5 \times (W_1 + W_2)}$$

R_s : Resolution factor

$R_{f(1)}$ & $R_{f(2)}$: R_f values of chromatographic peak 1 and 2

W_1 & W_2 : Width of chromatographic peak 1 and 2

2.3.1.2. Stationary phases

Stationary phase is a solid sorbent layer immobilized mobile phase components. The factors, such as size of particles, surface area, volume of pores and distribution of particle diameters, describe the properties of stationary phase. Development distance and analysis time are decreased by using the HPTLC plates consisting of smaller particles with a narrow size distribution (214).

The beginning of TLC is linked to aluminum oxide as the stationary phase. Nowadays, the most TLC separations are commonly performed on the inorganic adsorbent layers (silica or silica gel and alumina). Additionally, there are also organic layers (polyamide, cellulose); organic, polar covalently bonded modifications of the silica gel matrix (normal phase (NP): diol, cyanopropyl, and aminopropyl); and organic, nonpolar bonded stationary phases (reversed phase: RP-2, RP-8, RP-18) (Table 2.13). Reversed phases become more hydrophobic with the increase in chain length as follows: C2 > C8 > C18. Furthermore, other stationary phase contains cellulose, kieselguhr, magnesium silicate and polyamide, but they are not used in TLC in common (208). The selection of the sorbents for HPTLC method is important to analyze the compounds in different classes (Table 2.14) (208, 214, 215).

Table 2.13. Commercial bonded phases

Name	**Functionality**	**Polarity**	**Group**
RP 2	Dimethyl	Nonpolar	RP
RP 8	Octyl	Nonpolar	RP
RP 18	Octadecyl	Nonpolar	RP
Dipehnyl	Diphenyl	Nonpolar	RP
Amino	3-aminopropyl	Polar	RP/NP
Cyano	3-cyanopropyl	Polar	RP/NP
Diol	Spacer bonded propanediol	Polar	NP

Table 2.14. Selection of sorbents for analysis of compounds

Sorbent material	Substance class
Silica gel	Compounds in all classes, such as alkaloids, aflatoxins, fatty acids, antibiotics, lipids, pesticides, steroids, vitamins
Aluminum oxide	Steroids, alkaloids, carbohydrates, flavonoids, terpenes, aliphatic and aromatic hydrocarbons, pesticides
Kieselguhr	Herbicides, tetracyclines, carbohydrates, aflatoxins
Cellulose	Carbohydrates, amino acids, food dyes, amines, catechols, flavonoids, antibiotics, peptides, polyaromatic hydrocarbons
Polyamide	Phenolic and polyphenolic constituents, flavonoids, sugars, antioxidants, pesticides
Amino bonded modified silica gel	Carboxylic acids, carbohydrates, vitamins, pesticides, phenols, steroids, sulfonic acids
Cyano bonded modified silica gel	Pesticides, preservatives, phenols, steroids, carotenoids, flavonoids, quinolones
Diol bonded modified silica gel	Hormones, steroids, carbohydrates, phenolic acids, phenols
Chiral modified silica gel	Dipeptides, amino acids, lactose
Reversed phase modified silica gel	Lipids, fatty acids, antioxidants, capsaicin, amides, amines, amino acids, peptides, phenols, pesticides, antibiotics, steroids
Silica gel, impregnated with boric acid	Lipids, ascorbic acid derivatives, sugars, glycerides
Silica gel, impregnated with boric acid or phosphate	Carbohydrates
Silica gel, impregnated with caffeine	Polyaromatic hydrocarbons
Silica gel, impregnated with silver nitrate	Lipids

The comparison between the specifications of HPTLC and TLC plates is discussed in Table 2.15 (208, 216-218).

Table 2.15. Differences between HPTLC and TLC

Specification	**HPTLC**	**TLC**
Sample spotting	Automated/instrumental	Manual
Sample holder	Syringe	Capillary/pipette
Mean particle size	5-6 μm	10-12 μm
Particle size distribution	4-8 μm	5-20 μm
Plate height	12 μm	30 μm
Layer thickness	100 μm	250 μm
Sample volume	0.1-0.5 μL	1-5 μL
Development chamber	Less amount of mobile phase	Higher amount
Migration distance	3-7 cm	8-15 cm
Separation time	3-20 min	20-200 min
Scanning	UV + visible + fluorescence	Not possible
Detection limits in absorption	100-500 pg	1-5 ng
Detection limits in fluorescence	5-10 pg	50-100 pg
Quantitative analysis	Possible	Not possible

2.3.1.3. Mobile phases

The stationary phase must not be chemically affected or dissolved by mobile phases applied in HPTLC system because this causes the change of properties of chromatographic method. Chemical transformations of separated constituents must not be produced by mobile phases. The mobile phase in HPTLC analysis must be prepared used only once because an alteration of quantitative composition of mobile phase is

produced by the volatility of solvents and the chromatographic repeatability is affected negatively. Solvents used in mobile phases must be eliminated from the adsorbent layer in an easy way. Additionally, the solvents must be compatible with stationary phase.

According to selectivity characterization, solvents are categorized into eight groups (208, 219). Additionally, the characterization solvent polarity is called as polarity index (P′). The values of polarity index and selectivity groups of solvents utilized in HPTLC are given in Table 2.16 (116, 208, 219, 220).

Table 2.16. Polarity indices and selectivity groups of solvents

Solvent	**Polarity index (P′)**	**Selectivity group**
Pentane	0.0	-
Hexane	0.1	-
Cyclohexane	0.2	-
Carbon tetrachloride	1.6	-
Isopropyl ether	2.4	Aliphatic ethers
Methyl t-butyl ether	2.5	
Diethyl ether	2.8	
2-propanol	3.9	Aliphatic alcohols
Butanol	3.9	
1-propanol	4.0	
Ethanol	4.3	
Methanol	5.1	
Tetrahydrofuran	4.0	Tetrahydrofuran
Acetic acid	6.0	Acetic acid, glycols
Dichloromethane	3.1	Dichloromethane
Ethyl acetate	4.4	Aliphatic ketones and esters, acetonitrile
Methylethyl ketone	4.7	
Acetone	5.1	
Acetonitrile	5.8	
Toluene	2.4	Aromatic hydrocarbons
Benzene	2.7	
Chloroform	4.1	Chloroform, water
Water	10.2	

2.3.2. Practical Techniques of HPTLC

2.3.2.1. Sample preparation

Appropriate sample preparation process is an important step for HPTLC analysis. Extraction is a critical part of the herbal drug analysis. For fingerprint analysis, the extractions should be possibly simple, i.e. using ultrasonic bath. During method development, several extraction types, such as soxhlet extraction and digestion, should be evaluated. Besides, another important part of suitable extraction is the selection of the extraction solvent. In addition, the extract is separated from the insoluble matter by sonication or filtration or centrifugation to complete the extraction part. Furthermore, the significant factor influencing on sample spot is solvent type of sample solution. When elution strength of the solvent used for the sample preparation should be as low as possible, the sample dimension is very small. Volatile solvents, i.e. acetone, diethyl ether evaporate partially even before application. Therefore, the sample solvent is diluted with an organic polar solvent, involving methanol, ethanol or acetonitrile, which help application to the layer. Hence, the sample solution penetrates into the sorbent of the layer effectively (116, 208).

2.3.2.2. Sample application

The sample is applied on chromatographic plate by hand-operation with disposal micropipette or calibrated capillary or by a spray-on technique with a syringe. Manuel application is a simple technique but it should be performed with carefulness because the adsorbent layer is damaged by tip of capillary or syringe needle while pressing strongly over the layer. However, spray-on application prevents the adsorbent layer from the damage. Additionally, the precision and accuracy of application are increased and better resolution is produced for quantitative analysis by spray-on technique. The sample is applied in the form of band. The variance of sample volume applied on plate depends on the necessities of analysis, including components present in the extract for separation, linearity range and the development method (220). Furthermore, the sample is atomized by a stream of an inert gas (i.e. nitrogen) around the tip of the syringe and a band is created in the plate (217). The application as bands possesses a better separation and high response to densitometer (216).

2.3.2.3. Chromatogram development

Chromatogram is developed using various types of chambers and techniques. The different forms of development chambers include flat-bottom chamber [including the traditional "tank" in a variability of shapes and dimensions (vapor unsaturated or vapor saturated)], twin-through chamber (having a bottom with two compartments), horizontal developing chamber (double sided development, saturated or unsaturated), automatic development chamber [(ADC) full environmental control and automation] forced flow development chamber [(FFDC), development under pressure], vario chamber (multipurpose device, having six channels, saturated or unsaturated using six different mobile phases), and automated multiple development [(AMD), used for gradient high resolution chromatography] (116, 208). Besides, there are several types of development modes used in HTPLC method, as presented in Figure 2.3 (214). The two major development modes are conventional development and two-dimensional development. In conventional (one-dimensional) chromatography, solvent is developed by ascending chromatography. The lower edge of plate is dipped into the developing solvent. The chamber is saturated with vapors of mobile phase for effective reproducibility. In two-dimensional (bidirectional) chromatography, a single sample is spotted at the corner of a plate and developed in the conventional manner. After development, the plate is dried and rotated by 90° for a second solvent migration (209, 214).

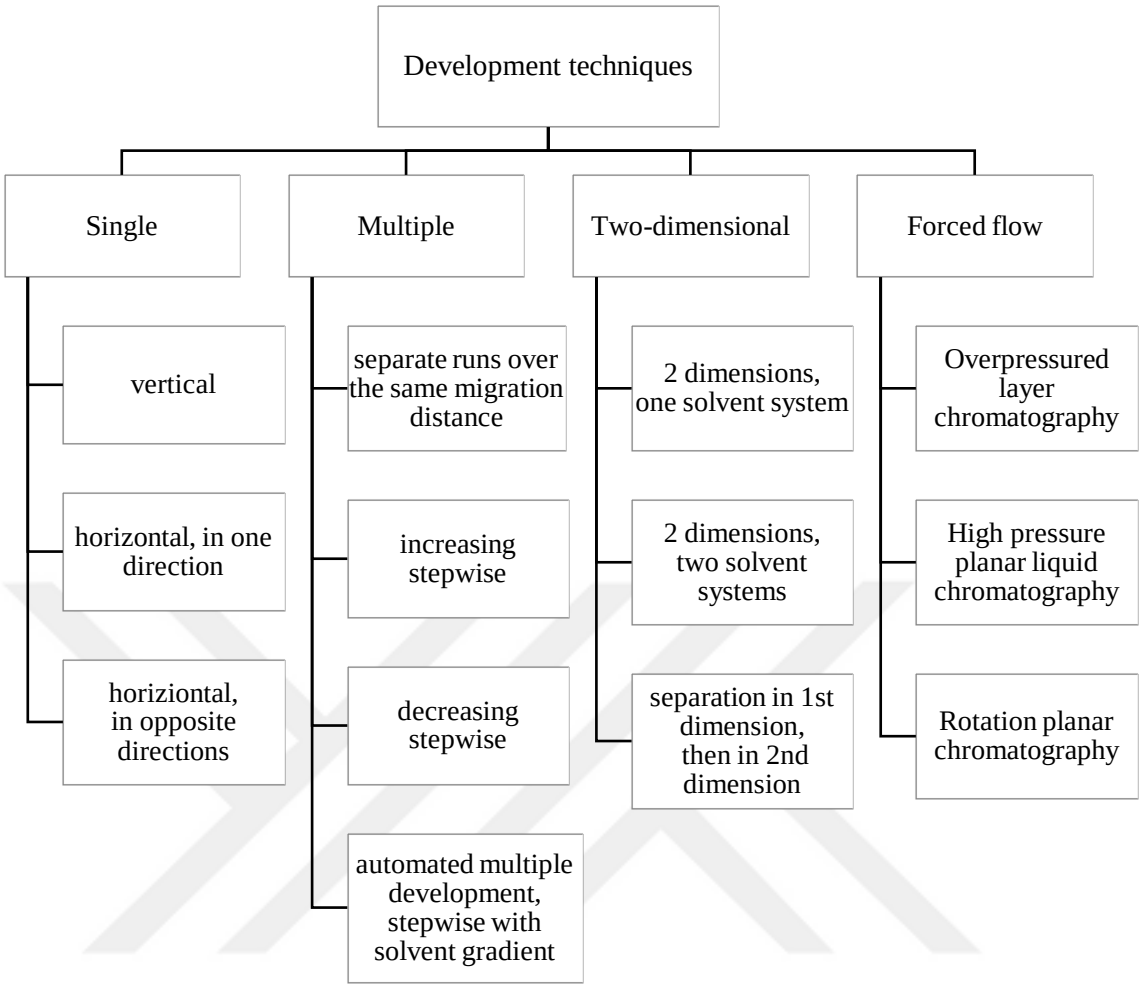


Figure 2.3. Techniques used for chromatogram development

2.3.2.4. Densitometry

Densitometry aids for the quantification of the compounds present in a sample on the HPTLC plate using scanning or video densitometry. A scanning densitometry provides reliable and accurate densitometric data for quantitative assessment and recording the absorbance spectra owing to multi-wavelength and fluorescence scanning as well as spectrum recording for identification (116, 208, 221). Optical instruments of the scanning densitometer are capable of absorbance, reflectance, or fluorescence measurements within a spectral range. The light sources used in the scanning densitometry are a deuterium lamp for absorption measurements in UV region (190-400 nm), a tungsten or tungsten-halogen lamp for visible region (400-800 nm), as well as a

mercury vapor lamp for providing high energy to measure by fluorescence (254-578 nm) (209, 214).

The scanning densitometer is more sensitive than a video densitometer because the digital imaging system is utilized for quantification with in limited parameters. Video densitometry is executed on electronic image of the chromatogram and represents a software-based evaluation. The pixels of image related to tracks are grouped and assessed on a gray scale by the video densitometer. However, the representation of the individual chromatogram is identified as a number of peaks quantified by the scanning densitometer. Video imaging is an easy and rapid technique to record the developed plate as a photographic image. A permanent visual record of the analysis is obtained (116, 208).

2.3.2.5. Visual detection

When the plate is dried after the development, compounds that have natural color are directly viewed in daylight (white light). Many substances are visualized using 254 nm-ultraviolet (UV) light (short-wavelength) with a fluorescence indicator embedded in the stationary phase. In addition, compounds that are naturally fluorescent are detected under 366 nm-UV light (long-wavelength). The image visualized at UV 254 nm is quenching of fluorescence of the indicator whereas visualization at UV 366 nm is related to emission of fluorescence (208, 214, 216).

2.3.2.6. Derivatization

Substances on the plate are derivatized by immersing the plate or spraying on the plate with an appropriate reagent to visualize chromatogram. Several derivatizing agents are used to detect the analyzed compound (Table 2.17) (116, 208, 210, 222). The derivatization is finalized by heating the plate. Time of spraying or dipping and heating, temperature, amount and concentration of the reagent solution affect the results of the derivatization (116).

Table 2.17. Derivatization reagents

Derivatization reagent	Detection of substances	Examination
Acetic anhydride	Ginkgolides	UV 254 nm, UV 366 nm
Acetic anhydride sulfuric acid (Liebermann-Burchard)	Sterols, terpenoids	White light
Acetic anhydride sulfuric acid copper	Universal reagent	
Aluminum chloride	Flavonoids, anthraquinones	UV 366 nm
Ammonia vapor	Opiates, mycotoxins, flavonoids, sennosides, valepotriates, anthracenes	White light, UV 366 nm
Aniline-diphenylamine-phosphoric acid	Sugars, glycosides	White light
Anisaldehyde-sulfuric acid	Terpenoids, saponins, sterols, iridoids, most lipophilic compounds	White light, UV 366 nm
Antimony-III-chloride (CarrPrice, $SbCl_3$)	Cardiac glycosides, saponins, sterols, flavonoids, double bonds in carotenoids	White light, UV 366 nm
Barton	Gingeroles	White light,
Benzidine	Aucubin	White light,
Chloramine-trichloroacetic acid	Cardiac glycosides	UV 366 nm
Copper (II) sulfate	Universal reagent	White light
2,6-dibromoquinone-4-chloroimide 2,6-dichloroquinone chloroimide (Gibb)	Arbutin, vitamin B_6, phenols, coumarins, thiols, thiones, antioxidants, capsaicin, barbiturates, amines (aliphatic and aromatic)	White light, UV 366 nm
2,4-dinitrophenylhydrazine (DNPH)	Ketones, aldehydes, alkaloids, silimarin	White light
DNPH-acetic acid-hydrochloric acid	Ketones, aldehydes	White light
Dragendorff Dragendorff followed by sodium nitrite Dragendorff followed by sulfuric acid	Alkaloids, heterocyclic nitrogen compounds	White light
4-dimethylamino benzaldehyde (EP)	Terpenes, sesquiterpene esters, proazulene	White light
4-dimethylamino benzaldehyde (Ehrlich)	Iridoids, proazulenes	White light
10% ethanolic KOH	Anthraquinones (emodin, rhein), anthrones (aloin, cascarosides), coumarins, scopoletin, umbelliferone	White light, UV 366 nm
Fast blue salt B	Phenolic compounds, tannins, cannabinoids	White light, UV 366 nm

Table 2.17. Derivatization reagents (continued)

Derivatization reagent	Detection of substances	Examination
Fast red salt	Amarogentin	UV 366 nm
Hydrochloric acid-acetic acid ($HCl+CH_3COOH$)	Valepotriates with diene structure	White light, UV 366 nm
Iodine (spraying solution), Iodine vapor	All lipophilic compounds, constituents containing conjugated double bonds, Lipids, carotenoids	White light, UV 366 nm
Iodine-chloroform	Ipecacuanha alkaloids	White light, UV 366 nm
Iodine-hydrochloric acid	Purine derivatives (caffeine, theophylline, theobromine)	White light, UV 254 nm
Iodine-starch	Conjugated double bonds, alkaloids, purine derivatives, lipids, carotenoids	White light
Iodoplatinate	Nitrogen-containing compounds (i.e. alkaloids)	White light
Iron-III-chloride ($FeCl_3$)	Phenols, ergot alkaloids, flavonoids, tannins, plant acids, enols, cholesteryl esters	White light
Kedde (3,5-dinitrobenzoic acid KOH)	Cardenolides	White light
Marquis	Morphine, codeine, thebaine	White light
Natural products/polyethylene glycol (NP/PEG) (NEU)	Flavonoids, carbohydrates, anthocyanines, plant acids	UV 366 nm
Nitric acid (HNO_3)	Ephedrine derivatives	UV 254 nm, UV 366 nm
Ninhydrin	Amino acids, peptides, biogenic amines, amino-sugars, ephedrine	White light
Palladium-II-chloride ($PdCl_2$)	*Allium* species	Visible
Phosphomolybdic acid [$H_3(P(Mo_3O_{10})_4$]	Fatty oils, phospholipids, reducing substances, steroids, essential oils compounds, morphine	White light, UV 366 nm
Potassium hydroxide (KOH, Bornträger)	Anthraquinones, anthrones, coumarins, flavonoids	White light , UV 366 nm
Sulfuric acid	General reagent	White light, UV 366 nm
Tin (II) chloride-hydrochloric acid	Aristolochic acids	UV 366 nm

Table 2.17. Derivatization reagents (continued)

Derivatization reagent	Detection of substances	Examination
Trichloroacetic acid-potassium hexacyanoferrate-iron-III-chloride	Sinalbin, sinigrin	Visible
Vanillin-glacial acetic acid	Terpenoids, sterols, ergot alkaloids, salicin, most lipophilic compounds	White light, UV 366 nm
Vanillin-phosphoric acid	Terpenoids, lignanes, cucurbitacins	White light , UV 366 nm
Vanillin-sulfuric acid	Essential oils (terpenoids, phenylpropanoids)	White light, UV 366 nm

2.3.2.7. Reporting and record keeping

Each HPTLC step should be reported in detail as well as the results are saved and stored in a proper and well-organized manner for ensuring the quality of the working instructions. Documenting is essential to compare different samples from different plates by exhibiting side by side and produce reproducible and reliable results. Record keeping is also useful to conduct the research and method development (116, 208).

2.3.3. Development and Validation of HPTLC System

There are several steps for method development and optimization of HPTLC system, as follows: analyzing the method in detailed, searching the studies and the available procedures reported in literature, conducting the initial works, selecting stationary phase, finding an appropriate mobile phase, development conditions, detection wavelength and derivatization reagent. After development and optimization of HPTLC system, method is validated because validation procedure confirms that an analytical method is accurate and repeatable to detect the analyte and produce the reliable results (116, 208).

The analytical process factors for the purpose of validation are designated in "Validation of Analytical Procedures Q2(R1)" provided by International Conference on Harmonization (ICH) (Table 2.18) (223).

Table 2.18. Parameters of analytical procedure for validation

Parameter \ Analysis		Qualitative (Identification)	Quantitative
Specificity		+	+
Limit of detection		-	-
Limit of quantitation		-	-
Linearity		-	+
Range		-	+
Precision	Repeatability	-	+
	Intermediate precision	-	+
Accuracy		-	+

2.3.3.1. Pre-validation parameters

2.3.3.1.1. Stability

The stability is important for HPTLC due to be an open-system. A sample component or a standard in the solution or on the plate can be affected by air, light, temperature, or humidity during the storage or during the chromatographic analysis. Additionally, the degradation can be investigated by the stability process (224-226).

2.3.3.1.2. Robustness

Robustness (ruggedness) is the capacity of an analytical technique to tolerate minor changes in system conditions is determined by robustness. Thus, the more reliably results can be produced by the robustness of the method (116, 224, 226).

Sample extraction time, sorbent type, composition of mobile phase, humidity, chamber saturation time, detection wavelength, developing distance, heating time and temperature during derivatization are the important factors that can affect the HPTLC performance (225, 227).

2.3.3.2. Validation parameters

When using HPTLC as a quantitative technique of analysis, it is necessary to quantify the bands, along with the validation parameters, namely specificity, limits of measurement, linearity, precision and accuracy. Validation is done by placing the plate into the scanner to record areas of peaks measured at one or several wavelengths.

2.3.3.2.1. Specificity

Specificity is the capability to measure an analyte in the existence of other constituents that are known to be existed in the sample (227). Electronic images of fingerprints are compared visually based on color, position, number and intensity of the zones (224).

2.3.3.2.2. Limit of measurement

The limit of measurement is categorized in two groups: detection limit and quantitation limit. The limit of detection (LOD) is the lowest quantity of a component in a sample that is determined but quantification is not necessary. LOD is a limit test which specifies whether or not a component is above or below a certain value. The limit of quantitation (LOQ) is the lowest quantity of a component in a sample that is quantitatively detected under the prescribed situations of analytical system (226, 228).

2.3.3.2.3. Linearity and range

Linearity is the capability of an analytical technique to reveal the consequences that are directly proportional to the amount of a constituent in a sample within a specified range (223, 228). The working range is obtained by diluting the sample and using the variable application volume (116, 208).

2.3.3.2.4. Precision

Precision measures the degree of repeatability and reproducibility of an analytical technique among series of multiple sampling results of the same sample under the stated circumstances of system (223, 228). In accordance with ICH, precision is classified at three levels: repeatability, intermediate precision, and reproducibility (223).

2.3.3.2.4.1. Repeatability

Repeatability (within-day or intra-assay precision) states the consequences of an analytical technique operating over a short time interval under the same circumstances (210, 229). For acceptance of repeatability, all fingerprints must match to each other. The changeability of the R_f values of 3 markers should not be more than 0.02 (224).

2.3.3.2.4.2. Intermediate precision

Intermediate precision (interday precision) states the outcomes from within-lab variations due to alterations in experimental equipments and periods (226, 229). For acceptance of intermediate precision, the changeability of the R_f values of 3 markers should not be more than 0.05 (224). The RSD values of intermediate precision range from 1.3 to 1.7 x RSD values of repeatability (225).

2.3.3.2.4.3. Reproducibility

Reproducibility refers to the results of collaborative studies among laboratories (223, 229). For acceptance of reproducibility, the changeability of the R_f values of 3 markers should not be more than 0.07 (224).

2.3.3.2.5. Accuracy

Accuracy (recovery) measures the exactness of an analytical technique or the closeness of the agreement between the mean value (measured value) and the value accepted either as a conventional, true value (standard) or an accepted reference value

after replicates (223, 227). The accuracy is stated as recovery % (210). For the assay of a plant sample, accuracy is assessed by inserting known quantities of the standard to the sample solution (116, 225). For acceptance of accuracy, RSD values should not be more than 10% as well as the relative recovery of the mean value range from 80% to 120% (225, 228).

3. MATERIALS and METHODS

3.1. Materials

3.1.1. Plant and Oily Materials

Flowering aerial parts of *H. perforatum* L. were collected by Prof. Dr. Yüksel Kan (Faculty of Agriculture, Selçuk University, Konya, Turkey) from the cultivation field of Selçuk University, Konya province of Turkey, in July, 2015.

Two samples of *H. perforatum* L. were collected from İstanbul and Tekirdağ provinces of Turkey by Esra Saçıcı. *H. triquetrifolium* Turra were collected from Adana province, *H. bithynicum* Boiss., *H. calycinum* L., *H. cerastoides* Spach and *H. perfoliatum* L. were collected from İstanbul province of Turkey by Esra Saçıcı. *H. kotschyanum* Boiss. and *H. thymopsis* Boiss. were collected by Prof. Dr. Şükran Kültür from Mersin and Malatya provinces of Turkey, respectively. *H. scabrum* L. was collected by Onur Altınbaşak from Sivas province of Turkey.

All plant materials were authenticated by Prof. Dr. Şükran Kültür (Faculty of Pharmacy, İstanbul University, İstanbul, Turkey). The voucher specimens of materials are deposited in the herbarium of Faculty of Pharmacy, İstanbul University (ISTE), İstanbul, Turkey. The identified *Hypericum* materials, locations and dates of collection are itemized in Table 3.1. All plant materials were dried in shade at room temperature, and later mechanically ground to powder in a laboratory mill in order to prepare a homogenous drug powder.

Besides, eight different brands of *H. perforatum* tea bags labeled as HT1-HT8 were purchased from food markets in İstanbul, Turkey. Additionally, packages of pulverized herba and bunches of dried herba were purchased from thirty-three different spice shops in İstanbul and Tekirdağ provinces of Turkey and coded as HS1-HS33. Moreover, capsules comprising the powder of *H. perforatum* extract encoded HC were purchased from a pharmacy in İstanbul, Turkey.

Table 3.1. Voucher specimens of *Hypericum* materials

Plant material	**Part**	**Location**	**Date**	**ISTE**
H. perforatum L.	Herba	İstanbul	06.2016	110603
H. perforatum L.	Herba	Tekirdağ	05.2016	110608
H. bithynicum Boiss.	Herba	İstanbul	04.2016	110609
H. calycinum L.	Herba	İstanbul	06.2016	110605
H. cerastoides Spach	Herba	İstanbul	04.2016	110602
H. perfoliatum L.	Herba	İstanbul	04.2016	110604
H. triquetrifolium Turra	Herba	Adana	06.2016	110613
H. kotschyanum Boiss.	Herba	Mersin	06.2015	98173
H. scabrum L.	Herba	Sivas	07.2016	115028
H. thymopsis Boiss.	Herba	Malatya	07.2015	99046

Furthermore, olive oil macerate of flowering aerial parts of *H. perforatum* L. was prepared regarding to traditional recipe by the Technological Development Region of Selçuk University (Konya, Turkey) in 2015. Hyperici oleum was prepared by putting the fresh flowering aerial parts of *H. perforatum* into olive oil in a glass jar and keeping the jar under the sun light for two months. Then the mixture was filtered to remove the solid residues to obtain red colored oily extract. The traditional recipe for the preparation of Hyperici oleum: "the content of jar should be exposed to the heat of the sun and the breeze of the night" (164). In addition, seven different marketed ready-made brands of *H. perforatum* oil macerates labeled as OE1-OE7 were purchased from a local pharmacy and several spice shops in İstanbul, Turkey.

3.1.2. Chemicals and Solvents

Table 3.2. Chemicals & solvents and their trademarks

Chemicals and Solvents	**Trademark**
Acetic acid	Riedel-de Haën
Acetone	Sigma-Aldrich
Acetonitrile	Sigma-Aldrich
Chloroform	Sigma-Aldrich
Dichloromethane	Sigma-Aldrich
Ethanol	Sigma-Aldrich
Ethyl acetate	Sigma-Aldrich
Ethyl formate	Merck
Ethyl methyl ketone	Merck
Formic acid	Sigma-Aldrich
Formic acid ethyl ester	Merck
n-Hexane	Sigma-Aldrich
Hyperforin	AppliChem PanReac
Hypericin	Sigma-Aldrich
Hyperoside	HWI group
Methanol	Sigma-Aldrich
Perchloric acid	Riedel-de Haën
Petroleum ether	Sigma-Aldrich
Phosphoric acid	Sigma-Aldrich
Sodium dihydrogen phosphate	Sigma-Aldrich
Sulfuric acid	Sigma-Aldrich
Tetrahydrofuran	HiPerSolv Chromanorm
Toluene	Sigma-Aldrich
Vanillin	Fluka

3.1.3. Equipments and Instruments

Table 3.3. Equipments & instruments and their trademarks

Equipments and instruments	**Trademark**
Automatic Developing Chamber 2	Camag
Balance	Ohaus Explorer
Centrifuge tubes	Isolab
Checktemp	Hanna
Chemstation software	Agilent
Filter paper	Munktell
Fume hood	Flores Valles
Glass laboratory equipments	Isolab
Grinding mill	IKA
Hairdryer	Rowenta
HPLC vacuum degasser	Agilent
HPLC quaternary pump	Agilent
HPLC auto-sampler	Agilent
HPLC column compartment	Agilent
HPLC diode array detector	Agilent
HPTLC dosage syringe (100 µL)	Hamilton
HPTLC glass and aluminum plates Si 60 F_{254}	Merck
Kettle	Arçelik
Linomat V	Camag
Lyophilizator	Christ Alpha 2-4 LD
Micropipettes	Rainin
Micropipette tips	Rainin

Table 3.3. Equipments & instruments and their trademarks (continued)

Equipments and instruments	**Trademark**
MilliQ H_2O device	Millipore
Oven	Binder
pH color-fixed indicator sticks	Macherey-Nagel
Poroshell 120 EC-18 column	Agilent
RC-disk filter (0.45 μm)	Sartorius stedim biotech
RC-syringe filter (0.45 μm)	Sartorius stedim biotech
Refrigerator	Arçelik
Rotary evaporator	Buchi, Heildoph
Screw caps	Agilent
Shaker	IKA-Werke
Single-use syringe	HSW soft-ject
Test tubes	Isolab
TLC aluminum plates Si 60 F_{254}	Merck
TLC scanner 3	Camag
TLC plate heater	Camag
TLC visualizer	Camag
Twin-through chamber	Camag
Ultrasonic bath	Sonorex
Vacuum filtration apparatus	Sartorius stedim biotech
Vial	Agilent
Vortex	IKA
WinCATS software	Camag
Zorbax Eclipse XDB-C18 column	Agilent

3.2. Methods

3.2.1. Extraction Methods

While performing the extraction and lyophilization procedure, all the laboratory glass equipments were covered with aluminum foil for the protection from daylight.

3.2.1.1. Selection of suitable alcoholic extraction methods for *H. perforatum*

The dried flowering aerial parts of *H. perforatum* L. cultivar (2 g) was powdered and extracted with ethanol (EtOH) or methanol (MeOH) (50 mL) by using various conditions as described below (Table 3.4). The extract was then filtered through a filter paper and evaporated under reduced pressure to dryness. The residue was redissolved in water, and lyophilized. The yield was calculated.

Table 3.4. Alcoholic extraction methods

Extraction method	Extraction solvent	Extraction technique	Extraction period	Extract code
I	EtOH 80%	by continuous stirring at 40 °C in a rotary evaporator	60 min	HP-E80-R
II	EtOH 100%	by continuous stirring at 40 °C in a rotary evaporator	60 min	HP-E100-R
III	EtOH 100%	in an ultrasonic bath by sonification	30 min	HP-E100-U
IV	EtOH 100%	in an orbital shaker	60 min	HP-E100-S
V	MeOH 80%	in an orbital shaker	60 min	HP-M80-S
VI	MeOH 100%	in an orbital shaker	60 min	HP-M100-S
VII	MeOH 100%	in an ultrasonic bath by sonification	30 min	HP-M100-U
VIII	MeOH 100%	by continuous stirring at 40 °C in a rotary evaporator	60 min	HP-M100-R

3.2.1.2. Aqueous extraction methods of *H. perforatum*

3.2.1.2.1. Aqueous extraction method I

The dried flowering aerial part of *H. perforatum* L. cultivar (2 g) was powdered and extracted with freshly boiled water (100 mL) (2% infusion). It was enclosed by watch glass, and left to steep at room temperature for 5 min. The extract was then filtered through a filter paper and the filtrate was frozen in a refrigerator and lyophilized. The yield was calculated and encoded HP-W5.

3.2.1.2.2. Aqueous extraction method II

The dried flowering aerial part of *H. perforatum* L. cultivar (2 g) was powdered and extracted with freshly boiled water (100 mL) (2% infusion). It was enclosed with a watch glass, and left to steep at room temperature for 10 min. The extract was then filtered through a filter paper and the filtrate was frozen in a refrigerator and lyophilized. The yield was calculated and encoded HP-W10.

3.2.1.3. Extraction methods from oily preparation of *H. perforatum*

In order to find the most convenient extraction technique to prepare the analytical samples from the oily extract of *H. perforatum* (Hyperici oleum) different organic solvents were used.

3.2.1.3.1. Extraction method I

H. perforatum oily preparation was extracted with acetonitrile (1:2.5, w/v) by continuous stirring in a rotary evaporator for 20 min. The suspension was then centrifuged at 4°C for 20 min at 769 *g*, and the supernatant was separated. This extraction process was repeated four times and the supernatants were combined. The extract was filtered through a filter (0.45 μm) (230).

3.2.1.3.2. Extraction method II

H. perforatum oily preparation (1 mL) was extracted with 5 mL of methanol-hexane (8:2, v/v) by shaker for 30 min. The methanol-hexane solution was collected in a separation funnel. The extract was filtered through a filter (0.45 μm) (231).

3.2.1.3.3. Extraction method III

Olive oil macerate of *H. perforatum* (1 mL) was mixed with acetone, and diluted to 5 mL with the same solvent. The extract was filtered through a filter (0.45 μm) (232).

3.2.1.3.4. Extraction method IV

Oily preparation of *H. perforatum* was extracted with methanol (5:10, w/v) for 30 min. The methanol extract was collected and filtered through a filter (0.45 μm) (233).

3.2.2. Preparation of Standard Solutions

Hyperforin (HyF), hypericin (HyP) and hyperoside (HyS) stock solution (1 mg/mL) were separately prepared in methanol (MeOH) and further diluted with the same solvent to prepare working solutions which were applied while developing and validating three HPTLC methods as well as used for HPLC analysis.

3.2.3. Preparation of Reference Test Solutions

In HPLC analysis of HyF, HyP and HyS, 1 mg of lyophilizate prepared from the reference sample *H. perforatum* L. cultivar was accurately weighed and dissolved with MeOH (1 mL) in an ultrasonic bath for 15 min. The concentration of sample test solution was 1 mg/mL.

For HPTLC analysis of HyF, HyP and HyS contents, each of lyophilizate extract obtained from *H. perforatum* L. cultivar, ten different *Hypericum* species, marketed *H. perforatum* (SJW) products, including a capsule formulation, eight herbal teas and

thirty-three herbal materials from stores and spice shops, as well as the aqueous extract from *H. perforatum* L. cultivar was accurately weighed in three replicates and dissolved with MeOH (2 mL) in an ultrasonic bath for 15 min. The concentration of each sample test solution was calculated and given in Table 3.5. Suspended particles were removed by filtration through a filter (0.45 μm).

Table 3.5. Concentration of each sample test solution for HPTLC analysis

HPTLC analysis	Sample	Concentration
HyF	Methanolic extract of *H. perforatum* L. cultivar *Hypericum* species Capsule formulation	5 mg/mL
	Tea products Marketed herbal materials Aqueous extracts of *H. perforatum* L. cultivar	10 mg/mL
HyP	Methanolic extract of *H. perforatum* L. cultivar *Hypericum* species Capsule formulation	10 mg/mL
	Tea products Marketed herbal materials Aqueous extracts of *H. perforatum* L. cultivar	20 mg/mL
HyS	Methanolic extract of *H. perforatum* L. cultivar *Hypericum* species Capsule formulation	5 mg/mL
	Tea products Marketed herbal materials Aqueous extracts of *H. perforatum* L. cultivar	10 mg/mL

3.2.4. HPTLC Method

In order to find suitable and effective solvent systems for the qualitative and quantitative analysis of hyperforin, hypericin and hyperoside contents in the *Hypericum* samples by HPTLC firstly suitability of the mobile phases which were practiced in the previous studies were firstly tested on the extract prepared from *H. perforatum* L. cultivar.

HPTLC analysis was conducted on HPTLC plates coated with silica gel 60 F_{254}. Solutions of samples and references, including hyperforin, hypericin and hyperoside, were implemented on the plates as 8.0 mm bands, 15.0 mm away from the left edge and 8.0 mm above from the bottom edge using Camag Linomat V sample applicator equipped with a 100 µL syringe connected to a nitrogen tank. After the application process, the plates were preconditioned with the mobile phase vapor [*n*-hexane-ethyl acetate (8:2, v/v) for HyF analysis; toluene-chloroform-ethyl acetate-formic acid (8:5:3.5:0.6, v/v/v/v) for HyP analysis; and ethyl acetate-formic acid-acetic acid-water (15:2:2:1, v/v/v/v) for HyS analysis], and developed to 70 mm in a saturated Automatic Developing Chamber 2 (ADC2) with the mobile phase. The developed plates were automatically dried, and scanned by Camag TLC Scanner 3. Thereafter, the plates were derivatized by reagents. Then, the derivatized plates were heated on Camag TLC plate heater, and the chromatograms were documented by Camag TLC Visualizer under 366 nm. The whole devices were managed by WinCATS v1.4.8 software.

3.2.5. HPLC Method

Quantitative analyses of hyperforin, hypericin and hyperoside were performed by HPLC system which was an Agilent HP1100 series with an auto-sampler, a thermo-stated column part, a quaternary pump, a vacuum degasser, and a DAD operated by Chemstation 10.01 software. The amounts of hyperforin, hypericin and hyperoside in extract from *H. perforatum* L. cultivar were detected by the method depicted under "Hyperici herbae extractum siccum quantificatum" in the Ph. Eur. (234) (Section 3.2.5.1, 3.2.5.2, and 3.2.5.3). Agilent Poroshell 120 EC-C18 column (2.7 µm, 150 x 3.0 mm) was employed for the separations of hyperforin and hypericin as well as Agilent Zorbax Eclipse XDB-C18 column (3.5 µm, 150 x 4.6 mm) was used for the separation

of hyperoside. Stock solutions of hyperforin, hypericin and hyperoside (1 mg/mL) were prepared in methanol and then diluted with methanol for the preparation of the working solutions. Each analyzed solution was implemented in triplicate. The percentage content of hyperforin, hypericin and hyperoside were computed with the below equalities (235, 236):

$$x = (A_1 \times c_2) / A_2 \quad (2.1)$$

$$e = (x / c_1) \times 100 \quad (2.2)$$

$$p = (y \times e) / 100 \quad (2.3)$$

A_1: standard peak area attained with test solution
A_2: standard peak area attained with reference solution
c_1: concentration of drug in test solution (mg/mL)
c_2: concentration of standard in reference solution (mg/mL)
y: yield of the drug extraction (%)
p: percentage content of standard in the drug (%)

3.2.5.1. HPLC method for hyperforin analysis

For hyperforin analyses the mobile phase A was phosphoric acid-water (3:1000, v/v) and the mobile phase B was phosphoric acid-acetonitrile (3:1000, v/v) were used. These two solvent systems were filtered and degassed before usage. The supervening gradient pattern was employed for the separation of hyperforin: 18% B (0-8 min), 18-53% B (8-18 min), 53-97% B (18-18.1 min), 97% B (18.1-29 min), 97-18% B (29-30 min). The injection volume was 10 µL, and the flow rate was 0.5 mL/min, as well as the detection was monitored at 275 nm. The peaks were identified with retention time (t_R) and DAD spectrum.

3.2.5.2. HPLC method for hypericin analysis

The solvent system employed for HPLC analysis of hypericin was the mix 39 volumes of ethyl acetate, 41 volumes of a 15.6 g/L solution of sodium dihydrogen phosphate (adjusted to pH 2 with phosphoric acid) and 160 volumes of methanol. This mobile phase was filtered and degassed before use. The method of hypericin was isocratic. The injection volume was 20 µL, and the flow rate was 0.3 mL/min, as well as the detection was monitored at 590 nm. The peaks were identified by t_R and DAD spectrum.

3.2.5.3. HPLC method for hyperoside analysis

For the analyses of hyperoside the mobile phase A which was composed of phosphoric acid and water (3:1000, v/v), and the mobile phase B was composed of phosphoric acid and acetonitrile (3:1000, v/v) were used. These two solvent systems were filtered and degassed before usage. The supervening gradient pattern was employed for the separation of hyperoside: 18% B (0-8 min), 18-53% B (8-18 min), 53-97% B (18-18.1 min), 97% B (18.1-29 min), 97-18% B (29-30 min). The injection volume was 10 µL, and the flow rate was 1.0 mL/min, as well as the detection was monitored at 360 nm. The peaks were identified with t_R and DAD spectrum.

3.2.6. Preparation of Detection Reagent for HPTLC

By continuously cooling the solution, 20 mL sulfuric acid (H_2SO_4) was carefully added to 120 mL methanol. Then, it was left to cool. After cooling, it was diluted to 200 mL with the same solvent (208).

3.2.7. Statistical Analysis

All analyzed solutions were implemented in 3 replicates. The findings were stated as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was employed for the statistical comparison among numerous average values. When a crucial discrimination was found amongst the results according to ANOVA, the difference was identified by conducting least significant difference (LSD) test.

Statistically important distinctness was described as $p<0.05$. The statistical analyses were conducted with IBM SPSS Statistics Data Editor V20.0 software.

4. RESULTS

4.1. Extract Yields and Extraction Method Selection

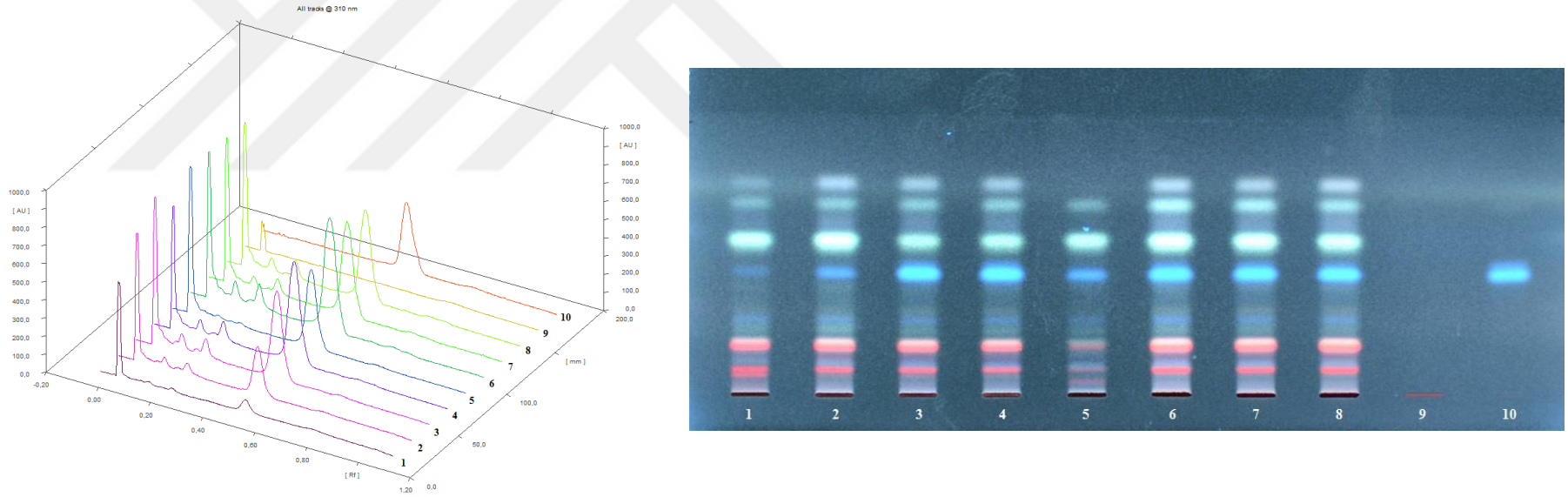
4.1.1. Yields of *H. perforatum* L. Cultivar Extract and Extraction Method Selection

The yields of eight alcoholic extracts and two aqueous extracts of *H. perforatum* L. cultivar were listed in Table 4.1.

Table 4.1. Yields of *H. perforatum* L. extracts

Extract	**Yield (%)**
HP-E80-R	21.83
HP-E100-R	19.14
HP-E100-U	17.10
HP-E100-S	15.28
HP-M80-S	19.59
HP-M100-S	14.34
HP-M100-U	16.54
HP-M100-R	18.49
HP-W5	25.23
HP-W10	25.40

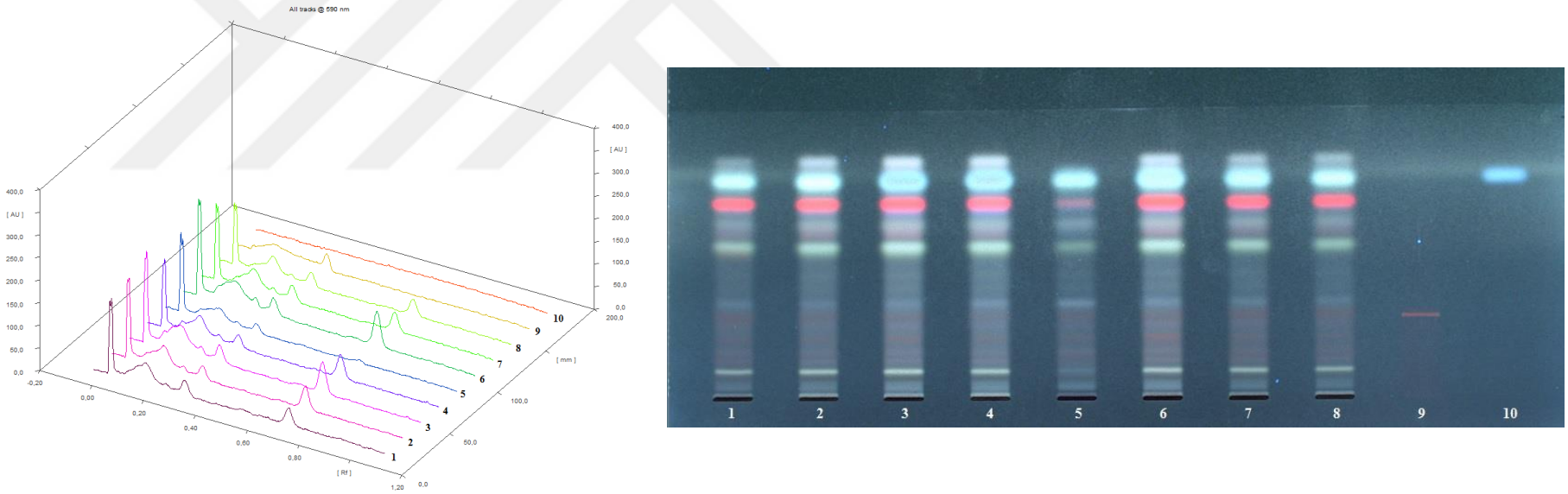
80% and 100% ethanolic and methanolic extracts of *H. perforatum* L. cultivar were prepared following the described processes in the section 3.2.1. Then, these extracts were dissolved in methanol (10 g/L). Hyperforin, hypericin and hyperoside were used as standards. HPTLC fingerprints after derivatization with H_2SO_4 reagent and three dimensional (3D) chromatograms belongs to eight extracts were presented in Figure 4.1 for HyF, Figure 4.2 for HyP and Figure 4.3 for HyS.



(a) **(b)**

Figure 4.1. (a) 3D overlay of HPTLC densitograms of the spots of hypericin and hyperforin as well as *H. perforatum* cultivar extracts at 310 nm. (b) HPTLC fingerprint analysis of *H. perforatum* cultivar extracts and hyperforin at 366 nm.

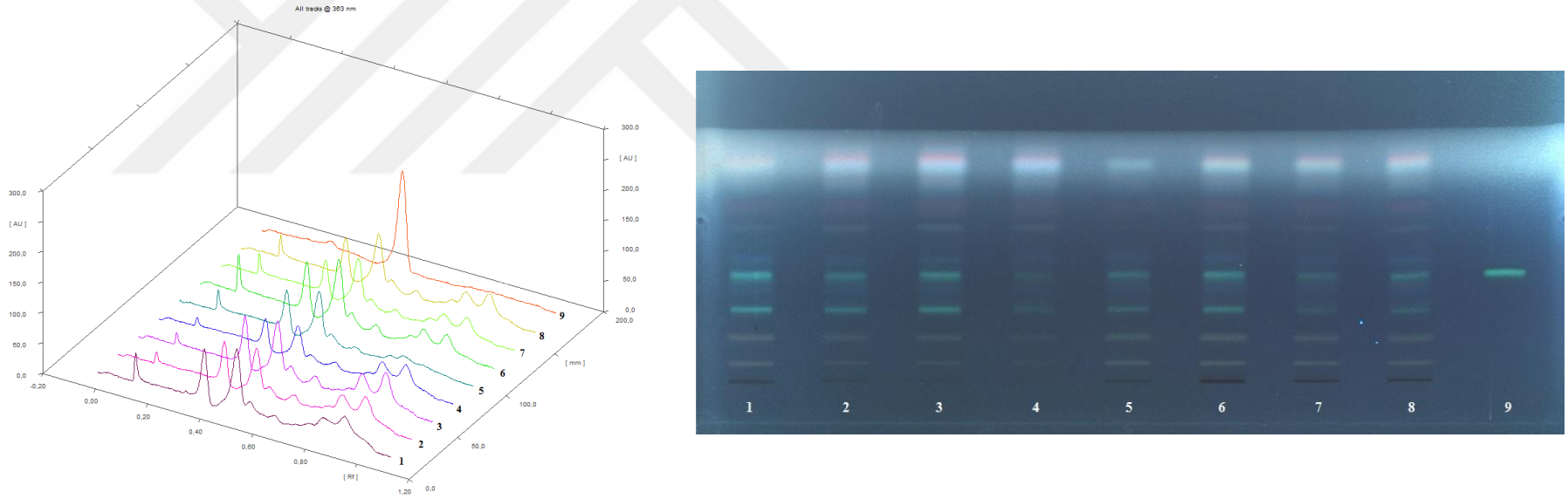
Derivatization: H_2SO_4 reagent. Mobile phase: *n*-hexane-ethyl acetate (8:2, v/v). Tracks: (1) HP-E80-R; (2) HP-E100-R; (3) HP-E100-U; (4) HP-E100-S; (5) HP-M80-S; (6) HP-M100-S; (7) HP-M100-U; (8) HP-M100-R; (9) Hypericin (reference); (10) Hyperforin (reference) (blue spot).



(a) **(b)**

Figure 4.2. (a) 3D overlay of HPTLC densitograms of the spots of hypericin and hyperforin as well as *H. perforatum* cultivar extracts at 590 nm. (b) HPTLC fingerprint analysis of *H. perforatum* cultivar extracts and hypericin at 366 nm.

Derivatization: H_2SO_4 reagent. Mobile phase: toluene-chloroform-ethyl acetate-formic acid (8:5:3.5:0.6, v/v/v/v). Tracks: (1) HP-E80-R; (2) HP-E100-R; (3) HP-E100-U; (4) HP-E100-S; (5) HP-M80-S; (6) HP-M100-S; (7) HP-M100-U; (8) HP-M100-R; (9) Hypericin (reference) (red spot); (10) Hyperforin (reference) (blue spot).



(a) **(b)**

Figure 4.3. (a) 3D overlay of HPTLC densitograms of the spots of hyperoside and *H. perforatum* cultivar extracts at 363 nm. (b) HPTLC fingerprint analysis of *H. perforatum* cultivar extracts and hyperoside at 366 nm.

Derivatization: H_2SO_4 reagent. Mobile phase: ethyl acetate-acetic acid-formic acid-water (15:2:2:1, v/v/v/v). Tracks: (1) HP-E80-R; (2) HP-E100-R; (3) HP-E100-U; (4) HP-E100-S; (5) HP-M80-S; (6) HP-M100-S; (7) HP-M100-U; (8) HP-M100-R; (9) Hyperoside (reference) (yellowish spot).

According to Figure 4.1, Figure 4.2, and Figure 4.3, it was observed that HyF, HyP, and HyS content in *H. perforatum* L. reference sample were found to be different depending on the extraction processes (Table 3.4). In this regard, method VI was selected for the extraction because of higher solubility of the investigated markers without applying heat.

4.1.2. Yields of *Hypericum* Species

The powdered samples of ten *Hypericum* species were extracted following the alcoholic extraction method VI as described in Section 3.2.1.1. The yields of each methanolic extract of ten *Hypericum* species were given in Table 4.2.

Table 4.2. Methanolic extract yields of various *Hypericum* species

***Hypericum* specimens**	**Yield (%)**
H. perforatum (İstanbul)	14.61
H. perforatum (Tekirdağ)	12.83
H. bithynicum	10.01
H. calycinum	21.50
H. cerastoides	18.34
H. perfoliatum	19.99
H. triquetrifolium	15.83
H. kotschyanum	11.39
H. scabrum	14.38
H. thymopsis	9.47

4.1.3. Yields of Commercial *H. perforatum* (SJW) Products

8 different brands of *H. perforatum* tea bags and 33 different *H. perforatum* materials bought from spice shops were extracted by the same alcoholic extraction method VI as described in Section 3.2.1.1. The yields of each methanolic extract of 41 samples were shown in Table 4.3.

Table 4.3. Yields of each marketed *H. perforatum* (SJW) product

Commercial product	Yield (%)
HT1	10.31
HT2	9.76
HT3	13.32
HT4	12.06
HT5	12.00
HT6	12.54
HT7	10.98
HT8	13.88
HS1	10.00
HS2	9.83
HS3	8.77
HS4	9.61
HS5	10.47
HS6	5.22
HS7	8.74
HS8	4.15
HS9	7.30

Table 4.3. Yields of each marketed *H. perforatum* (SJW) product (continued)

Commercial product	Yield (%)
HS10	5.94
HS11	8.95
HS12	14.32
HS13	5.34
HS14	7.20
HS15	8.89
HS16	10.67
HS17	12.75
HS18	9.99
HS19	9.79
HS20	8.12
HS21	7.78
HS22	9.12
HS23	9.44
HS24	12.26
HS25	8.19
HS26	7.68
HS27	8.17
HS28	10.68
HS29	11.06
HS30	11.08
HS31	11.31
HS32	8.43
HS33	10.43

4.2. Analysis of Hyperforin Content in Samples

4.2.1. HPLC Analysis of Hyperforin

The system suitability was confirmed for HPLC method of hyperforin standard according to the Ph. Eur. (234). In this study, retention time (t_R) of hyperforin in the reference solution was determined to be 26.18±0.00 (n=3) (Figure 4.4). The specification of hyperforin in the sample solution was justified by matching t_R with that of the reference solution and it was found as 26.19±0.01 (n=3) (Figure 4.5). Furthermore, the percentage content of hyperforin in *H. perforatum* L. cultivar was determined as 3.44% and it was higher than 2.0 per cent (13). For that reason, *H. perforatum* cultivar was considered as a reference material for hyperforin analyses.

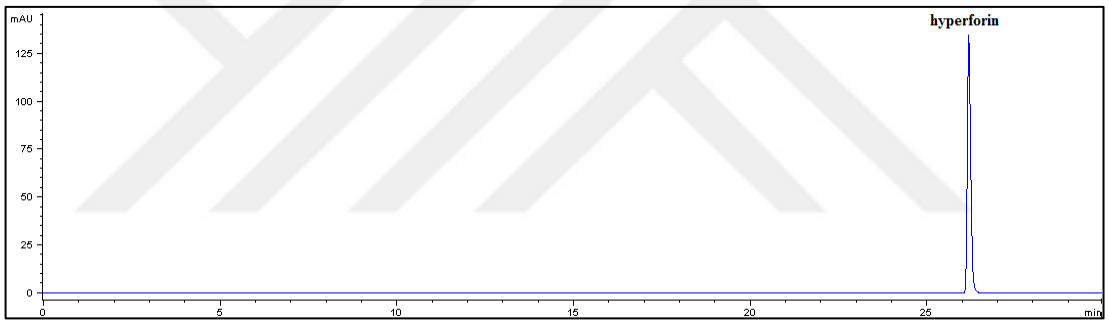


Figure 4.4. HPLC chromatogram of hyperforin reference solution at 275 nm

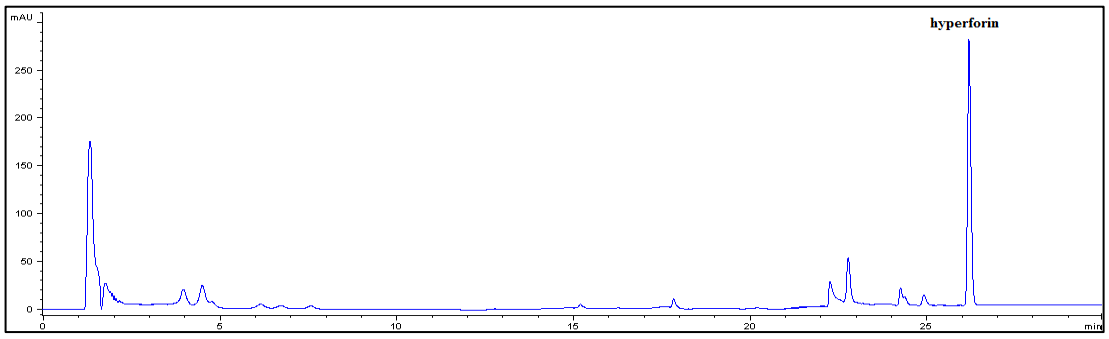


Figure 4.5. HPLC chromatogram of hyperforin in *H. perforatum* L. cultivar test solution at 275 nm

4.2.2. HPTLC Analysis of Hyperforin Content in Samples

4.2.2.1. Method development for hyperforin

While testing the solvent systems stated in the former studies, 20 min saturated twin-through chamber was used. The results of the literature survey which include the mobile phases used in TLC and HPTLC methods for detection of hyperforin (MP-HyF) were listed in Table 4.4 and HPTLC plates after derivatization obtained from five mobile phases were exhibited in Figure 4.6a-e.

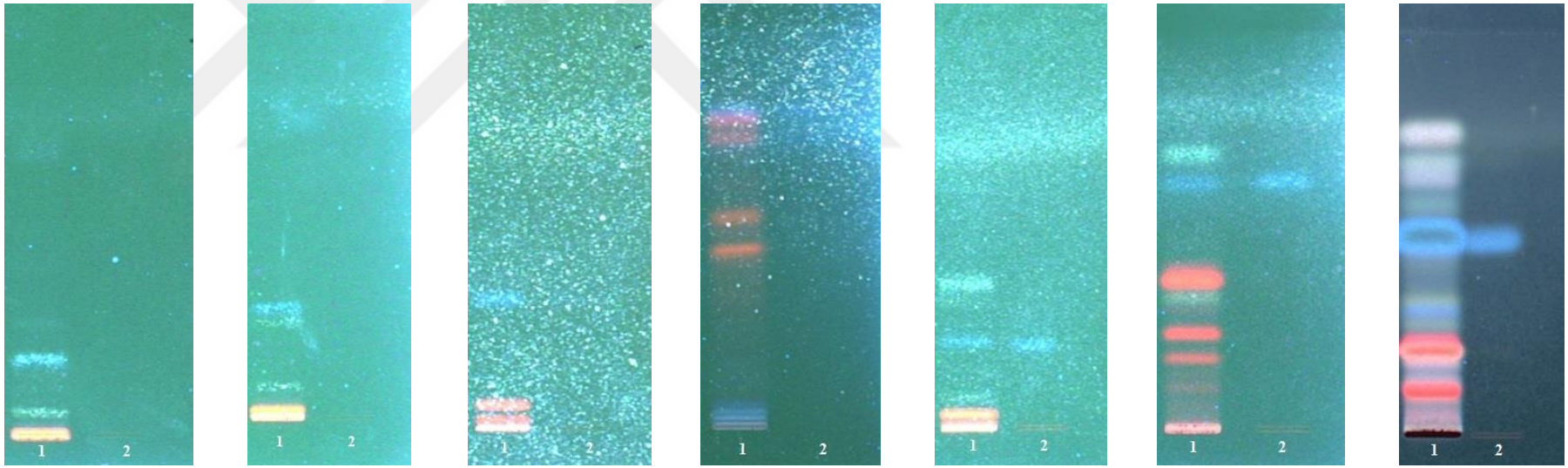
Table 4.4. Solvent systems tested in HPTLC analysis of hyperforin

Mobile phase	Solvent system	Reference
MP-HyF-1	toluene-dichloromethane (4:1)	(116, 123, 125)
MP-HyF-2	toluene-dichloromethane (4:2)	(124)
MP-HyF-3	petroleum ether-ethyl acetate (9:1)	(119)
MP-HyF-4	toluene-formic acid-formic acid ethyl ester (5:1:4)	(75)
MP-HyF-5	*n*-hexane-ethyl acetate (9:1)	(52)

Several mobile systems reported previously were tested in order to find their suitability for detection of hyperforin spots in HPTLC analysis (Table 4.4). After visualization with H_2SO_4 reagent, MP-HyF-5 showed the finest blue spots than other mobile phases, but its R_f value was less than 0.3. Therefore further attempts have been made to develop new and more efficient mobile phases which would provide better resolution for hyperforin analysis as well as to increase the R_f value. These solvent systems were given in Table 4.5 and HPTLC plates after derivatization were displayed in Figure 4.6f-g. The best result was established by MP-HyF-7.

Table 4.5. New mobile systems developed for HPTLC analysis of hyperforin

Mobile phase	Solvent system
MP-HyF-6	*n*-hexane-ethyl acetate (3:1)
MP-HyF-7	*n*-hexane-ethyl acetate (8:2)



(a) **(b)** **(c)** **(d)** **(e)** **(f)** **(g)**

Figure 4.6. HPTLC chromatograms of HyF (reference) and the extract from the flowering aerial parts of *H. perforatum* L. cultivar obtained with various mobile systems at 366 nm.

HPTLC plates were developed by (a) MP-HyF-1; (b) MP-HyF-2; (c) MP-HyF-3; (d) MP-HyF-4; (e) MP-HyF-5; (f) MP-HyF-6; (g) MP-HyF-7. Tracks: (1) *H. perforatum* extract; (2) HyF (reference); derivatization: H_2SO_4 reagent.

4.2.2.2. Method validation of hyperforin

The developed HPTLC process was verified following the ICH guidelines for the points of stability, robustness, specificity, detection and quantitation limits, linearity, intra- and interday precisions as well as recovery (223).

4.2.2.2.1. Stability

The stability of HyF in methanol at several days and different temperatures, stability of HyF reference solutions adsorbed to the plate prior to chromatography, and stability of sample test solution through development were explored to assess the stability of the components during the chromatographic processing.

4.2.2.2.1.1. Stability of hyperforin solution during storage period

The stability of HyF standard in methanol (0.2 mg/mL) was tested to verify whether spontaneous degradation occurred within 21 days after preparation and during storage at -20°C and 4°C. All experiments were iterated three times. Each peak area of the working solutions was compared with that of the fresh preparation of HyF standard in methanol at all time intervals. No chromatographic differences and no important degradation were detected. Thus, each stock solution prepared in methanol was found to be stable during storage at -20°C as well as 4°C for 3-week period, $F_{(3,20)}$=1.087, p=0.378 [$F_{crit\ (3,20)}$=3.098].

4.2.2.2.1.2. Stability of hyperforin solution on the stationary phase

The freshly prepared HyF reference solution (0.2 mg/mL) was applied on plate every 30 minutes up to 2 hours prior the development. The plate was left uncovered, subjected to daylight and air throughout these periods. This procedure was repeated in triplicate. One-way ANOVA was employed to assure the interruptions in time intervals. The difference was seen between the mean areas at time intervals, $F_{(4,10)}$=3.643, p=0.044 [$F_{crit\ (4,10)}$=3.478]. Therefore, LSD test was conducted to compare the average areas and find the discriminations. There was no difference between the mean areas of HyF reference solution at time zero and 30 minutes (p=0.769). Nonetheless, the average

area of HyF reference solution significantly differed from the mean area of HyF reference solutions applied at the interval of 60 minutes (p=0.044), 90 minutes (p=0.026), and 120 minutes (p=0.016).

4.2.2.2.1.3. Stability of sample test solution during migration with the mobile phase

Stability of the components in *H. perforatum* sample solution on the plate during the migration was investigated by two-dimensional separation process. After first migration of the test solution, the plate was turned 90° and developed in the same mobile system at diagonal direction. All the components in sample were situated on the diagonal of the chromatogram demonstrating their stability during the migration (Figure 4.7).

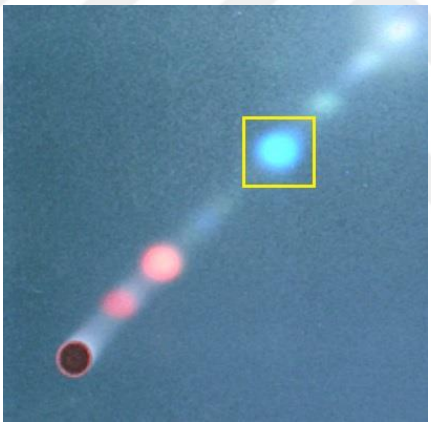


Figure 4.7. 2D-development of *H. perforatum* sample test solution for stability testing of components in *n*-hexane-ethyl acetate (8:2, v/v) at 366 nm. Derivatization: H_2SO_4 reagent.

4.2.2.2.2. Robustness

Some modifications in system conditions were made to evaluate the robustness of the method. In this regard, time of chamber saturation and humidity control, plate preconditioning time as well as time from development to scanning were slightly altered and then, the proportions of the peak area of HyF reference (0.2 mg/mL) and sample

solutions (5 mg/mL) were examined for the observation of the alterations. One-way ANOVA was used to compare the results. Accordingly, no significant differences were detected in the mean rates across the conditional changes, including saturation ($F_{(6,14)}$=2.125, p=0.115), humidity control ($F_{(6,14)}$=1.628, p=0.212), preconditioning ($F_{(6,14)}$=1.834, p=0.164), and time from chromatography to scan ($F_{(6,14)}$=2.488, p=0.075) [($F_{crit\,(6,14)}$=2.848)].

4.2.2.2.3. Specificity

Specificity of the solvent system was assessed by examining HyF reference (0.2 mg/mL) and sample solution (5 mg/mL). Within this context, R_f value of HyF in *H. perforatum* test solution was compared with that of HyF in reference solution and the R_f value was found to be 0.49±0.01 (Figure 4.8). Additionally, HPTLC chromatogram visualized under 366 nm and the densitograms of reference and sample solution containing HyF were employed for the approval.

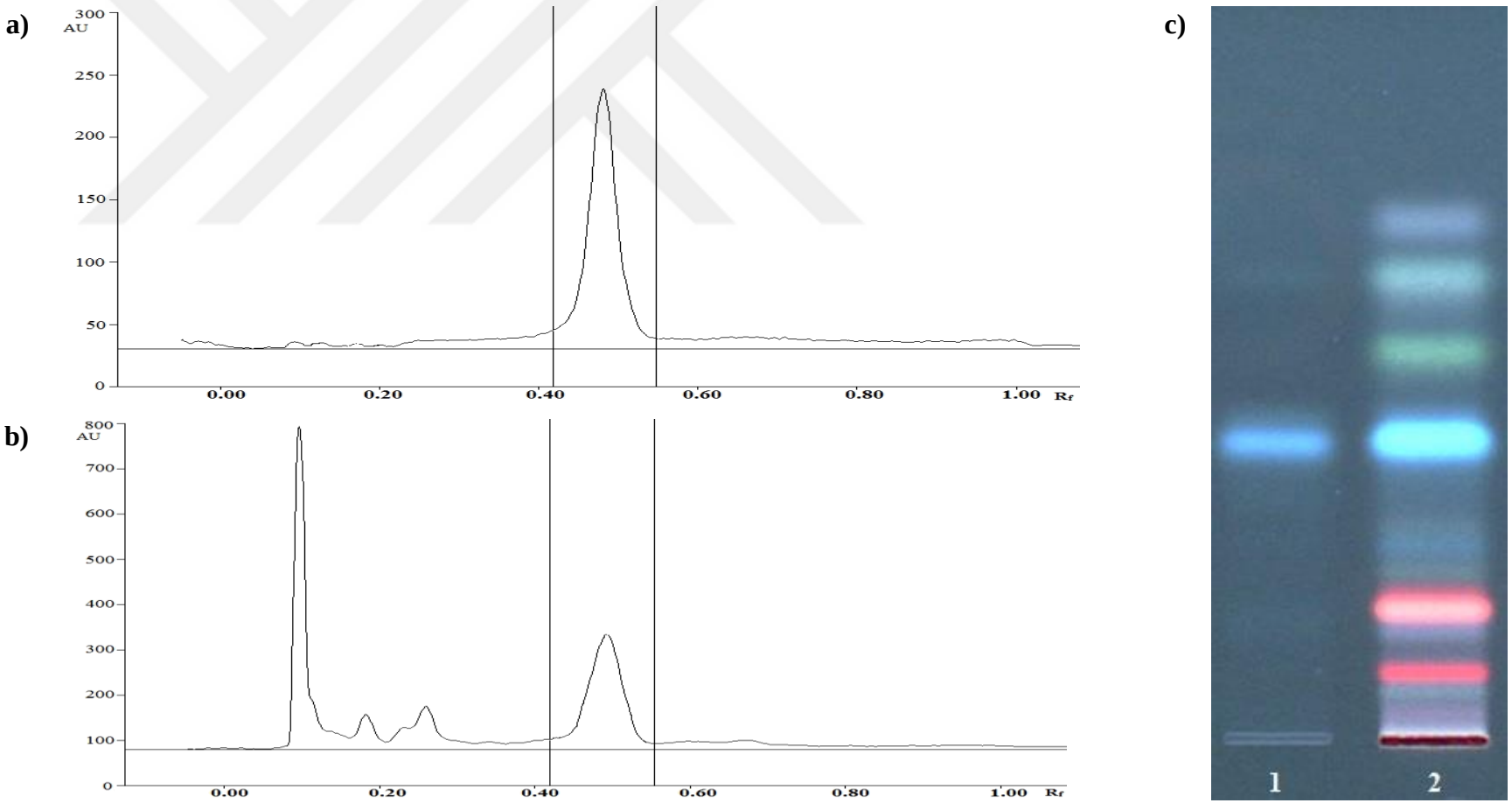


Figure 4.8. For specificity of the developed HPTLC process, HPTLC densitograms from (a) HyF reference solution (2 µL); (b) *H. perforatum* L. sample solution (2 µL) at 310 nm, and (c) HPTLC fingerprints at 366 nm.

Tracks: (1) HyF reference solution (2 µL); (2) *H. perforatum* L. sample solution (2 µL); derivatization: H_2SO_4 reagent.

4.2.2.2.4. Limits of detection (LOD) and quantitation (LOQ)

HyF reference solutions in different concentrations (0.02 mg/mL, 0.05 mg/mL, and 0.1 mg/mL) and in increasing volumes (1-10 µL) were applied on several plates and migrated to estimate the detection limit and quantitation limit. Then the values of LOD and LOQ were found to be 120 and 400 ng/band, respectively.

4.2.2.2.5. Linearity and calibration curve

Linearity of the developed HPTLC system was evaluated at the applied values within the range of 0.4-1.4 µg/band of reference solutions and calibration curves were constructed with three independent injections. The linear correlation between the injected volume and the peak area were obtained from the applied concentrations of HyF solution and the coefficient of linear correlation was estimated to be 0.999±0.001. The concentration of HyF was proportional to the peak area and the regression equation was y=-681.2+10.08x [y=peak area; x=concentration in ng/band].

4.2.2.2.6. Precision

4.2.2.2.6.1. Repeatability

The intra-assay precision of the developed HPTLC process was determined in three replicates of the experiment with *H. perforatum* samples prepared three time intervals on the same day. The quantities of HyF in samples expressed as mean± SD as well as the RSD values were given in Table 4.6.

4.2.2.2.6.2. Intermediate precision

The interday precision of the newly developed HPTLC system was carried out with the *H. perforatum* samples prepared on three different days. Analysis of each sample was repeated thrice and the amount of HyF in each sample was stated as mean± SD. The results of interday precision were similar to those of intraday precision and the findings were given in Table 4.6. Accordingly, the outcomes presented that the values

of RSD for intra- and interday precisions were within the expected range of assessment criteria (225).

Table 4.6. Intra- and interday precision results of developed HPTLC method for HyF

Precision analysis		**HyF (mg/g)** (*n*=3)		
		sample-I	**sample-II**	**sample-III**
Within-day	analysis-I	34.65±0.05	35.11±0.07	36.59±0.11
	analysis-II	35.57±0.11	34.28±0.05	36.43±0.13
	analysis-III	35.50±0.08	34.68±0.09	37.52±0.08
	RSD (%)	1.28	1.05	1.41
Between-days	analysis-I	36.42±0.17	36.97±0.09	37.05±0.14
	analysis-II	36.95±0.14	36.05±0.11	37.58±0.18
	analysis-III	35.53±0.19	35.58±0.13	36.04±0.17
	RSD (%)	1.76	1.71	1.88

4.2.2.2.7. Recovery

The accuracy of the developed HPTLC system was validated by the standard addition method. The known quantities of HyF reference (0.1, 0.2 and 0.4 µg/band) were added to pre-analyzed sample solutions. Three replicates of each concentration were tested. The amount of HyF detected in mixed solution was expressed as mean±SD as well as the mean recovery and RSD were calculated as per cent (Table 4.7). The results presented that the means of percentage recoveries were within the expected range of assessment criteria (225).

Table 4.7. Recovery findings of HPTLC system of HyF

HyF added (µg/band)	**HyF found (µg/band)**	**RSD (%)**	**Recovery (%)**
0.1	0.101±0.001	1.19	101.05
0.2	0.201±0.003	1.35	100.28
0.4	0.405±0.008	1.93	101.22

4.2.3. Hyperforin Assay in Various *Hypericum* Species

The presence of HyF in the extracts from the flowering aerial parts of different *Hypericum* species was detected by the comparison of their R_f values and corresponding peaks related to retention spots with those of HyF reference. The analysis was performed in triplicate. The quantities of HyF determined in various *Hypericum* specimens were given as mean±SD in Table 4.8.

Table 4.8. HyF contents in various *Hypericum* species

***Hypericum* specimen**	**HyF (mg/g)**
H. perforatum (İstanbul)	26.20±0.02
H. perforatum (Tekirdağ)	26.40±0.07
H. bithynicum	ndt
H. calycinum	ndt
H. cerastoides	ndt
H. perfoliatum	ndt
H. triquetrifolium	6.40±0.05
H. kotschyanum	ndt
H. scabrum	9.70±0.10
H. thymopsis	ndt

ndt: HyF not detected

Furthermore, the explanations of these findings as well as the designation of differentiation between these samples were supported by HPTLC densitograms (Figure 4.9) and fingerprint (Figure 4.10) of *Hypericum* species.

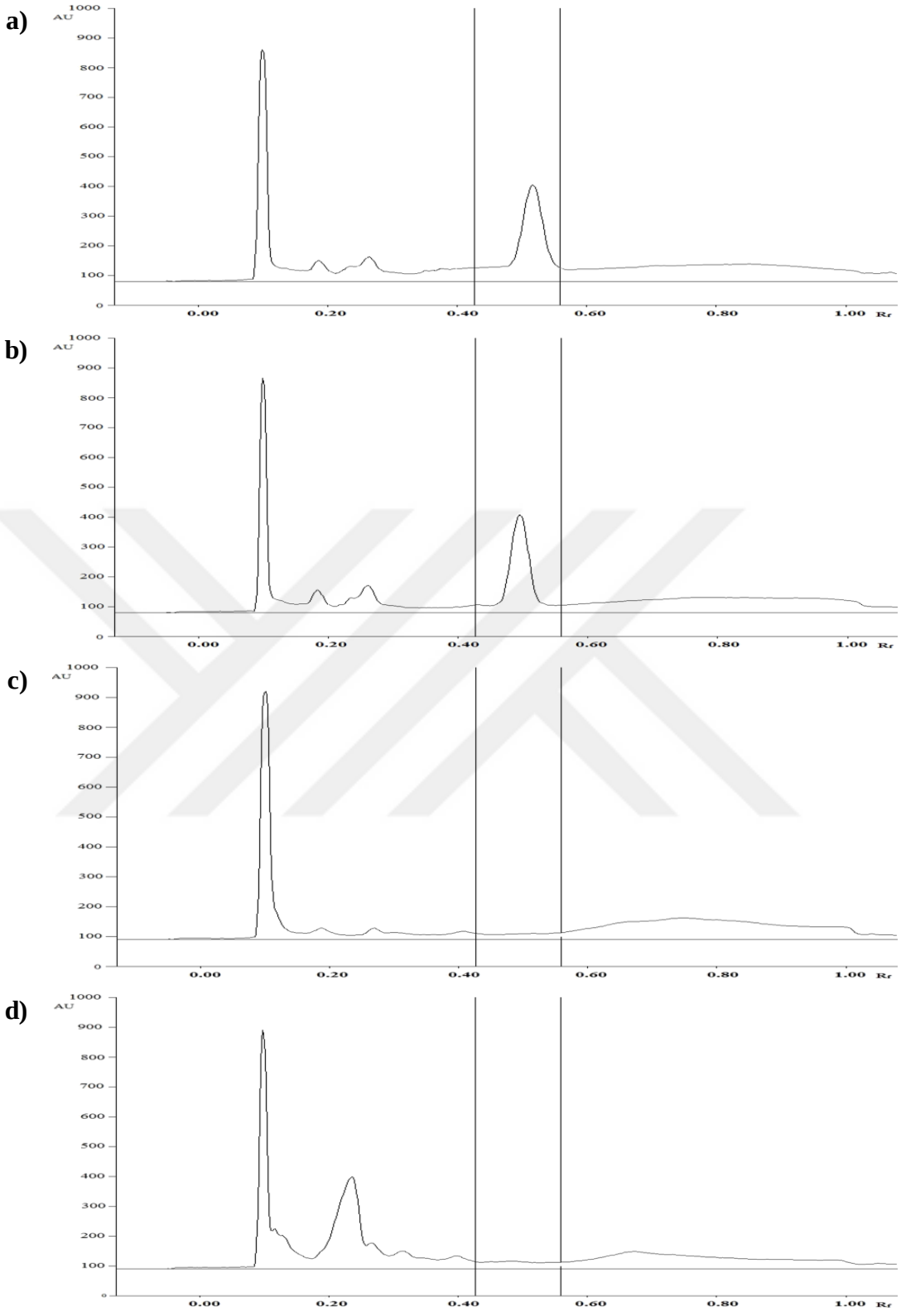


Figure 4.9. HPTLC densitograms of the extracts from different *Hypericum* species at 310 nm. (a) *H. perforatum* (İstanbul); (b) *H. perforatum* (Tekirdağ); (c) *H. bithynicum*; (d) *H. calycinum* (10 µg/band).

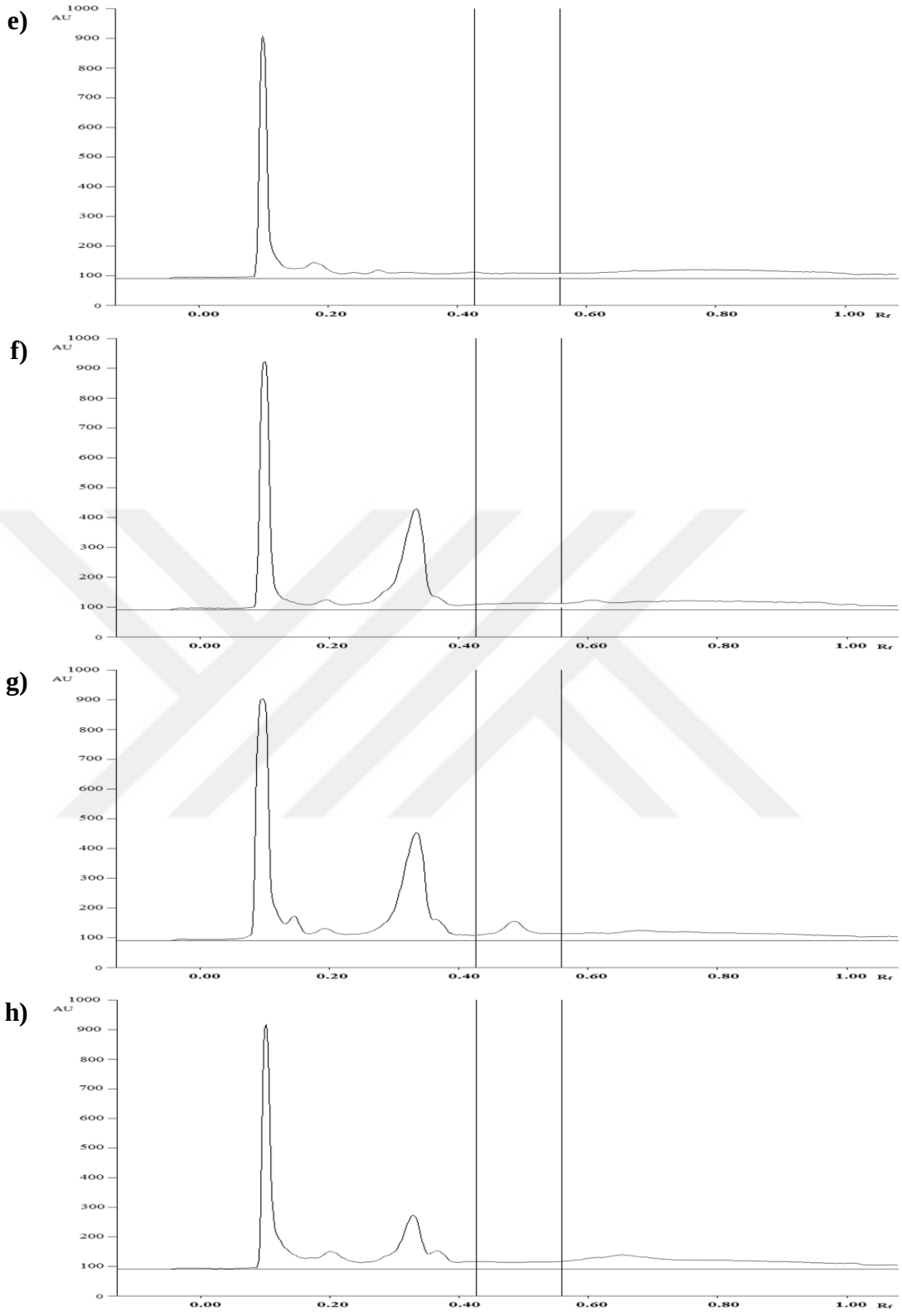


Figure 4.9. HPTLC densitograms of the extracts from different *Hypericum* species at 310 nm (continued). (e) *H. cerastoides*; (f) *H. perfoliatum*; (g) *H. triquetrifolium*; (h) *H. kotschyanum* (10 µg/band).

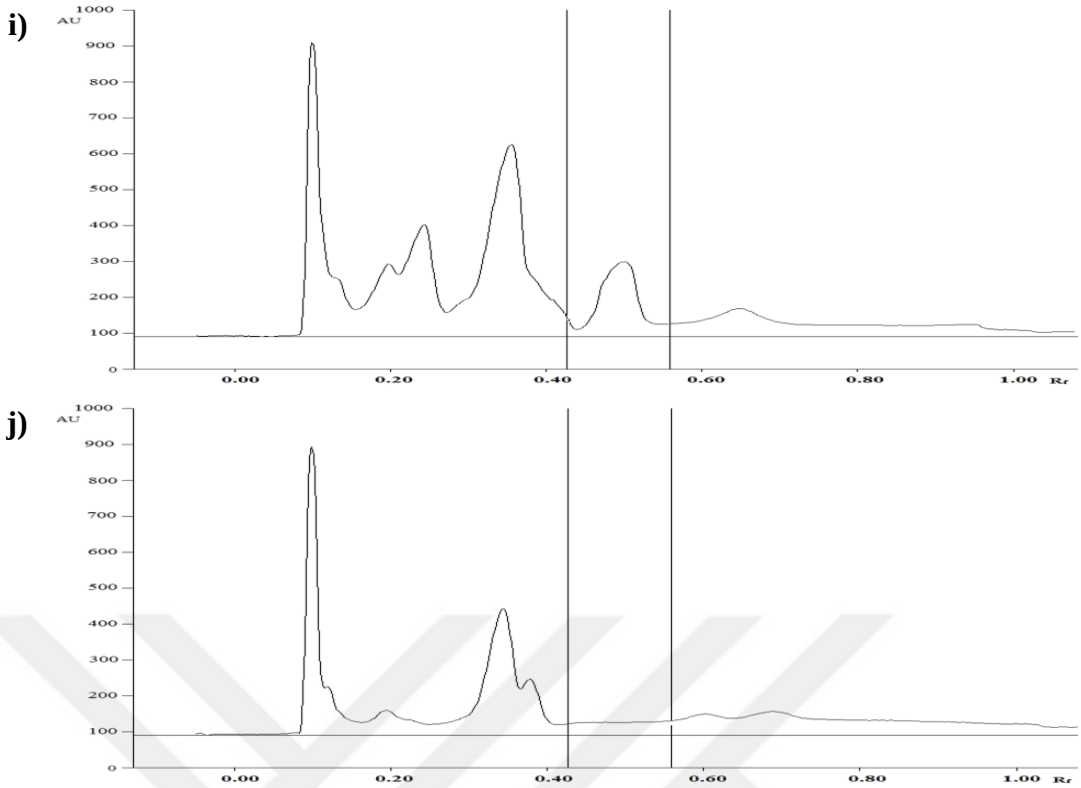


Figure 4.9. HPTLC densitograms of the extracts from different *Hypericum* species at 310 nm (continued). (i) *H. scabrum*; (j) *H. thymopsis* (10 µg/band).

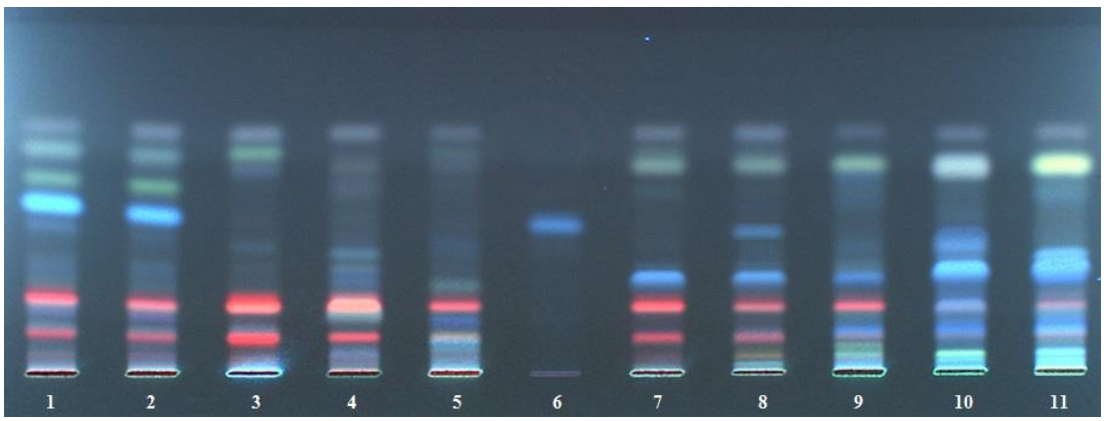


Figure 4.10. HPTLC chromatogram of the extracts from *Hypericum* species at 366 nm. Tracks: (1) *H. perforatum* (İstanbul); (2) *H. perforatum* (Tekirdağ); (3) *H. bithynicum*; (4) *H. calycinum*; (5) *H. cerastoides*; (6) HyF (reference) (0.4 µg/band); (7) *H. perfoliatum*; (8) *H. triquetrifolium*; (9) *H. kotschyanum*; (10) *H. scabrum*; (11) *H. thymopsis*; applied volume of *Hypericum* species: 10 µg/band; derivatization: H_2SO_4 reagent.

According to HPTLC densitograms and chromatogram, it was indicated that HyF was identified in *H. perforatum* obtained from two different locations, *H. triquetrifolium* and *H. scabrum* specimens between the ranges from 6.40 to 26.40 mg/g (Table 4.8). Although the amount of HyF in two *H. perforatum* samples was quite similar [26.20 mg/g for İstanbul and 26.40 mg/g for Tekirdağ], these values were lower than that of the *H. perforatum* L. reference sample [36.03 mg/g]. In addition, the densitograms and chromatograms of these two *H. perforatum* specimens were overlapping with those of *H. perforatum* L. reference sample. Nevertheless, HyF was not found in *H. bithynicum*, *H. calycinum*, *H. cerastoides*, *H. perfoliatum*, *H. kotschyanum* and *H. thymopsis* specimens.

4.2.4. Hyperforin Assay in the Marketed *H. perforatum* (SJW) Products

The presence of HyF in the *Hypericum* products marketed as tea, capsule or dried herbal material was detected by comparison of their R_f values and relevant peaks with those of HyF reference. The analysis was conducted in triplicate. The amounts of HyF determined in these products were given as mean±SD in Table 4.9, while related HPTLC densitograms (Figure 4.11, Figure 4.13a, Figure 4.14) and fingerprints (Figure 4.12, Figure 4.13b, Figure 4.15) were also presented below.

Table 4.9. HyF contents in marketed *H. perforatum* (SJW) products

Commercial product	HyF (mg/g)	Commercial product	HyF (mg/g)
HT1	8.20±0.03	HS13	<LOQ
HT2	8.90±0.01	HS14	20.77±0.04
HT3	<LOQ	HS15	<LOQ
HT4	14.30±0.01	HS16	<LOQ
HT5	6.20±0.07	HS17	ndt
HT6	ndt	HS18	<LOQ
HT7	<LOQ	HS19	23.76±0.02
HT8	ndt	HS20	<LOQ
HC	17.90±0.02	HS21	<LOQ
HS1	10.17±0.01	HS22	26.52±0.07
HS2	ndt	HS23	ndt
HS3	14.95±0.03	HS24	ndt
HS4	<LOQ	HS25	14.41±0.01
HS5	27.95±0.01	HS26	ndt
HS6	30.57±0.05	HS27	35.36±0.02
HS7	<LOQ	HS28	ndt
HS8	ndt	HS29	<LOQ
HS9	14.88±0.01	HS30	<LOQ
HS10	<LOQ	HS31	<LOQ
HS11	9.10±0.04	HS32	16.50±0.01
HS12	26.39±0.05	HS33	10.39±0.03

ndt: HyF not detected; <LOQ: under the limit of quantitation

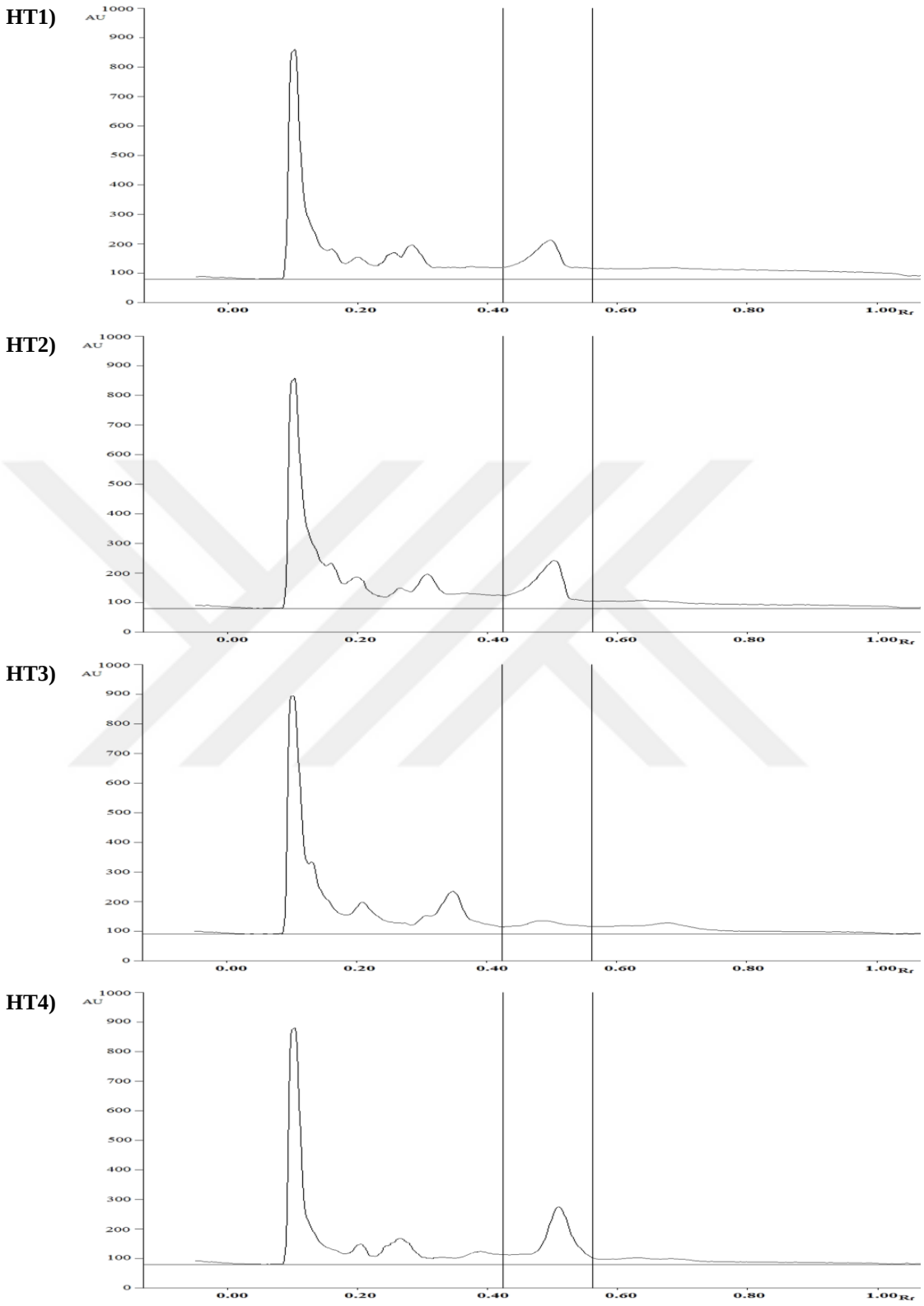


Figure 4.11. HPTLC densitograms from *H. perforatum* (SJW) tea products at 310 nm. Test solutions of tea products: 20 µg/band.

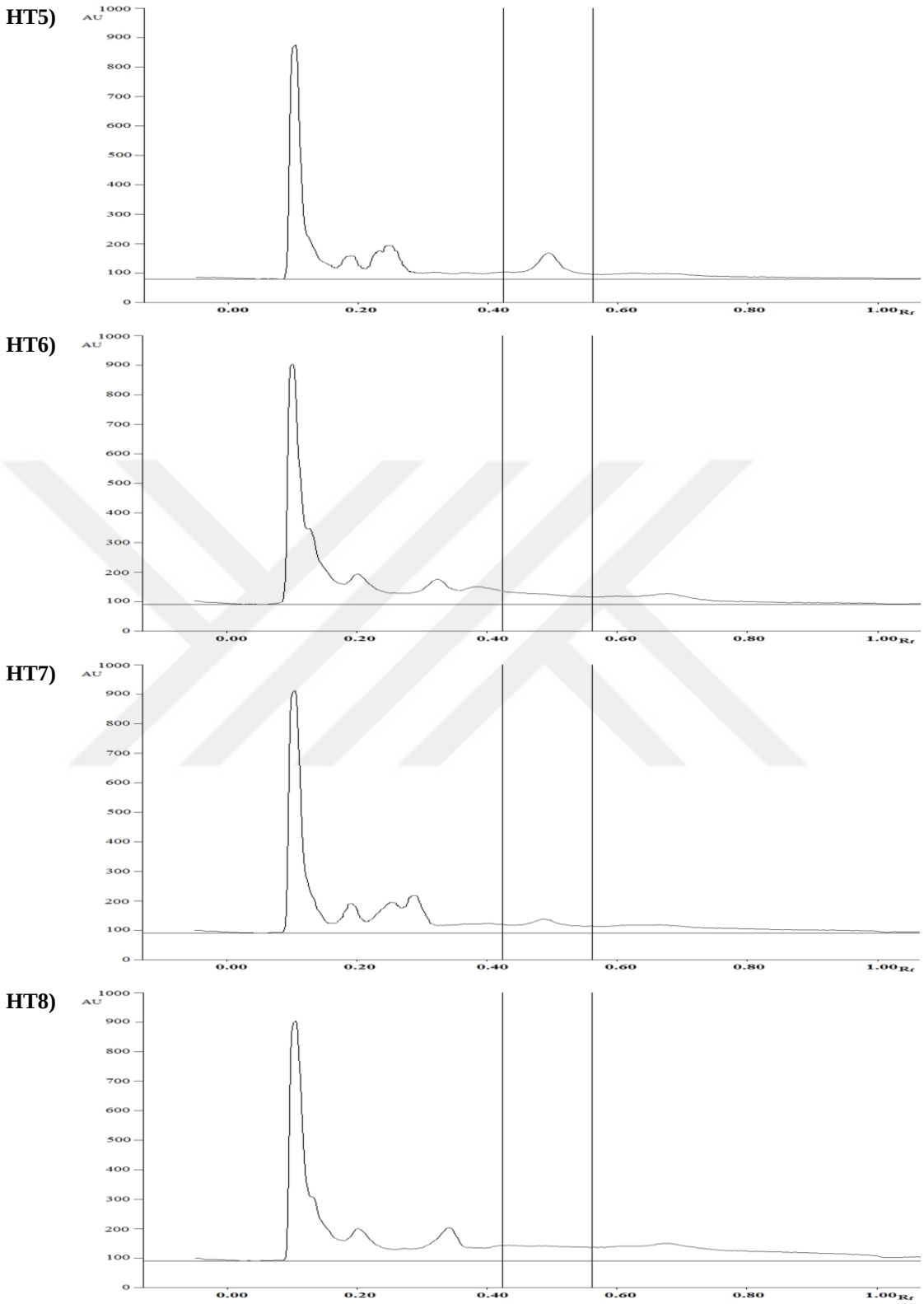


Figure 4.11. HPTLC densitograms from *H. perforatum* (SJW) tea products at 310 nm (continued). Test solutions of tea products: 20 µg/band.

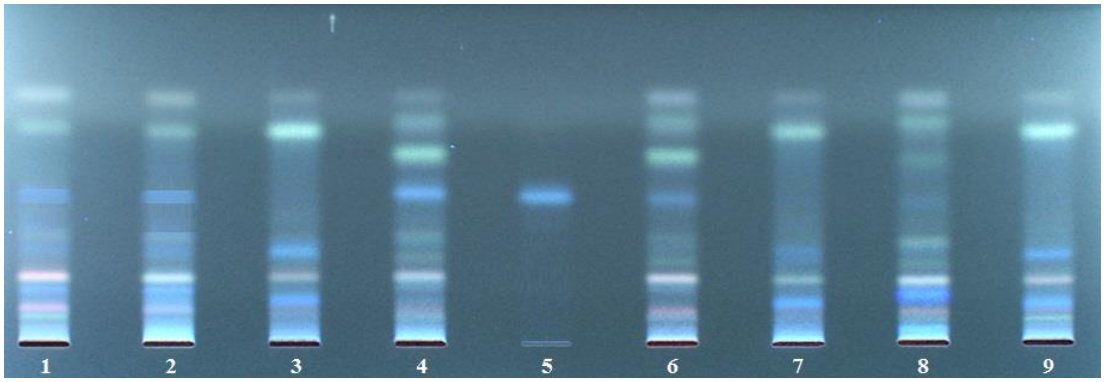
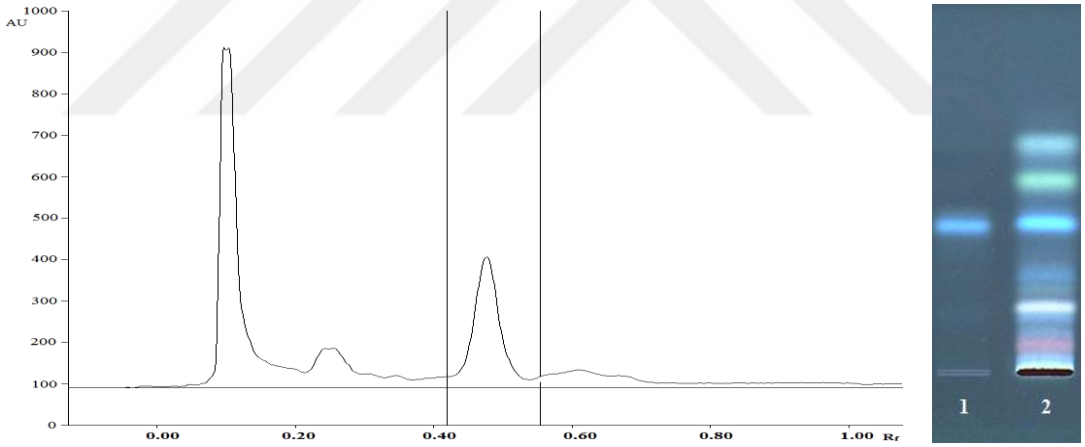


Figure 4.12. HPTLC chromatogram from *H. perforatum* (SJW) tea products at 366 nm. Tracks: (1) HT1; (2) HT2; (3) HT3; (4) HT4; (5) HyF reference (0.4 µg/band); (6) HT5; (7) HT6; (8) HT7; (9) HT8; applied volume of *H. perforatum* (SJW) tea products: 20 µg/band; derivatization: H_2SO_4 reagent.



(a) **(b)**

Figure 4.13. (a) HPTLC densitogram obtained from *H. perforatum* (SJW) capsule formulation at 310 nm (10 µg/band) and (b) HPTLC chromatogram of *H. perforatum* (SJW) capsule formulation at 366 nm. Tracks: (1) HyF reference (0.4 µg/band); (2) HC (10 µg/band); derivatization: H_2SO_4 reagent.

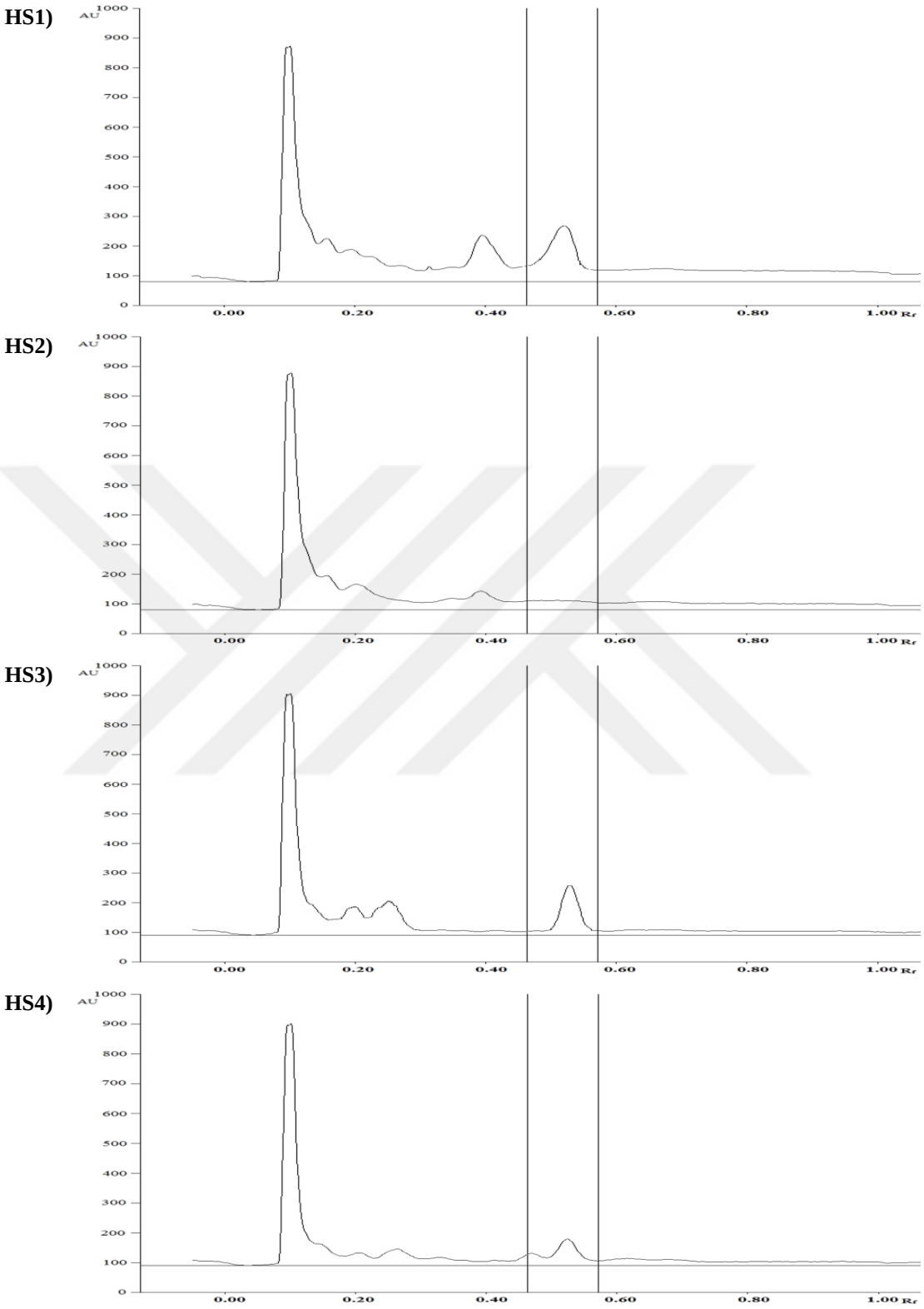


Figure 4.14. HPTLC densitograms from the marketed *H. perforatum* (SJW) herbal materials at 310 nm. Sample test solutions: 20 µg/band.

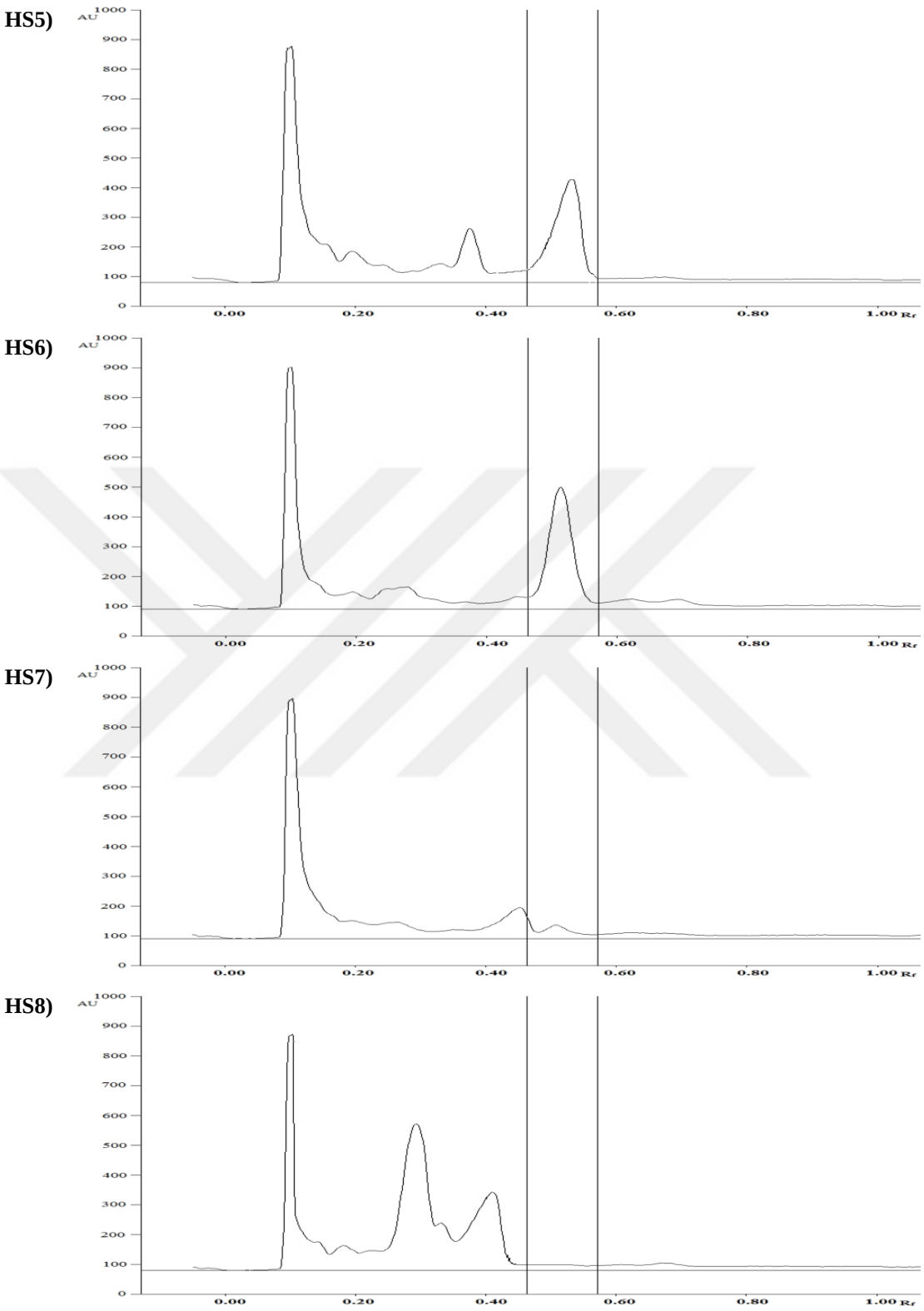


Figure 4.14. HPTLC densitograms from the marketed *H. perforatum* (SJW) herbal materials at 310 nm (continued). Sample test solutions: 20 µg/band.

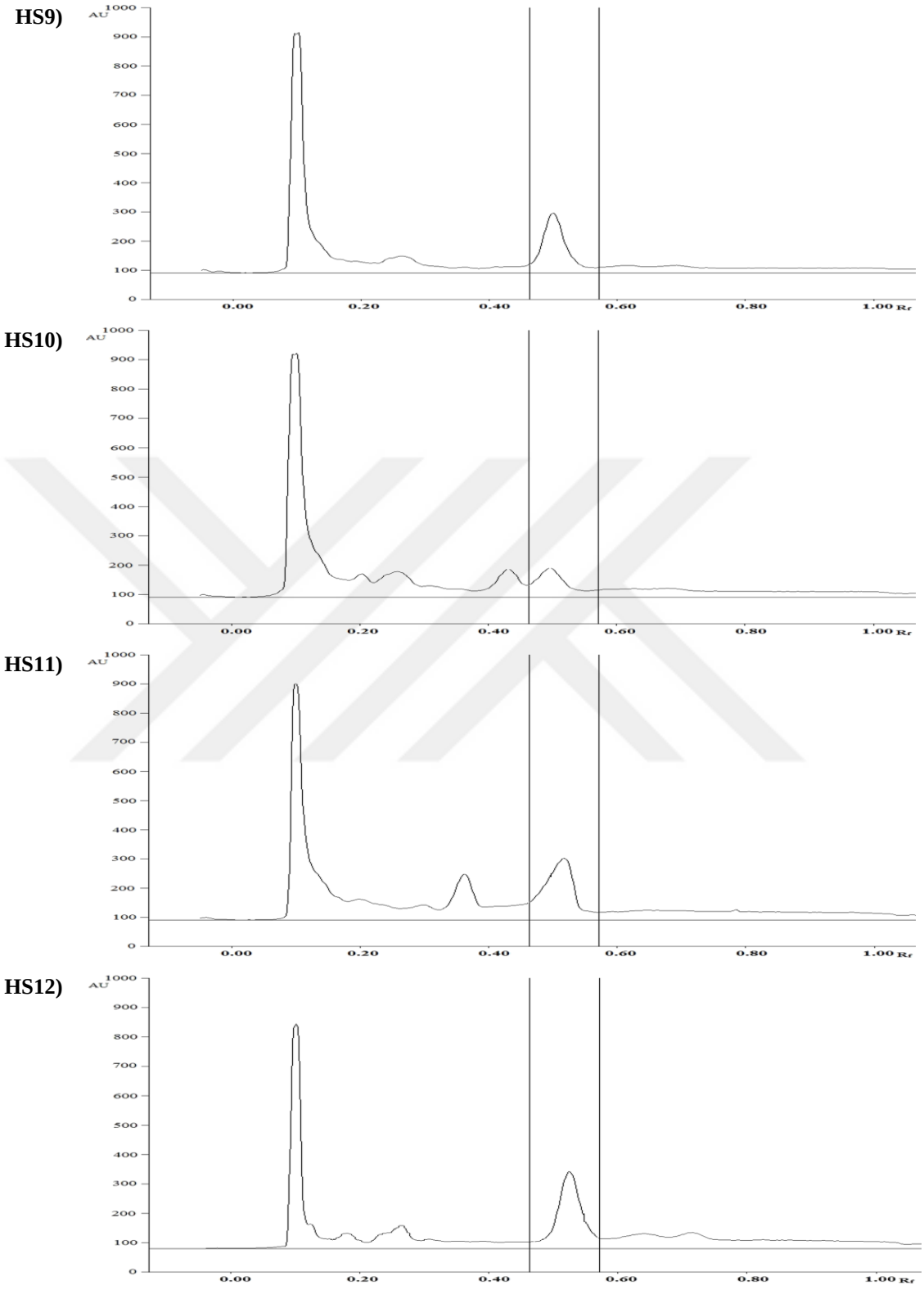


Figure 4.14. HPTLC densitograms from the marketed *H. perforatum* (SJW) herbal materials at 310 nm (continued). Sample test solutions: 20 µg/band.

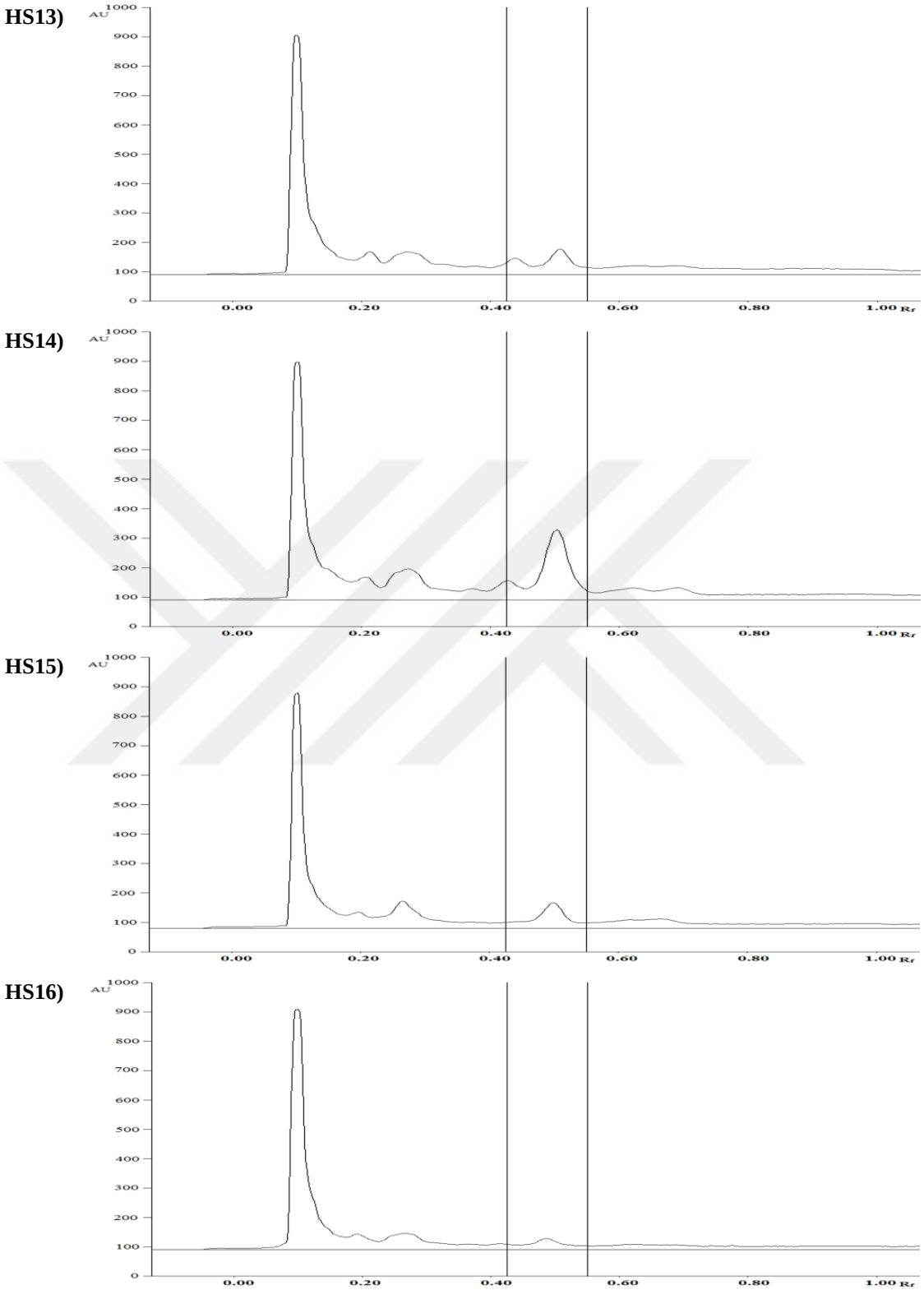


Figure 4.14. HPTLC densitograms from the marketed *H. perforatum* (SJW) herbal materials at 310 nm (continued). Sample test solutions: 20 µg/band.

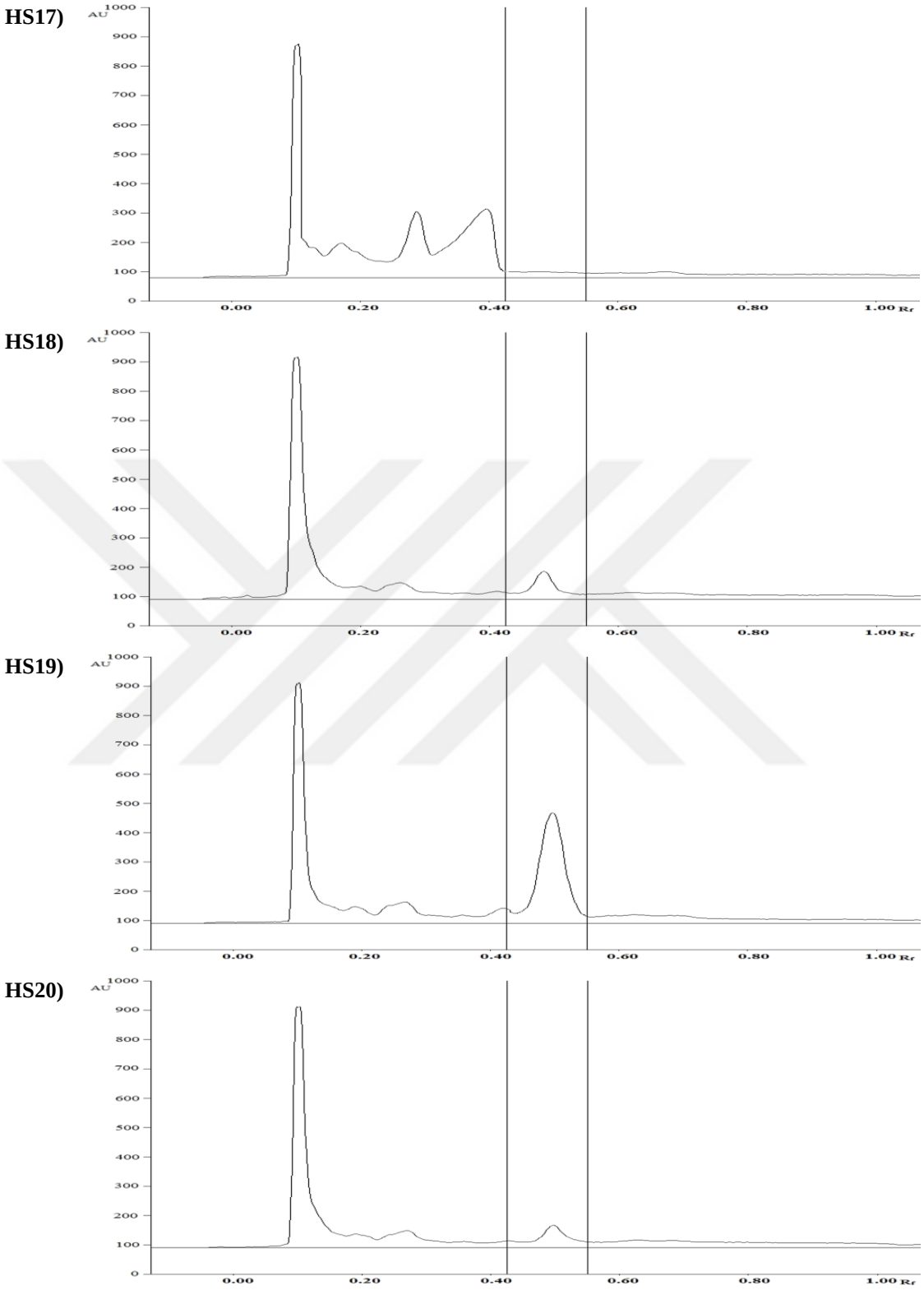


Figure 4.14. HPTLC densitograms from the marketed *H. perforatum* (SJW) herbal materials at 310 nm (continued). Sample test solutions: 20 µg/band.

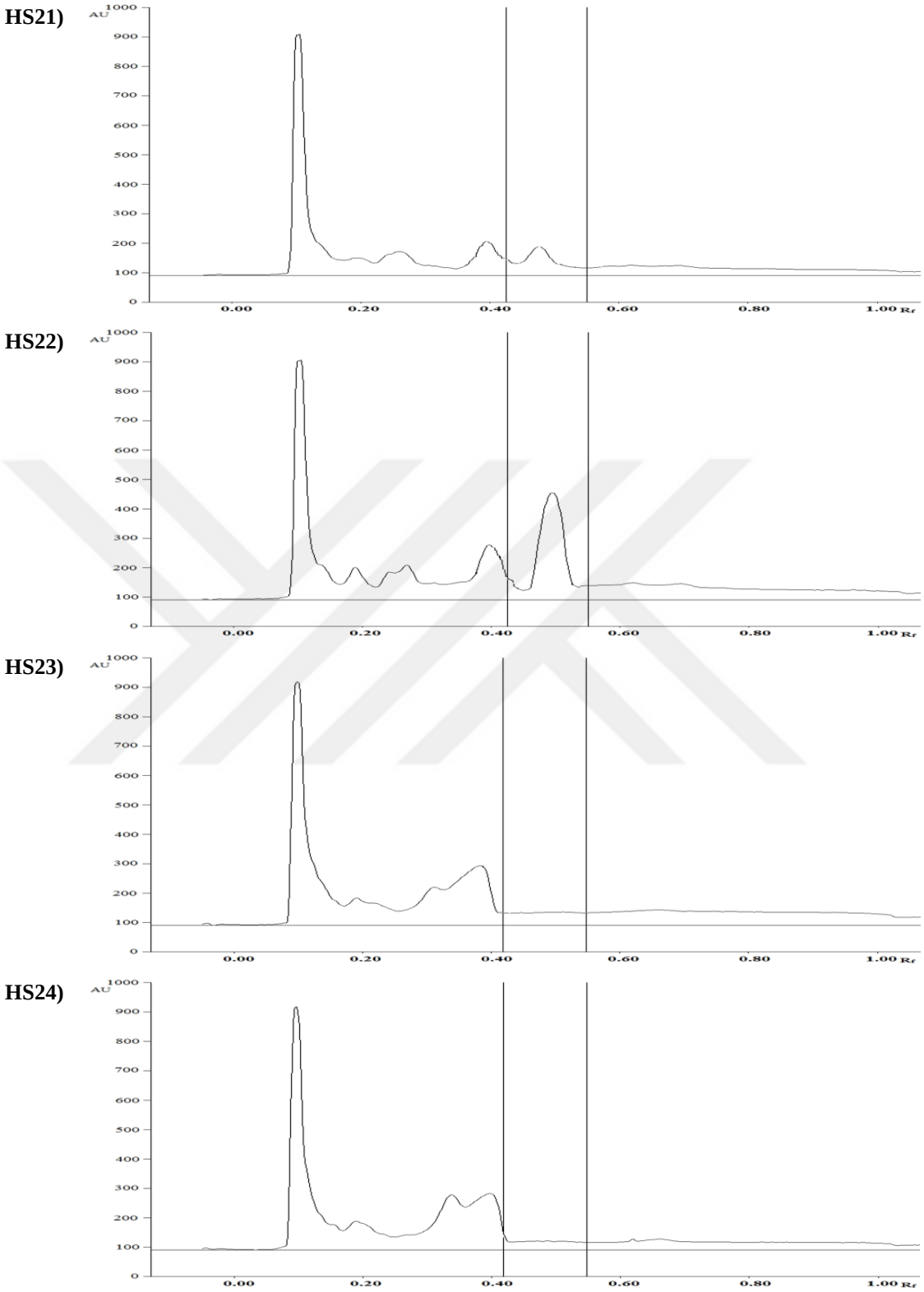


Figure 4.14. HPTLC densitograms from the marketed *H. perforatum* (SJW) herbal materials at 310 nm (continued). Sample test solutions: 20 µg/band.

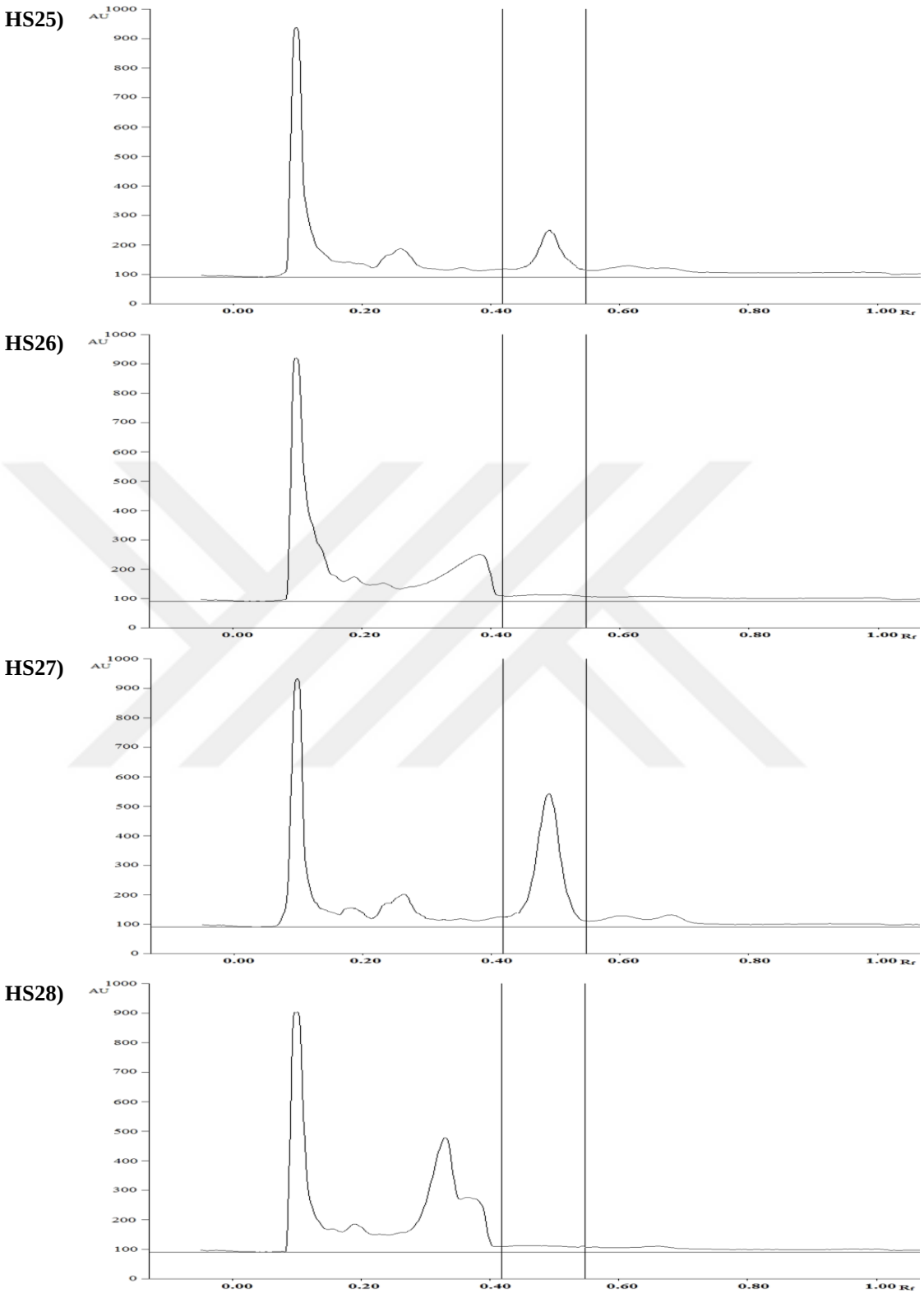


Figure 4.14. HPTLC densitograms from the marketed *H. perforatum* (SJW) herbal materials at 310 nm (continued). Sample test solutions: 20 µg/band.

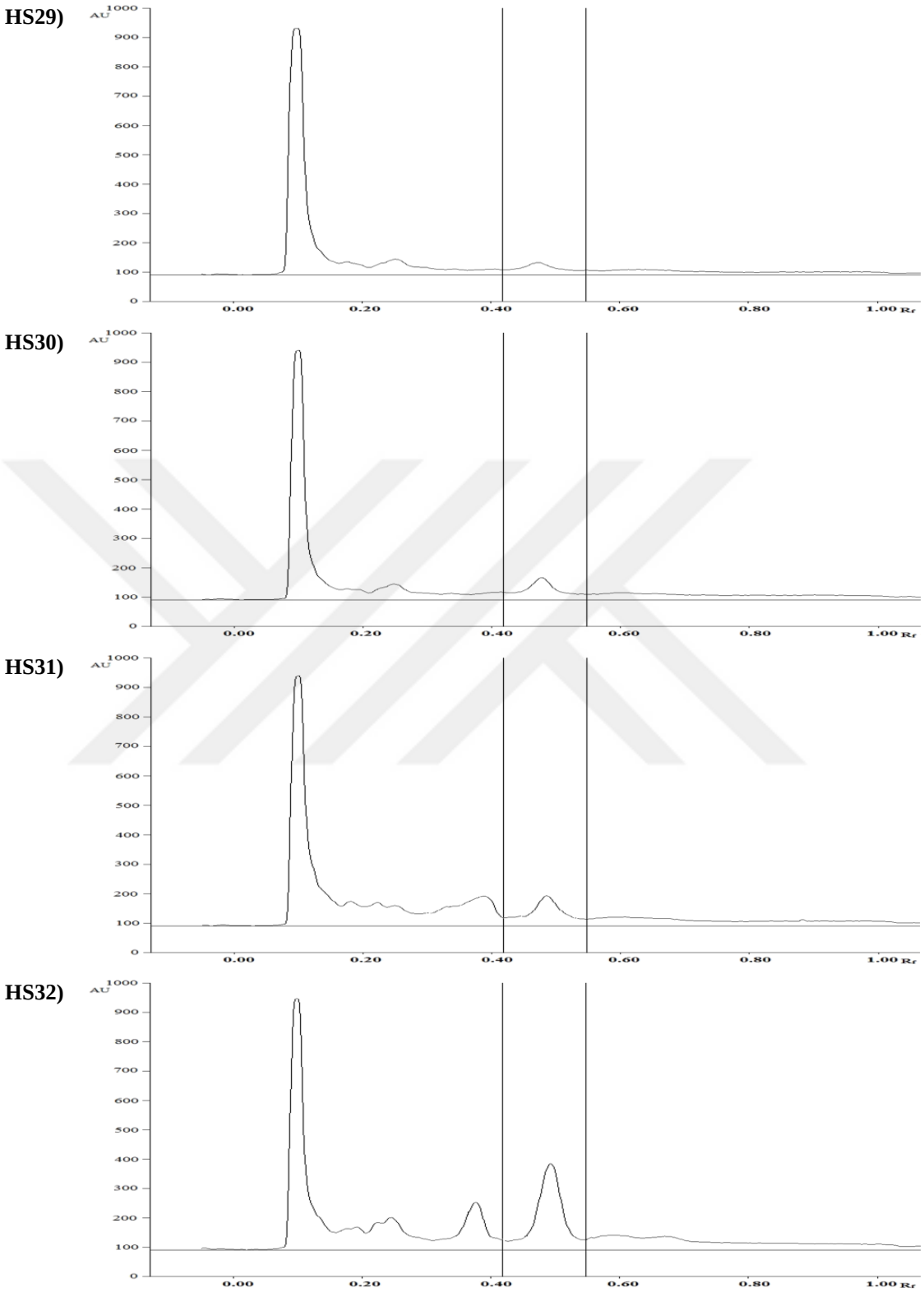


Figure 4.14. HPTLC densitograms from the marketed *H. perforatum* (SJW) herbal materials at 310 nm (continued). Sample test solutions: 20 µg/band.

HS33)

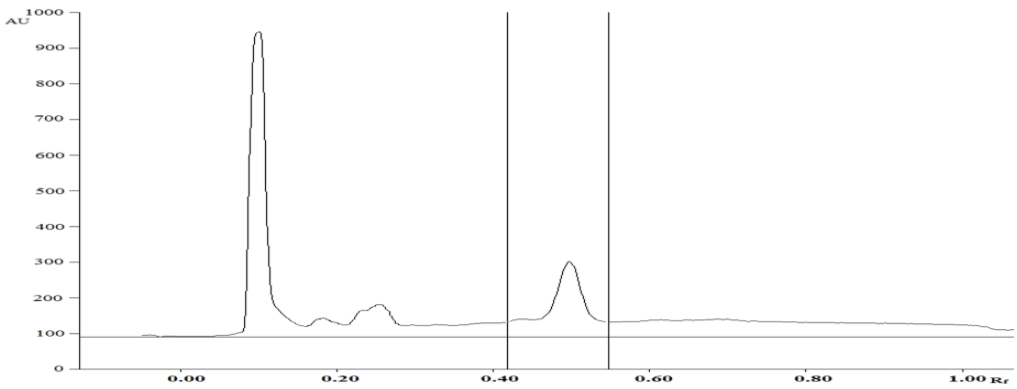


Figure 4.14. HPTLC densitograms from the marketed *H. perforatum* (SJW) herbal materials at 310 nm (continued). Sample test solutions: 20 µg/band.

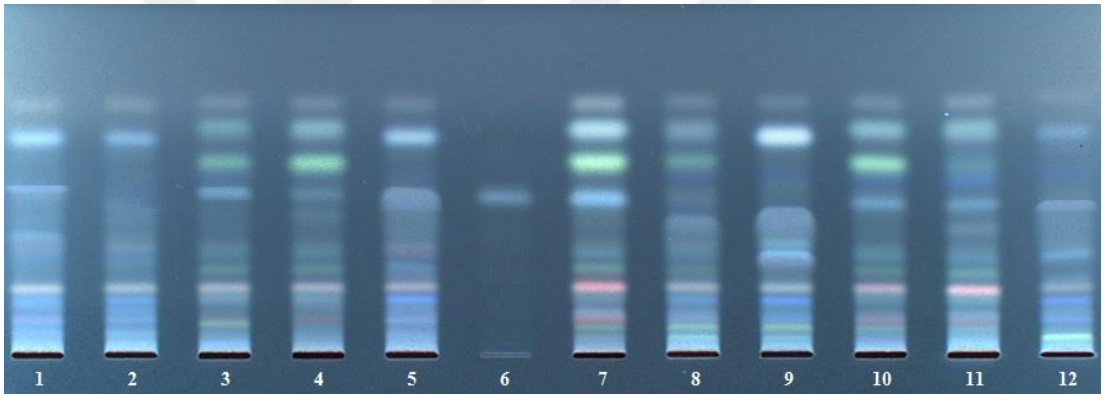


Figure 4.15. HPTLC chromatogram of the marketed *H. perforatum* (SJW) herbal materials at 366 nm. Tracks: (1) HS1; (2) HS2; (3) HS3; (4) HS4; (5) HS5; (6) HyF reference (0.4 µg/band); (7) HS6; (8) HS7; (9) HS8; (10) HS9; (11) HS10; (12) HS11; applied volume of *H. perforatum* (SJW) herbal samples: 20 µg/band; derivatization: H_2SO_4 reagent.

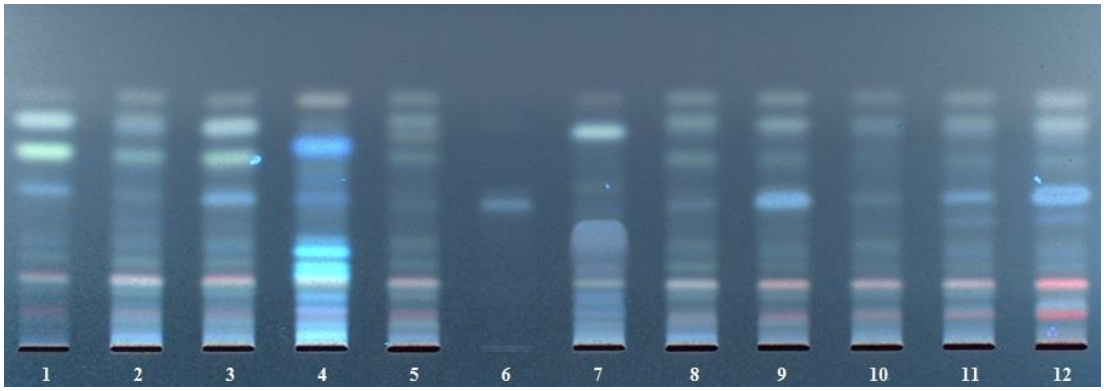


Figure 4.15. HPTLC chromatogram of the marketed *H. perforatum* (SJW) herbal materials at 366 nm (continued). Tracks: (1) HS12; (2) HS13; (3) HS14; (4) HS15; (5) HS16; (6) HyF reference (0.4 µg/band); (7) HS17; (8) HS18; (9) HS19; (10) HS20; (11) HS21; (12) HS22; applied volume of *H. perforatum* (SJW) herbal samples: 20 µg/band; derivatization: H_2SO_4 reagent.

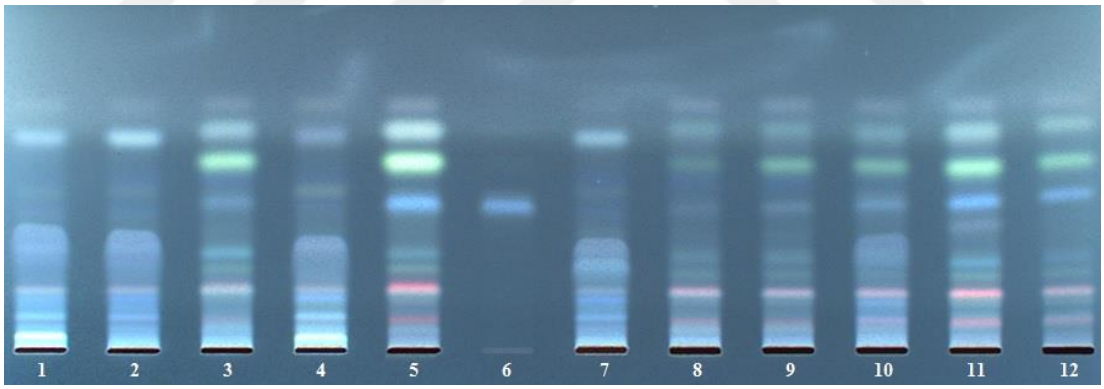


Figure 4.15. HPTLC chromatogram of the marketed *H. perforatum* (SJW) herbal materials at 366 nm (continued). Tracks: (1) HS23; (2) HS24; (3) HS25; (4) HS26; (5) HS27; (6) HyF reference (0.4 µg/band); (7) HS28; (8) HS29; (9) HS30; (10) HS31; (11) HS32; (12) HS33; applied volume of *H. perforatum* (SJW) herbal samples: 20 µg/band; derivatization: H_2SO_4 reagent.

As shown in Figure 4.11 to Figure 4.15, HyF was detected in HT1-5, HT7, HC, HS1, HS3-7, HS9-16, HS18-22, HS25, HS27, and HS29-33. Furthermore, HT1-5 and HT7, HS3, HS6, HS12, HS27 and HS33 displayed similar HPTLC densitograms to those of the reference *H. perforatum* and wild *H. perforatum* samples under 310 nm-light. Additionally, HT6, HT8 and the remaining 28 *Hypericum* samples exhibited different densitograms with those of the reference *H. perforatum* and wild *H. perforatum* samples. On the other hand, a quite similar fingerprint densitogram and chromatogram was observed for HS28 with that of *H. thymopsis* sample. Besides, the chromatograms and densitograms of each group of HS5 and HS11; HS9 and HS25; HS10 and HS21; HS14 and HS19; HS16, HS18, HS20, HS29 and HS30; HS23 and HS24 were found to be almost identical, respectively.

Regarding to Table 4.9, in several commercial *Hypericum* (SJW) products, HyF was not detected or remained below the LOQ. In addition, the amount of HyF detected by the developed HPTLC system in four SJW tea products was between 6.20 to 14.30 mg/g. However, HyF content determined in SJW capsule formulation [17.90 mg/g] was found to be higher comparing to the tea products. Besides, HyF contents of marketed *H. perforatum* (SJW) herbal materials were ranging from 9.10 to 35.36 mg/g and the amount quantified in HS3, HS5, HS6, HS9, HS12, HS14, HS19, HS22, HS25, HS27 and HS32 samples was higher than those in the tea products.

4.2.5. Evaluation of Hyperforin Content in Aqueous Extracts from *H. perforatum*

The presence of HyF in aqueous extracts from the flowering aerial parts of *H. perforatum* L. cultivar was detected by the comparison of their R_f values and relevant peaks with that of HyF reference. The analysis was performed with three replicates. The results were also supported by HPTLC densitograms and fingerprints from the aqueous extracts of *H. perforatum* L. reference sample (Figure 4.16). As shown in HPTLC densitograms and chromatogram there was no spot corresponding to HyF in HP-W5 and HP-W10 samples.

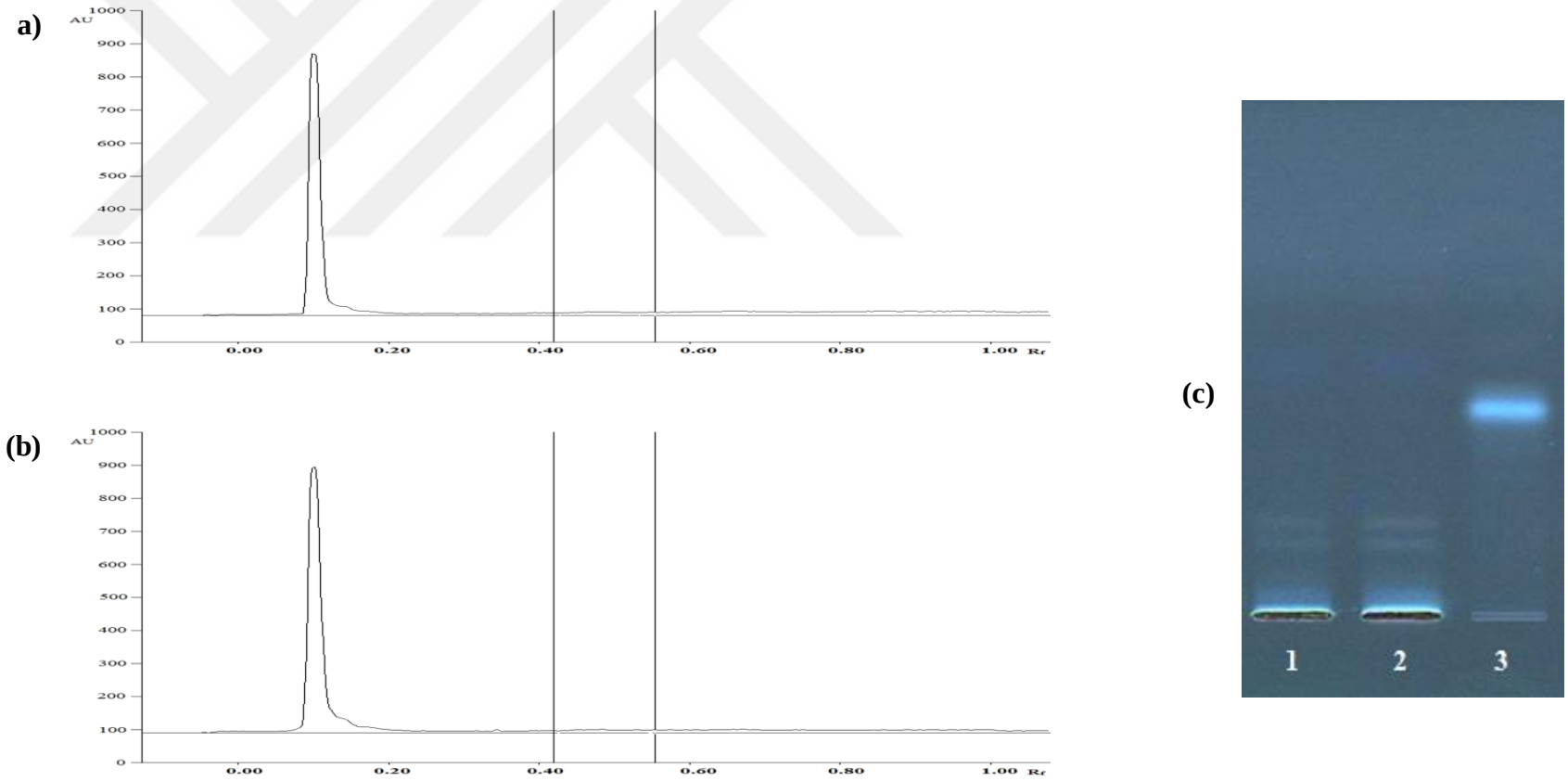


Figure 4.16. HPTLC densitograms obtained from the aqueous extracts of *H. perforatum* L. cultivar: (a) HP-W5 and (b) HP-W10 at 310 nm (20 µg/band); (c) HPTLC chromatogram of the aqueous extracts of *H. perforatum* L. cultivar at 366 nm.

Tracks: (1) HP-W5 (20 µg/band); (2) HP-W10 (20 µg/band); (3) HyF reference (0.4 µg/band); derivatization: H_2SO_4 reagent.

4.2.6. Evaluation of Hyperforin Content in Solvent Extracts from Oily Preparation of *H. perforatum*

Quantification of HyF contents in acetonitrile, methanol-hexane, acetone, and methanol extracts from the oily preparation of *H. perforatum* were examined by the developed HPTLC method. The analysis was conducted in triplicate. The amounts of HyF detected in four different extracts were given as mean±SD as the average of triplicate analysis in Table 4.10.

Table 4.10. HyF contents in different solvent extracts from the oily preparation of *H. perforatum*

Solvent extract	**HyF (µg/100 mg oil)**
Acetonitrile	2.94±0.10
Methanol-hexane	1.68±0.24
Acetone	<LOQ
Methanol	2.56±0.17

<LOQ: under the limit of quantitation

Moreover, the HPTLC densitograms (Figure 4.17) and chromatographic image (Figure 4.18) of these solvent extracts from the oily preparation of *H. perforatum* were displayed below.

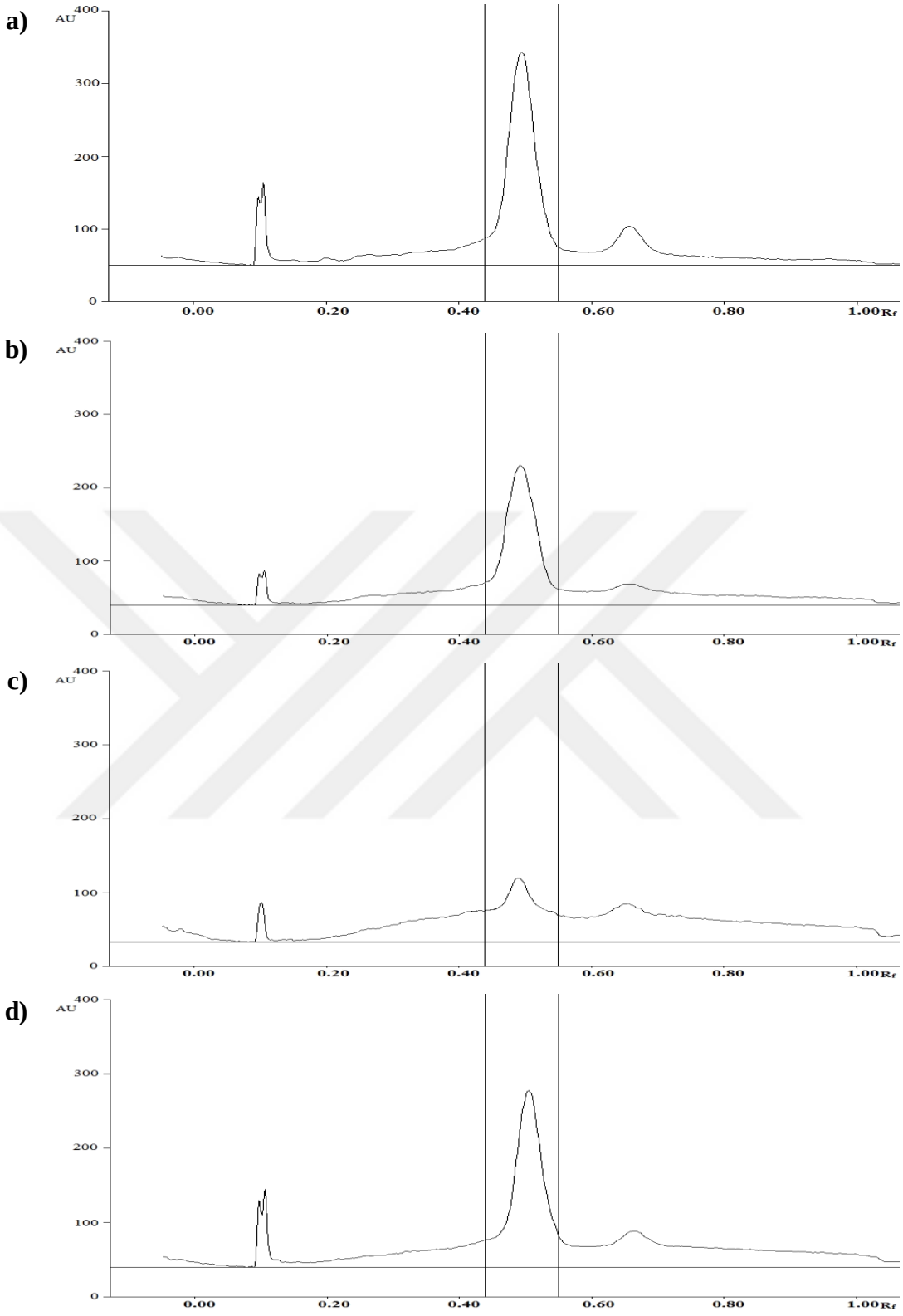


Figure 4.17. HPTLC densitograms obtained with the developed method for HyF at 310 nm extracted with various solvents from the oily preparation of *H. perforatum* (0.2 mg/band): (a) acetonitrile extract; (b) methanol-hexane extract; (c) acetone extract; (d) methanol extract.

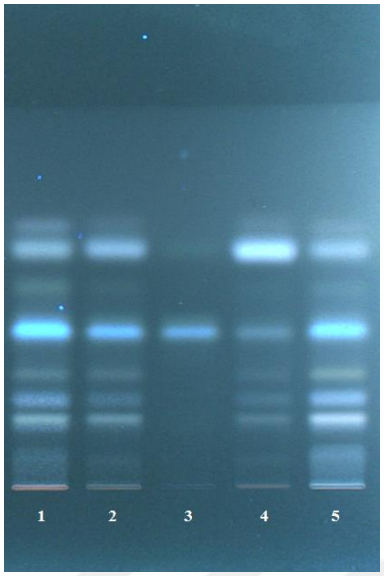


Figure 4.18. HPTLC chromatogram obtained with the developed method for HyF at 366 nm extracted with various solvents from the oily preparation of *H. perforatum*. Tracks: (1) acetonitrile extract; (2) methanol-hexane extract; (3) HyF (reference); (4) acetone extract; (5) methanol extract; derivatization: H_2SO_4 reagent.

The presence of HyF in the oily preparation of *H. perforatum* was investigated by the comparison of their R_f values and peaks with that of HyF reference. According to Figure 4.18, when HPTLC chromatogram was examined, it was observed that all analyzed solvent extracts from the oily preparation of *H. perforatum* were found to contain HyF. As shown in Table 4.10, HyF contents in four different solvent extracts from the oily preparation of *H. perforatum* showed significant variations. In fact, the determined quantity of HyF was less than the LOQ in acetone extract, while the highest HyF content was determined in acetonitrile extract (Figure 4.17). Therefore, acetonitrile was employed for the extraction of HyF from the oily preparations of *H. perforatum* in analytical assays.

4.2.7. Evaluation of Hyperforin Content in Commercial *H. perforatum* (SJW) Oily Products

The presence of HyF in the acetonitrile extracts obtained from the commercial *Hypericum* oily products was identified by comparison of their R_f values and peaks with that of HyF reference. The HPTLC analysis was carried out in three replicates and the amounts of HyF determined in these products were expressed as mean±SD in Table 4.11. HPTLC densitograms (Figure 4.19) and the related chromatographic image (Figure 4.20) were given below.

Table 4.11. HyF contents in the acetonitrile extracts from marketed SJW oily products

Oily product	HyF (µg/100 mg oil)
OE1	1.17±0.19
OE2	1.52±0.41
OE3	1.23±0.29
OE4	0.74±0.16
OE5	<LOQ
OE6	<LOQ
OE7	ndt

ndt: HyF not detected; <LOQ: under the limit of quantitation

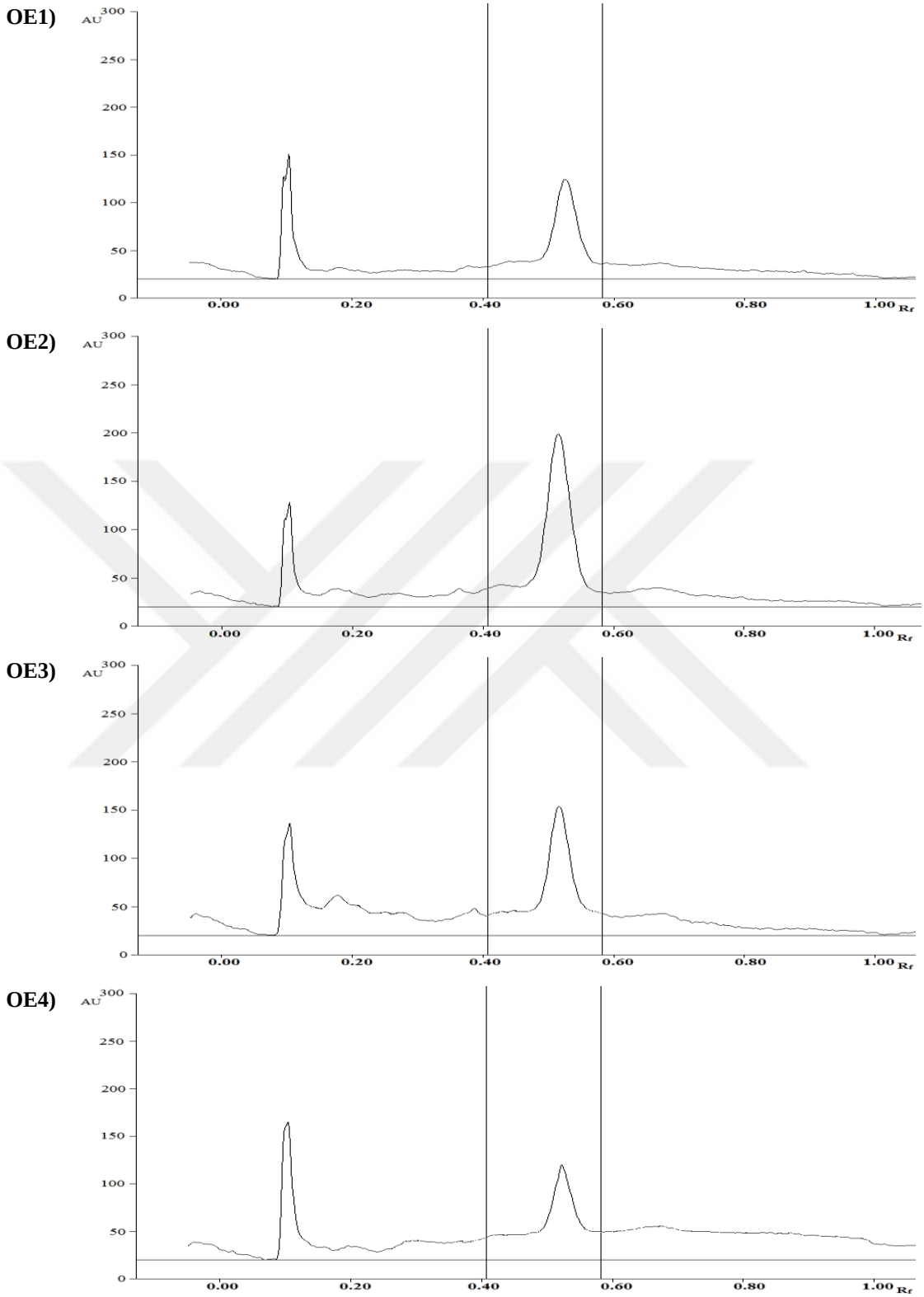


Figure 4.19. HPTLC densitograms from *H. perforatum* (SJW) oily products at 310 nm. Test solutions of oily products: 0.4 mg/band.

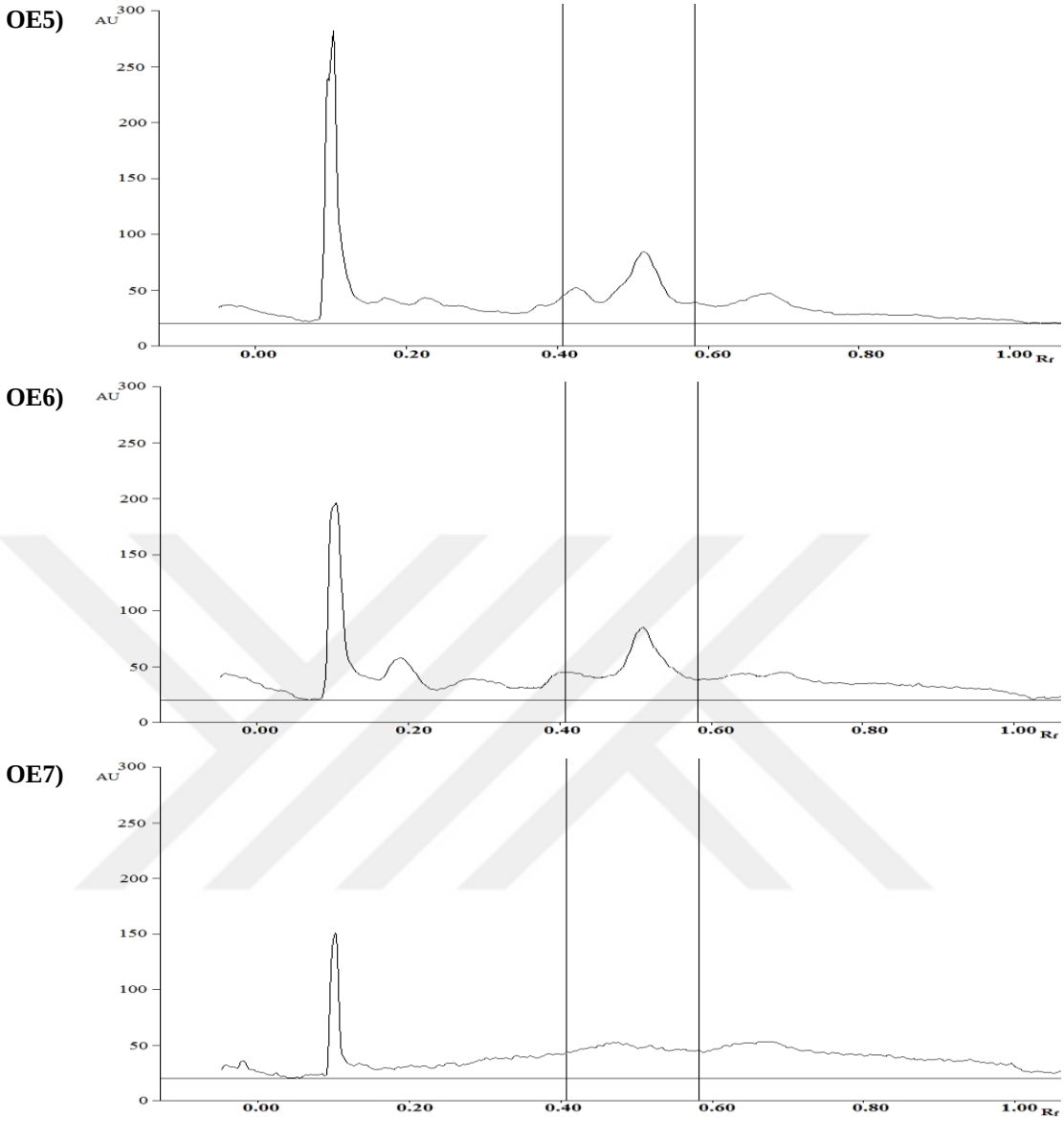


Figure 4.19. HPTLC densitograms from *H. perforatum* (SJW) oily products at 310 nm (continued). Test solutions of oily products: 0.4 mg/band.

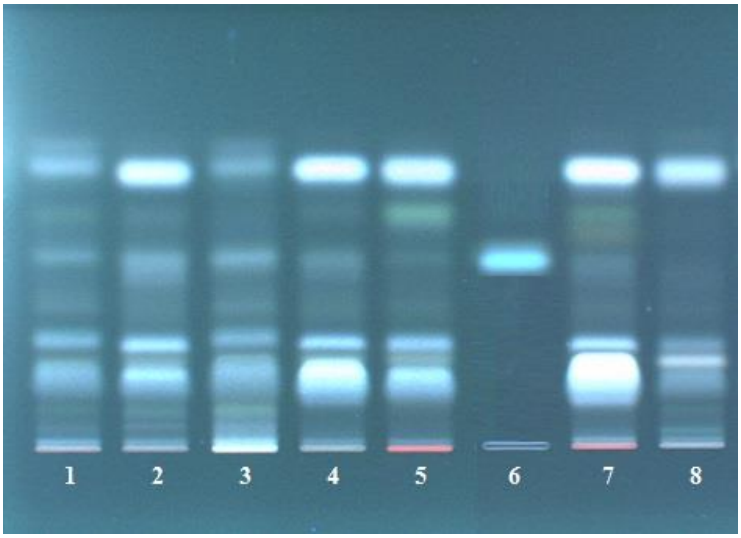


Figure 4.20. HPTLC chromatogram of the marketed *H. perforatum* (SJW) oily products at 366 nm. Tracks: (1) OE1; (2) OE2; (3) OE3; (4) OE4 ; (5) OE5; (6) HyF (reference); (7) OE6 ; (8) OE7; derivatization: H_2SO_4 reagent.

The existence of HyF in commercial *H. perforatum* (SJW) oily products was investigated by the comparison of their R_f values and peaks with that of HyF reference. As shown in Figure 4.20, it was detected that OE1-6 extracts were found to contain HyF whereas HyF was not observed from HPTLC fingerprint of OE7 at 366 nm. The highest HyF content was determined in OE2 (Figure 4.19 and Table 4.11).

4.3. Analysis of Hypericin Content in Samples

4.3.1. HPLC Analysis of Hypericin

The system suitability was confirmed for HPLC method of hypericin standard according to the Ph. Eur. (234). In this study, retention time (t_R) of hypericin in the reference solution was determined to be 10.36±0.04 (n=3) (Figure 4.21). The identity of hypericin in the sample solution was approved by competing t_R with that of the standard solution and it was found as 10.28±0.02 (n=3) (Figure 4.22). Additionally, the percentage content of hypericin in *H. perforatum* cultivar was determined as 0.19% which was higher than the official limit set at minimum 0.08 per cent (234). For that reason, *H. perforatum* cultivar was considered as a reference material for hypericin analyses.

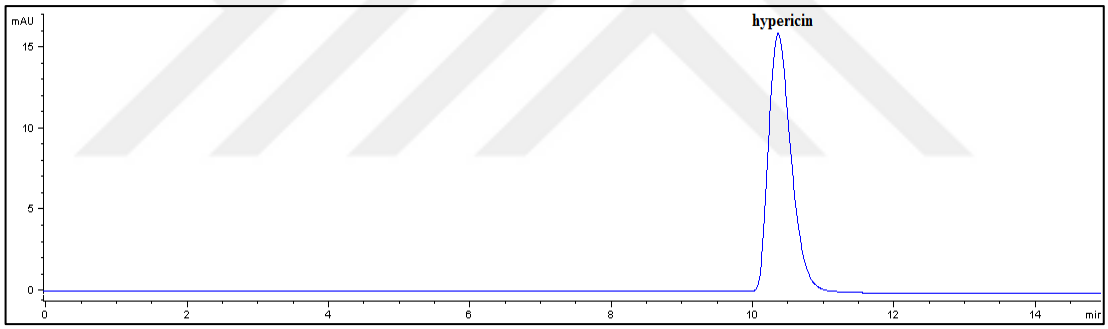


Figure 4.21. HPLC chromatogram of hypericin reference solution at 590 nm

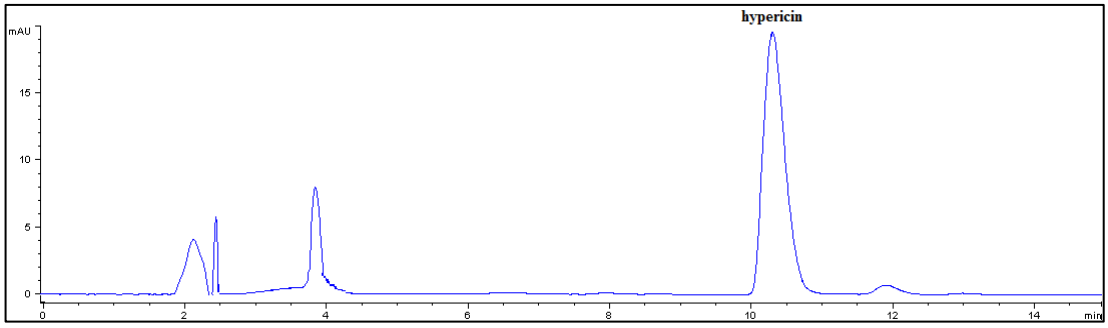


Figure 4.22. HPLC chromatogram of hypericin in *H. perforatum* L. cultivar test solution at 590 nm

4.3.2. HPTLC Analysis of Hypericin Content in Samples

4.3.2.1. Method development for hypericin

The list of mobile phases used in TLC and HPTLC analysis of hypericin (MP-HyP) which were employed in the previously published studies was given in Table 4.12. HPTLC plate images obtained from these eighteen mobile phases were demonstrated in Figure 4.23. During the evaluation of these developing solvent systems 20 min saturated twin-through chamber was utilized.

Table 4.12. Solvent systems tested in HPTLC analysis of hypericin

Mobile phase	Solvent system	Reference
MP-HyP-1	ethyl acetate-formic acid-water (30:2:3)	(234)
MP-HyP-2	ethyl acetate-formic acid-acetic acid-water (100:11:11:26)	(115)
MP-HyP-3	formic acid-water-ethyl acetate (1:0.5:10)	(116)
MP-HyP-4	ethyl acetate-dichloromethane-formic acid-acetic acid-water (100:25:10:10:11)	(116, 122, 124, 125)
MP-HyP-5	ethyl acetate-formic acid-acetic acid-water (100:11:11:27)	(116)
MP-HyP-6	formic acid-water-ethyl acetate (3:4:43)	(116)
MP-HyP-7	ethyl acetate-methanol-formic acid (9:0.8:0.2)	(116)
MP-HyP-8	formic acid-water-ethyl acetate (2:1:17)	(51, 116, 121)
MP-HyP-9	toluene-ethyl acetate-formic acid (5:4:1)	(74, 113)
MP-HyP-10	chloroform-methanol-water (9:4:1)	(116)
MP-HyP-11	water-formic acid-ethyl methyl ketone-ethyl acetate (1:1:3:5)	(116)

Table 4.12. Solvent systems tested in HPTLC analysis of hypericin (continued)

Mobile phase	Solvent system	Reference
MP-HyP-12	formic acid-water-ethyl acetate (1.5:1:15)	(112, 114)
MP-HyP-13	ethyl acetate-formic acid (50:6)	(113)
MP-HyP-14	ethyl acetate-water-methanol (100:16.5:13.5)	(118)
MP-HyP-15	chloroform-water-methanol (61:7:32)	(118)
MP-HyP-16	chloroform-water-methanol (7:0.1:3)	(118)
MP-HyP-17	chloroform-methanol (8:2)	(118)
MP-HyP-18	chloroform-methanol (9:1)	(118)

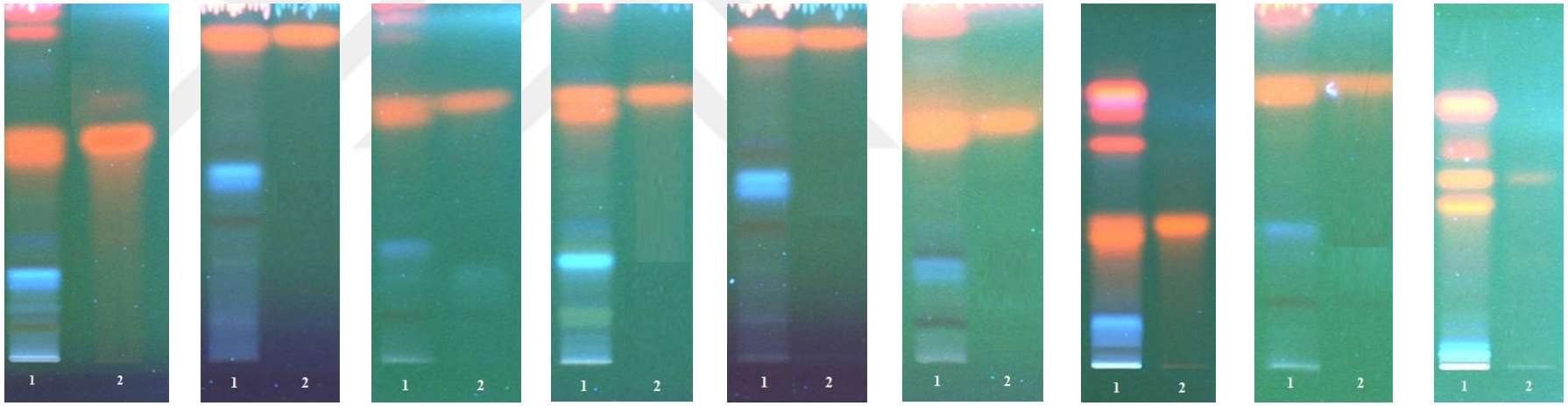
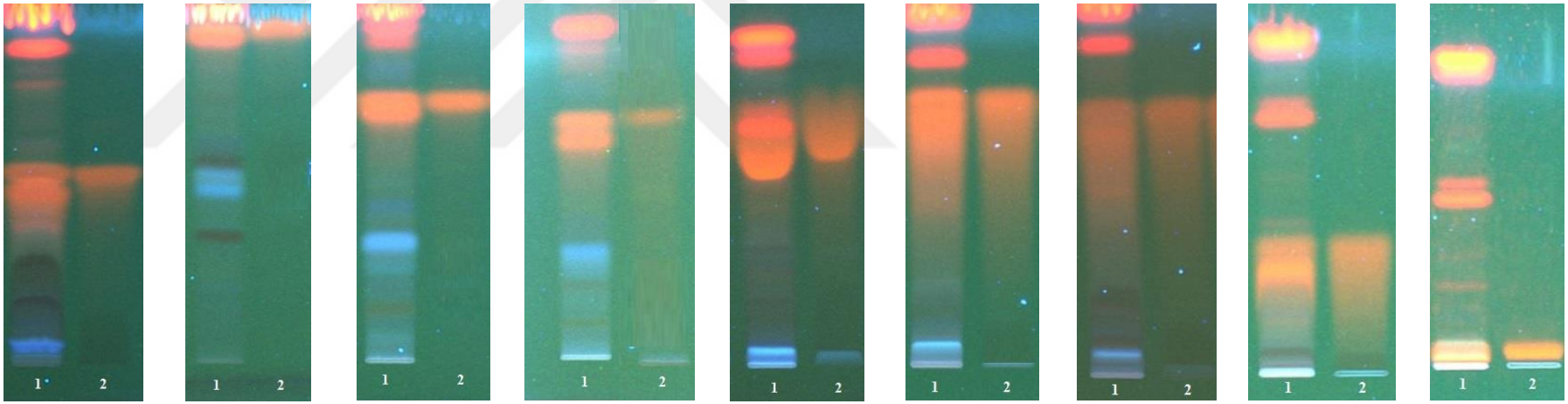


Figure 4.23. HPTLC chromatograms of HyP (reference) and the extract from the flowering aerial parts of *H. perforatum* L. cultivar obtained with various mobile systems at 366 nm.

HPTLC plates were developed by (a) MP-HyP-1; (b) MP-HyP-2; (c) MP-HyP-3; (d) MP-HyP-4; (e) MP-HyP-5; (f) MP-HyP-6; (g) MP-HyP-7; (h) MP-HyP-8; (i) MP-HyP-9. Tracks: (1) *H. perforatum* extract; (2) HyP (reference).



(j) **(k)** **(l)** **(m)** **(n)** **(o)** **(p)** **(q)** **(r)**

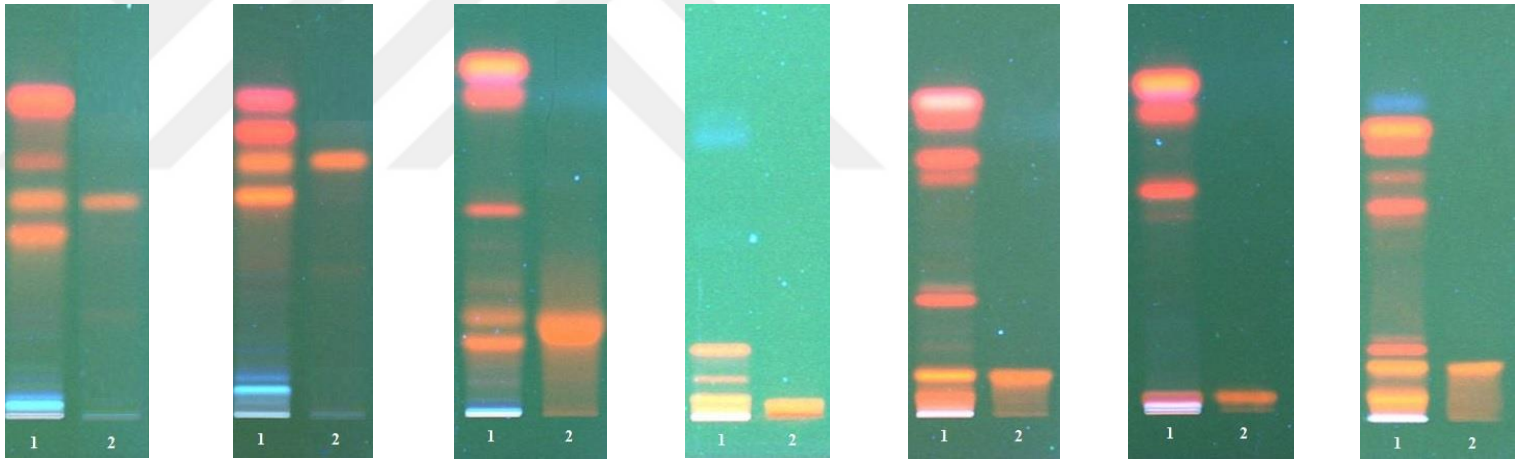
Figure 4.23. HPTLC chromatograms of HyP (reference) and the extract from the flowering aerial parts of *H. perforatum* L. cultivar obtained with various mobile systems at 366 nm (continued).

HPTLC plates were developed by (j) MP-HyP-10; (k) MP-HyP-11; (l) MP-HyP-12; (m) MP-HyP-13; (n) MP-HyP-14; (o) MP-HyP-15; (p) MP-HyP-16; (q) MP-HyP-17; (r) MP-HyP-18. Tracks: (1) *H. perforatum* extract; (2) HyP (reference).

Among the mobile systems reported in the previously published studies (Table 4.12) the best separation was obtained with MP-HyP-9 (Figure 4.23). However, further attempts have been made to improve the separation potential of this solvent system by fluctuations in the ratios of solvents or composition. These solvent systems were given in Table 4.13 and images of the HPTLC plates were presented in Figure 4.24. The best result was established by MP-HyP-30.

Table 4.13. New mobile systems developed for HPTLC analysis of hypericin

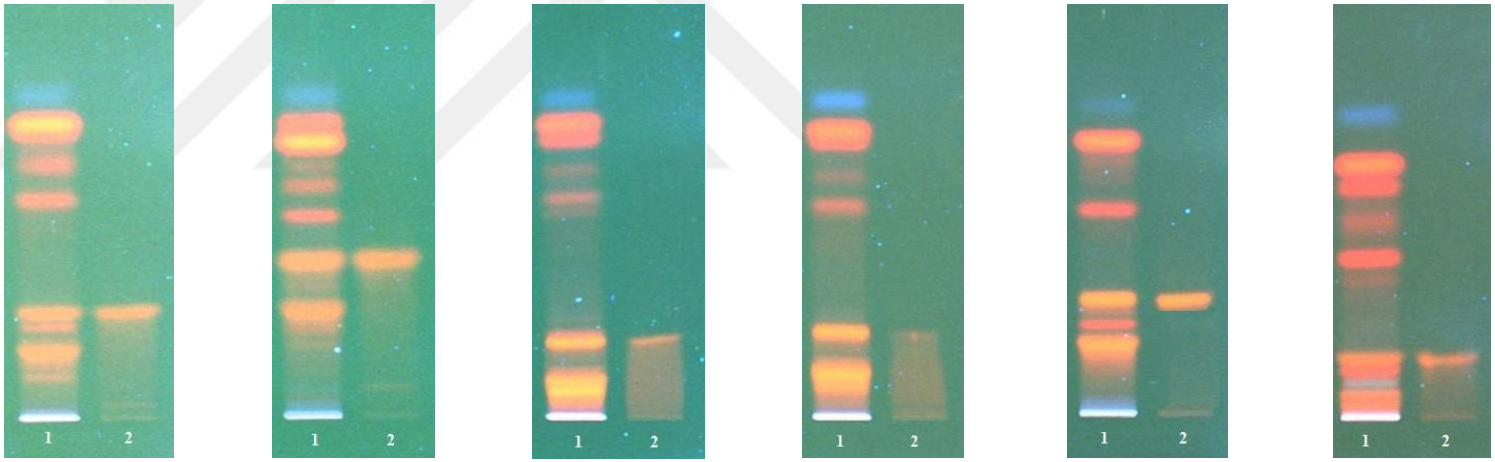
Mobile phase	**Solvent system**
MP-HyP-19	ethyl acetate-formic acid-toluene (4:1:6)
MP-HyP-20	ethyl acetate-formic acid-toluene (5:2:7)
MP-HyP-21	toluene-chloroform-formic acid-acetone (8:5:0.2:7)
MP-HyP-22	toluene-formic acid-chloroform (8:0.2:5)
MP-HyP-23	toluene-chloroform-formic acid-ethyl acetate (8:5:0.2:7)
MP-HyP-24	toluene-chloroform-tetrahydrofuran-formic acid (8:5:7:0.2)
MP-HyP-25	toluene-chloroform-formic acid-ethyl acetate (8:5:0.2:3.5)
MP-HyP-26	toluene-chloroform-formic acid-ethyl acetate (8:5:0.5:3.5)
MP-HyP-27	toluene-chloroform-formic acid-ethyl acetate (8:5:1:3.5)
MP-HyP-28	toluene-chloroform-formic acid-ethyl acetate (8:5:0.3:3.5)
MP-HyP-29	toluene-chloroform-formic acid-ethyl acetate (8:5:0.4:3.5)
MP-HyP-30	toluene-chloroform-formic acid-ethyl acetate (8:5:0.6:3.5)
MP-HyP-31	*n*-hexane-chloroform-formic acid-ethyl acetate (8:5:0.5:3.5)



(a) **(b)** **(c)** **(d)** **(e)** **(f)** **(g)**

Figure 4.24. HPTLC chromatograms of HyP (reference) and the extract from the flowering aerial parts of *H. perforatum* L. cultivar obtained with various new mobile systems at 366 nm.

HPTLC plates were developed by (a) MP-HyP-19; (b) MP-HyP-20; (c) MP-HyP-21; (d) MP-HyP-22; (e) MP-HyP-23; (f) MP-HyP-24; (g) MP-HyP-25. Tracks: (1) *H. perforatum* extract; (2) HyP (reference).



(h) **(i)** **(j)** **(k)** **(l)** **(m)**

Figure 4.24. HPTLC chromatograms of HyP (reference) and the extract from the flowering aerial parts of *H. perforatum* L. cultivar obtained with various mobile systems at 366 nm (continued).

HPTLC plates were developed by (h) MP-HyP-26; (i) MP-HyP-27; (j) MP-HyP-28; (k) MP-HyP-29; (l) MP-HyP-30; (m) MP-HyP-31. Tracks: (1) *H. perforatum* extract; (2) HyP (reference).

4.3.2.2. Method validation of hypericin

The developed HPTLC process was verified from the points of stability, robustness, specificity, detection and quantitation limits, linearity, intra- and interday precisions as well as recovery predicating in ICH guidelines (223).

4.3.2.2.1. Stability

The stability of HyP in methanol at several days and different temperatures, stability of HyP reference solutions adsorbed to the plate prior to chromatography, and stability of sample test solution through development were explored to assess the stability of the components during the chromatographic processing.

4.3.2.2.1.1. Stability of hypericin solution during storage period

The stability of HyP standard in methanol (0.02 mg/mL) was tested to verify whether spontaneous degradation occurred within 21 days after preparation and during storage at -20°C and 4°C. All experiments were iterated three times. Each peak area of the working solutions was compared with that of the fresh preparation of HyP standard in methanol at all time intervals. No chromatographic differences and no noteworthy degradation were detected. Thus, each stock solution prepared in methanol was found to be stable during storage at -20°C and 4°C for 3-week period, $F_{(3,20)}$=2.342, p=0.104 [$F_{crit\,(3,20)}$=3.098].

4.3.2.2.1.2. Stability of hypericin solution on the stationary phase

The freshly prepared HyP reference solution (0.02 mg/mL) was applied on plate every 30 minutes up to 2 hours prior the development. The plate was left uncovered, subjected to daylight and air throughout these periods. This procedure was repeated in triplicate. One-way ANOVA was employed to assure the interruptions in time intervals. The difference was seen between the mean areas at time intervals, $F_{(4,10)}$=25.716, p=0.000 [$F_{crit(4,10)}$=3.478]. Therefore, LSD test was conducted to compare the average areas and find the discriminations. There was no difference between the mean areas of HyP reference solution at time zero and 30 minutes (p=0.370). Nonetheless, the average

area of HyP reference solution significantly differed from the mean area of HyP reference solutions applied at the interval of 60 minutes (p=0.013), 90 minutes (p=0.000), and 120 minutes (p=0.000).

4.3.2.2.1.3. Stability of sample test solution during migration with the mobile phase

Stability of the components in *H. perforatum* sample solution on the plate during the migration was investigated by two-dimensional separation process. After first migration of the test solution, the plate was turned 90° and developed in the same mobile system at diagonal direction. All the components in sample were situated on the diagonal of the chromatogram demonstrating their stability during the migration (Figure 4.25).

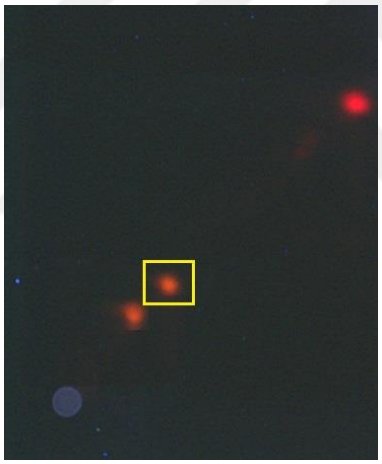


Figure 4.25. 2D-development of *H. perforatum* sample test solution for stability testing of components in toluene-chloroform-ethyl acetate-formic acid (8:5:3.5:0.6, v/v/v/v) at 366 nm. Derivatization: H_2SO_4 reagent.

4.3.2.2.2. Robustness

Some modifications in system conditions were made to evaluate the robustness of the method. In this regard, time of chamber saturation and humidity control, plate preconditioning time as well as time from development to scanning were slightly

changed and then, the proportions of the peak area of HyP reference (0.02 mg/mL) and sample solutions (10 mg/mL) were examined for the observation of the modifications. One-way ANOVA was used to compare the results. Accordingly, no significant differences were detected in the mean rates across the conditional changes, including saturation ($F_{(6,14)}$=2.465, p=0.077), humidity control ($F_{(6,14)}$=1.987, p=0.136), preconditioning ($F_{(6,14)}$=2.122, p=0.115), and time from chromatography to scan ($F_{(6,14)}$=2.663, p=0.061) [($F_{crit\,(6,14)}$=2.848)].

4.3.2.2.3. Specificity

Specificity of the solvent system was assessed by examining HyP reference (0.02 mg/mL) and sample solution (10 mg/mL). Within this context, R_f value of HyP in *H. perforatum* test solution was compared with that of the HyP in reference solution and the R_f value was found to be 0.35±0.03 (Figure 4.26). Additionally, HPTLC chromatogram visualized under 366 nm and the densitograms of reference and sample solution containing HyP were employed for approval.

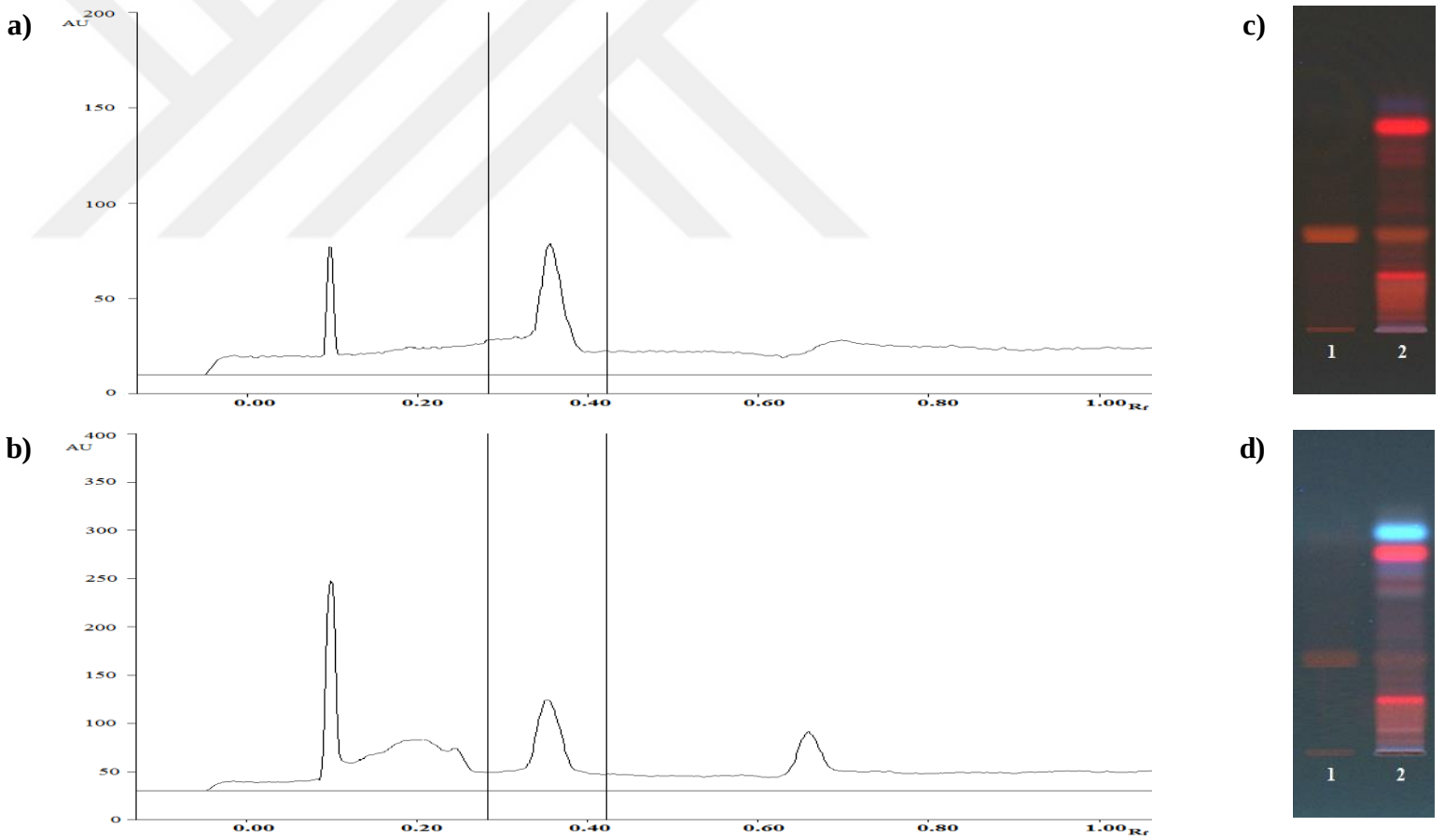


Figure 4.26. For specificity of the developed HPTLC process, HPTLC densitograms from (a) HyP reference solution (1 µL); (b) *H. perforatum* L. sample solution (2 µL) at 590 nm, and HPTLC fingerprints (c) before derivatization (d) after derivatization with H_2SO_4 reagent at 366 nm. Tracks: (1) HyP reference solution (1 µL); (2) *H. perforatum* L. sample solution (2 µL).

4.3.2.2.4. Limits of detection (LOD) and quantitation (LOQ)

HyP reference solutions in different concentrations (0.002 mg/mL, 0.01 mg/mL, and 0.02 mg/mL) and in increasing volumes (1-5 µL) were applied on numerous plates and migrated to estimate the detection limit and quantitation limit. Then the values of LOD and LOQ were found to be 6 and 20 ng/band, respectively.

4.3.2.2.5. Linearity and calibration curve

Linearity of the developed HPTLC system was evaluated at the applied values within the range of 20-100 ng/band of reference solutions and calibration curves were constructed with three independent injections. The quadratic correlation between the injected volumes and the peak area were obtained from the applied concentrations of HyP solution and the correlation coefficient was estimated to be 0.999±0.001. The regression equation was y=-85.47+23.46x-0.14x^2 [y=peak area; x=concentration in ng/band].

4.3.2.2.6. Precision

4.3.2.2.6.1. Repeatability

The intra-assay precision of the developed HPTLC process was conducted in three replicates of the experiment with the *H. perforatum* samples prepared three time intervals on the same day. The quantities of HyP in samples expressed as mean± SD as well as the RSD values were given in Table 4.14.

4.3.2.2.6.2. Intermediate precision

The interday precision of the newly developed HPTLC system was carried out with the *H. perforatum* samples prepared on three different days. Analysis of each sample was repeated thrice and the amount of HyP in each sample was stated as mean± SD. The results of interday precision were similar to those of intraday precision and the findings were given in Table 4.14. Accordingly, the outcomes presented that the values

of RSD for intra- and interday precisions were within the expected ranges of assessment criteria (225).

Table 4.14. Intra- and interday precision results of developed HPTLC method for HyP

Precision analysis		**HyP (mg/g)** (*n*=3)		
		sample-I	**sample-II**	**sample-III**
Within-day	analysis-I	1.59±0.03	1.62±0.02	1.59±0.02
	analysis-II	1.57±0.02	1.60±0.04	1.61±0.02
	analysis-III	1.60±0.01	1.64±0.01	1.58±0.01
	RSD (%)	1.44	1.77	1.26
Between-day	analysis-I	1.57±0.04	1.58±0.04	1.58±0.03
	analysis-II	1.61±0.02	1.63±0.06	1.62±0.02
	analysis-III	1.58±0.05	1.60±0.03	1.59±0.04
	RSD (%)	2.40	2.79	2.09

4.3.2.2.7. Recovery

The accuracy of the developed HPTLC system was validated by the standard addition method. The known quantities of HyP reference (10, 20 and 50 ng/band) were added to pre-analyzed sample solutions. Three replicates of each concentration were tested. The amount of HyP detected in mixed solution was expressed as mean±SD as well as the mean recovery and RSD were calculated as per cent (Table 4.15). The results presented that the means of percentage recoveries were within the expected ranges of assessment criteria (225).

Table 4.15. Recovery findings of HPTLC system of HyP

HyP added (ng/band)	**HyP found (ng/band)**	**RSD (%)**	**Recovery (%)**
10	10.01±0.11	1.14	100.06
20	19.88±0.32	1.61	99.42
50	49.42±0.65	1.32	98.84

4.3.3. Hypericin Assay in Various *Hypericum* Species

The presence of HyP in the extracts from flowering aerial parts of different *Hypericum* species was detected by the comparison of their R_f values and peaks related to retention spots with those of HyP reference. The analysis was performed in triplicate. The quantities of HyP determined in various *Hypericum* specimens were exhibited as mean±SD in Table 4.16.

Table 4.16. HyP contents in various *Hypericum* species

***Hypericum* specimen**	**HyP (mg/g)**
H. perforatum (İstanbul)	1.41±0.04
H. perforatum (Tekirdağ)	1.28±0.05
H. bithynicum	0.81±0.07
H. calycinum	ndt
H. cerastoides	<LOQ
H. perfoliatum	1.09±0.08
H. triquetrifolium	0.83±0.03
H. kotschyanum	ndt
H. scabrum	ndt
H. thymopsis	ndt

ndt: HyP not detected; <LOQ: under the limit of quantitation

Furthermore, the explanations of these findings as well as the designation of differentiation between these samples were supported by HPTLC densitograms (Figure 4.27) and fingerprint (Figure 4.28) of *Hypericum* species.

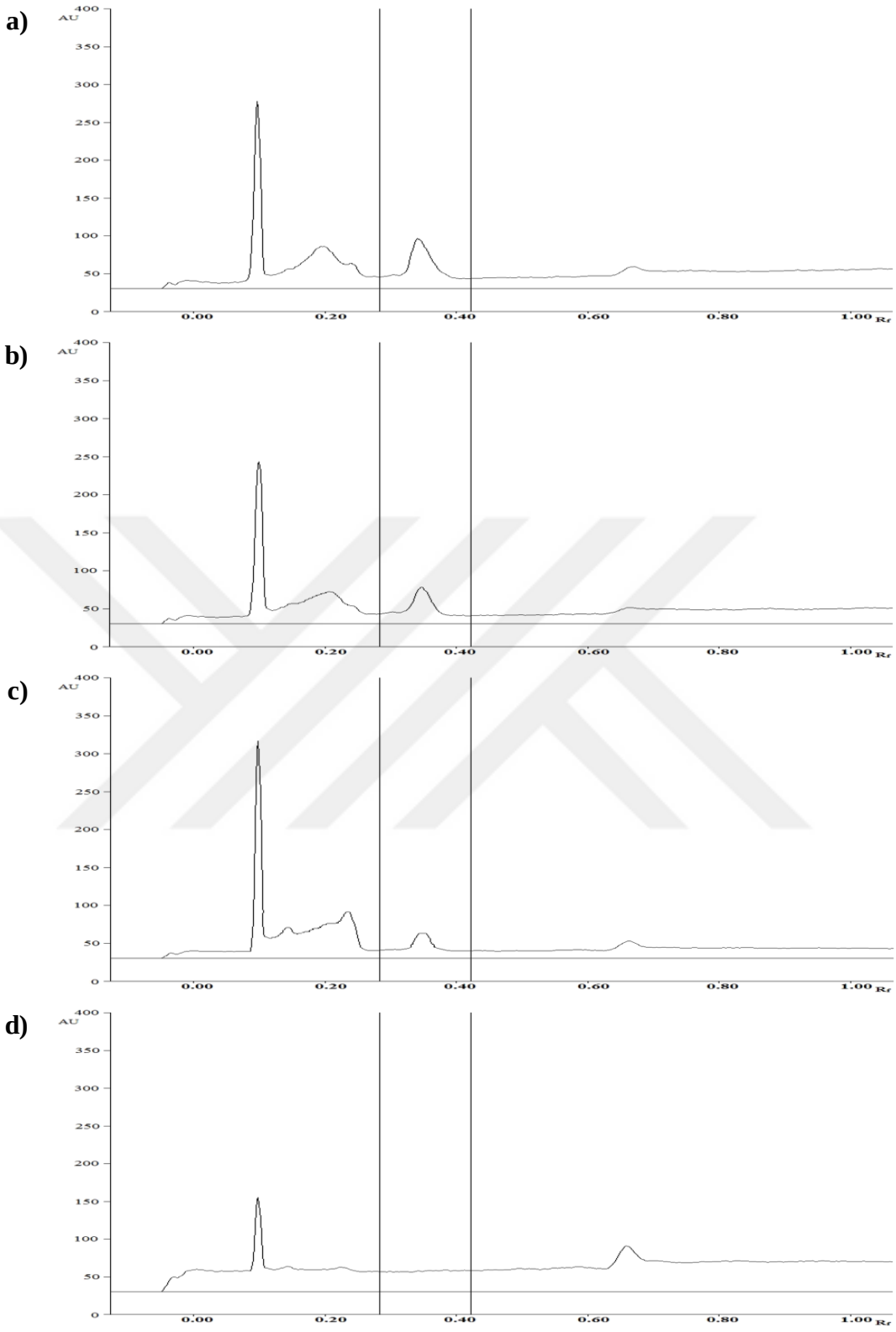


Figure 4.27. HPTLC densitograms of the extracts from different *Hypericum* species at 590 nm. (a) *H. perforatum* (İstanbul); (b) *H. perforatum* (Tekirdağ); (c) *H. bithynicum*; (d) *H. calycinum* (20 µg/band).

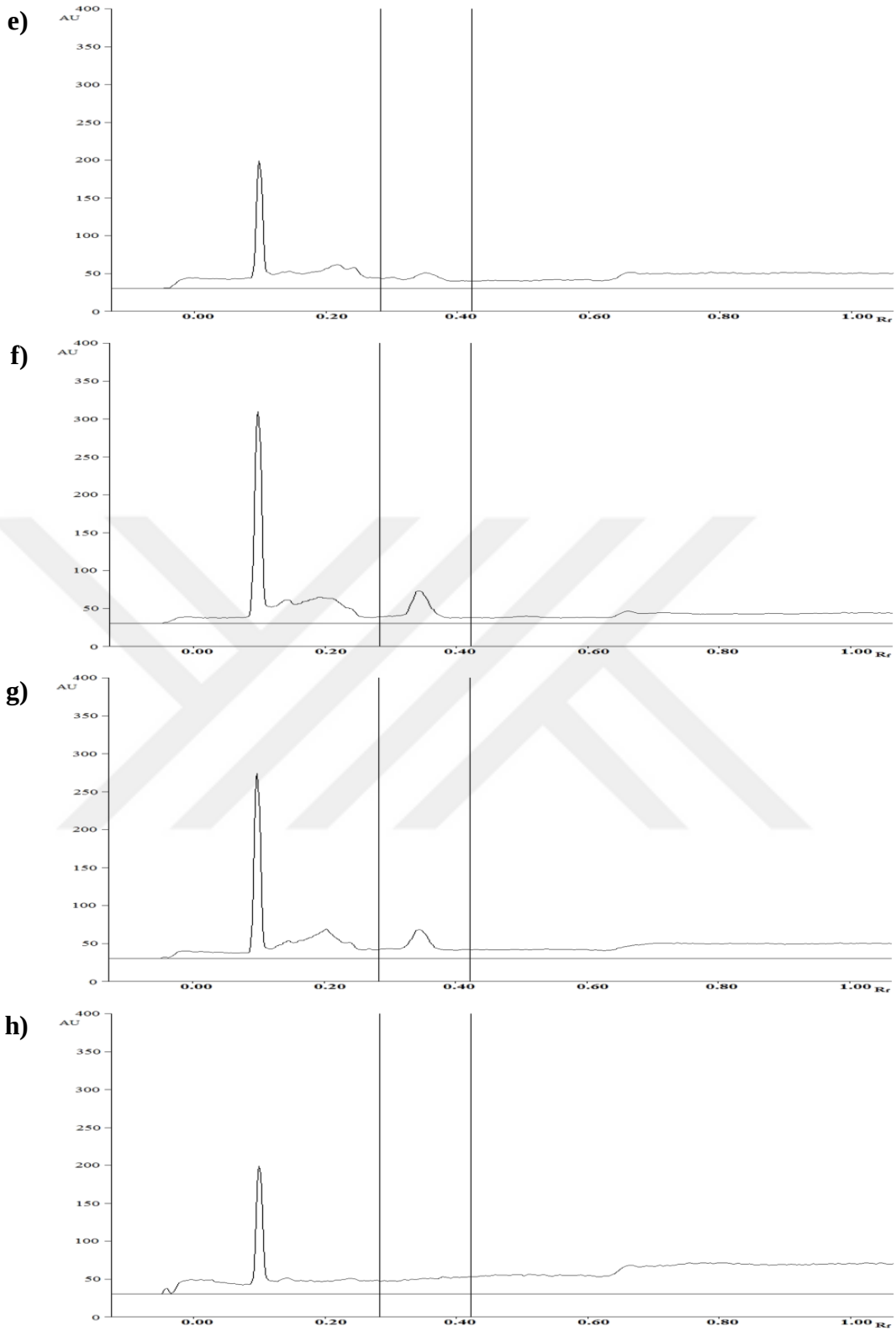


Figure 4.27. HPTLC densitograms of the extracts from different *Hypericum* species at 590 nm (continued). (e) *H. cerastoides*; (f) *H. perfoliatum*; (g) *H. triquetrifolium*; (h) *H. kotschyanum* (20 µg/band).

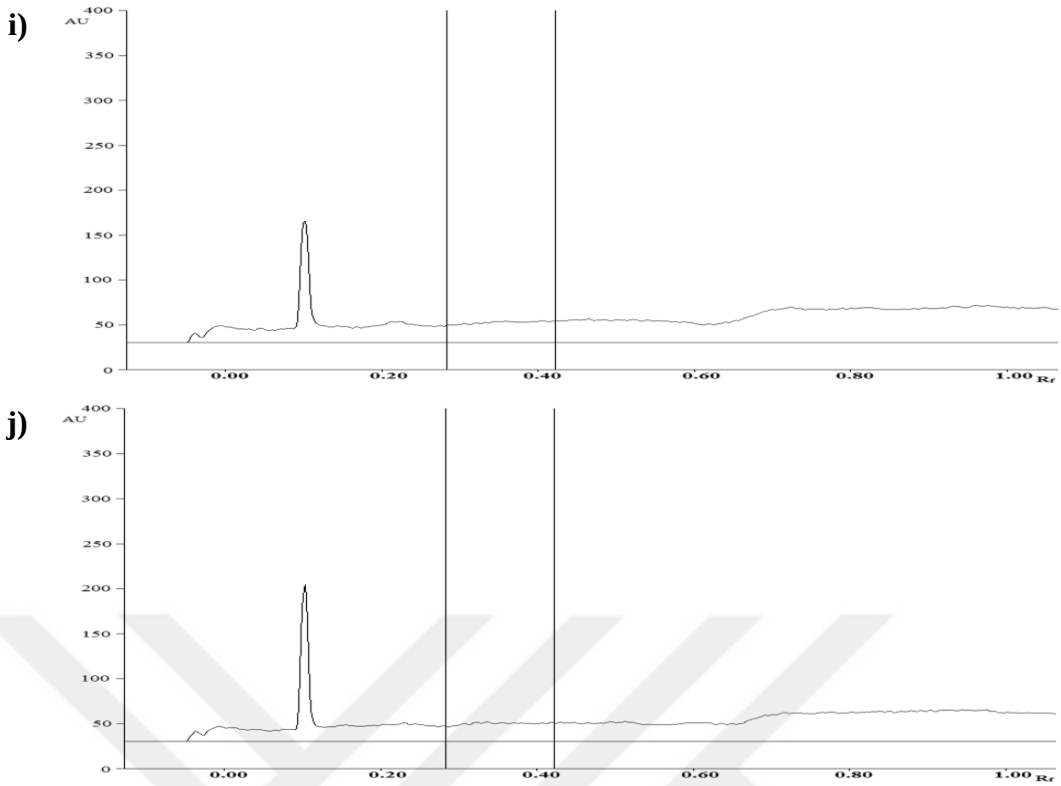


Figure 4.27. HPTLC densitograms of the extracts from different *Hypericum* species at 590 nm (continued). (i) *H. scabrum*; (j) *H. thymopsis* (20 µg/band).

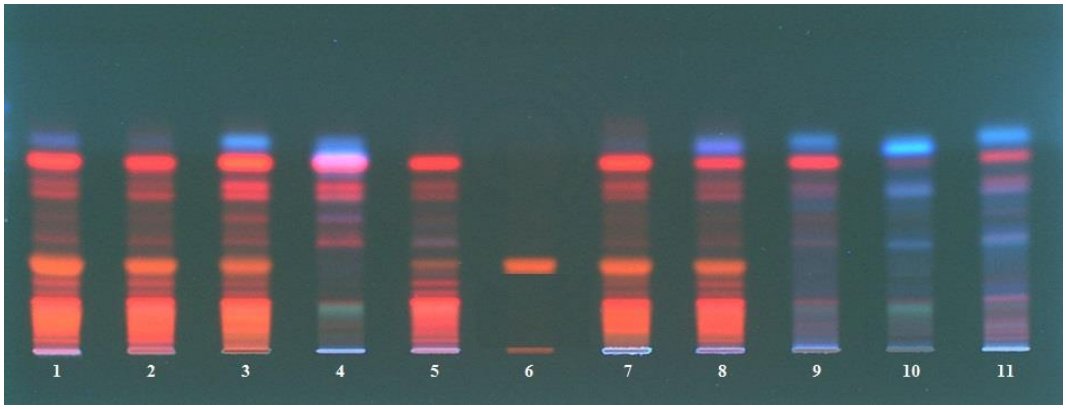


Figure 4.28. HPTLC chromatogram of the extracts from *Hypericum* species at 366 nm. Tracks: (1) *H. perforatum* (İstanbul); (2) *H. perforatum* (Tekirdağ); (3) *H. bithynicum*; (4) *H. calycinum*; (5) *H. cerastoides*; (6) HyP (reference) (20 ng/band); (7) *H. perfoliatum*; (8) *H. triquetrifolium*; (9) *H. kotschyanum*; (10) *H. scabrum*; (11) *H. thymopsis*; applied volume of *Hypericum* species: 20 µg/band.

According to HPTLC densitograms and chromatogram, it was indicated that HyP was identified in *H. perforatum* obtained from two different locations, *H. bithynicum*, *H. perfoliatum* and *H. triquetrifolium* specimens between the ranges from 0.81 to 1.41 mg/g (Table 4.16). Although the densitograms and chromatograms of two *H. perforatum* samples were overlapping with those of *H. perforatum* L. reference sample, their HyP contents were lower; 1.41 mg/g for İstanbul and 1.28 mg/g for Tekirdağ samples vs. 1.60 mg/g in *H. perforatum* L. reference sample. On the other hand, HyP concentration in *H. cerastoides* was less than the LOQ. However, HyP was not found in *H. calycinum*, *H. kotschyanum*, *H. scabrum* and *H. thymopsis* specimens.

4.3.4. Hypericin Assay in the Marketed *H. perforatum* (SJW) Products

The presence of HyP in the *Hypericum* products marketed as tea, capsule or dried herbal material was detected by the comparison of their R_f values and relevant peaks with those of HyP reference. The analysis was conducted in triplicate. The amounts of HyP determined in these products were presented as mean±SD in Table 4.17, while related HPTLC densitograms (Figure 4.29, Figure 4.31a, Figure 4.32) and fingerprints (Figure 4.30, Figure 4.31b, Figure 4.33) were also presented below.

Table 4.17. HyP contents in marketed *H. perforatum* (SJW) products

Commercial product	HyP (mg/g)	Commercial product	HyP (mg/g)
HT1	<LOQ	HS13	1.32±0.05
HT2	<LOQ	HS14	1.28±0.04
HT3	0.76±0.09	HS15	0.42±0.07
HT4	1.40±0.03	HS16	1.39±0.05
HT5	0.56±0.07	HS17	0.10±0.01
HT6	0.50±0.04	HS18	1.49±0.09
HT7	1.49±0.03	HS19	1.34±0.03
HT8	0.85±0.01	HS20	1.35±0.06
HC	0.91±0.07	HS21	1.37±0.09
HS1	<LOQ	HS22	1.31±0.06
HS2	0.35±0.09	HS23	0.13±0.02
HS3	1.30±0.01	HS24	<LOQ
HS4	0.92±0.04	HS25	1.40±0.14
HS5	<LOQ	HS26	<LOQ
HS6	0.73±0.07	HS27	1.37±0.04
HS7	0.86±0.09	HS28	<LOQ
HS8	<LOQ	HS29	1.42±0.09
HS9	1.07±0.10	HS30	1.44±0.03
HS10	1.36±0.06	HS31	0.56±0.05
HS11	<LOQ	HS32	1.38±0.08
HS12	1.29±0.08	HS33	1.46±0.03

<LOQ: under the limit of quantitation

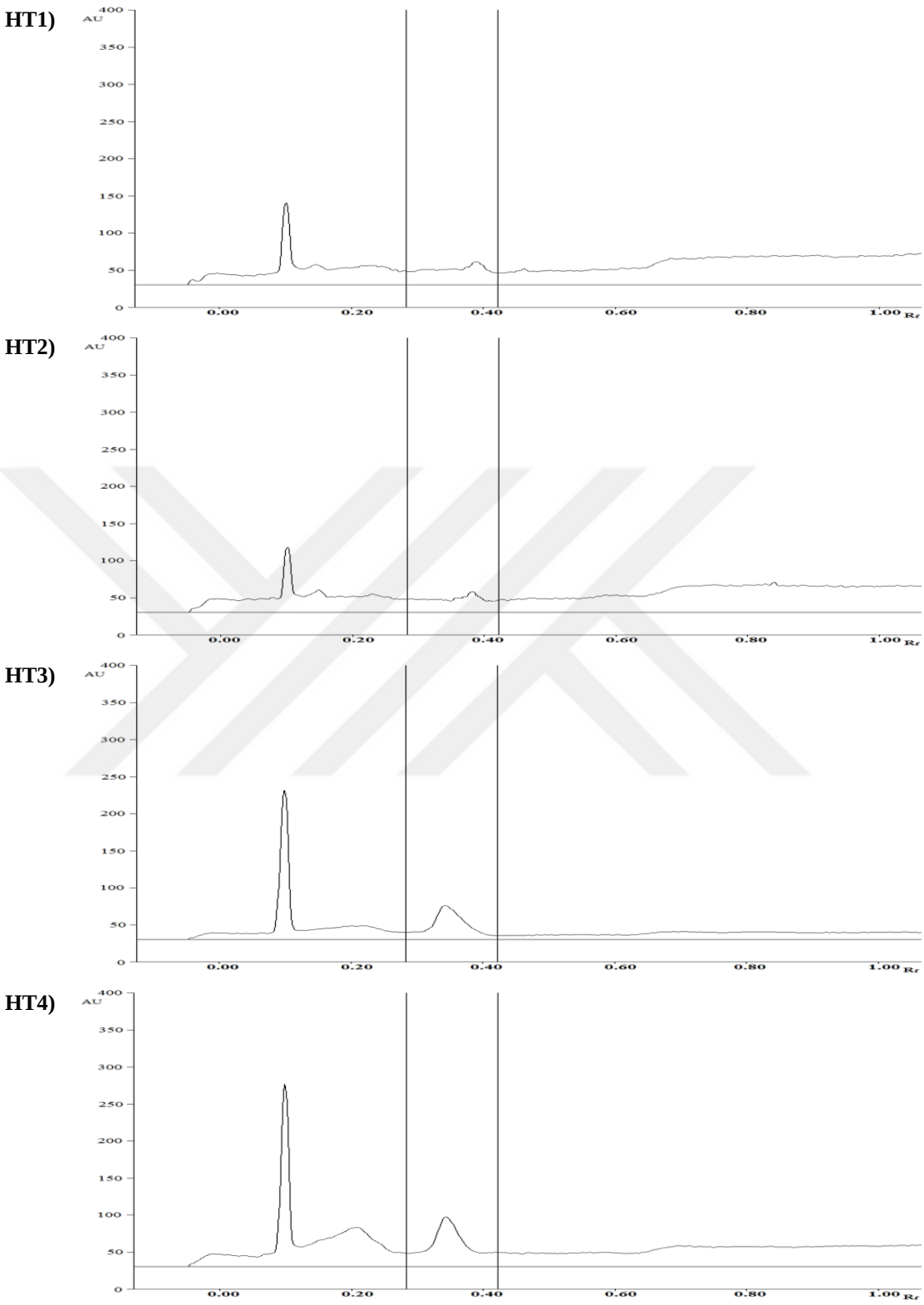


Figure 4.29. HPTLC densitograms from *H. perforatum* (SJW) tea products at 590 nm. Test solutions of tea products: 40 µg/band.

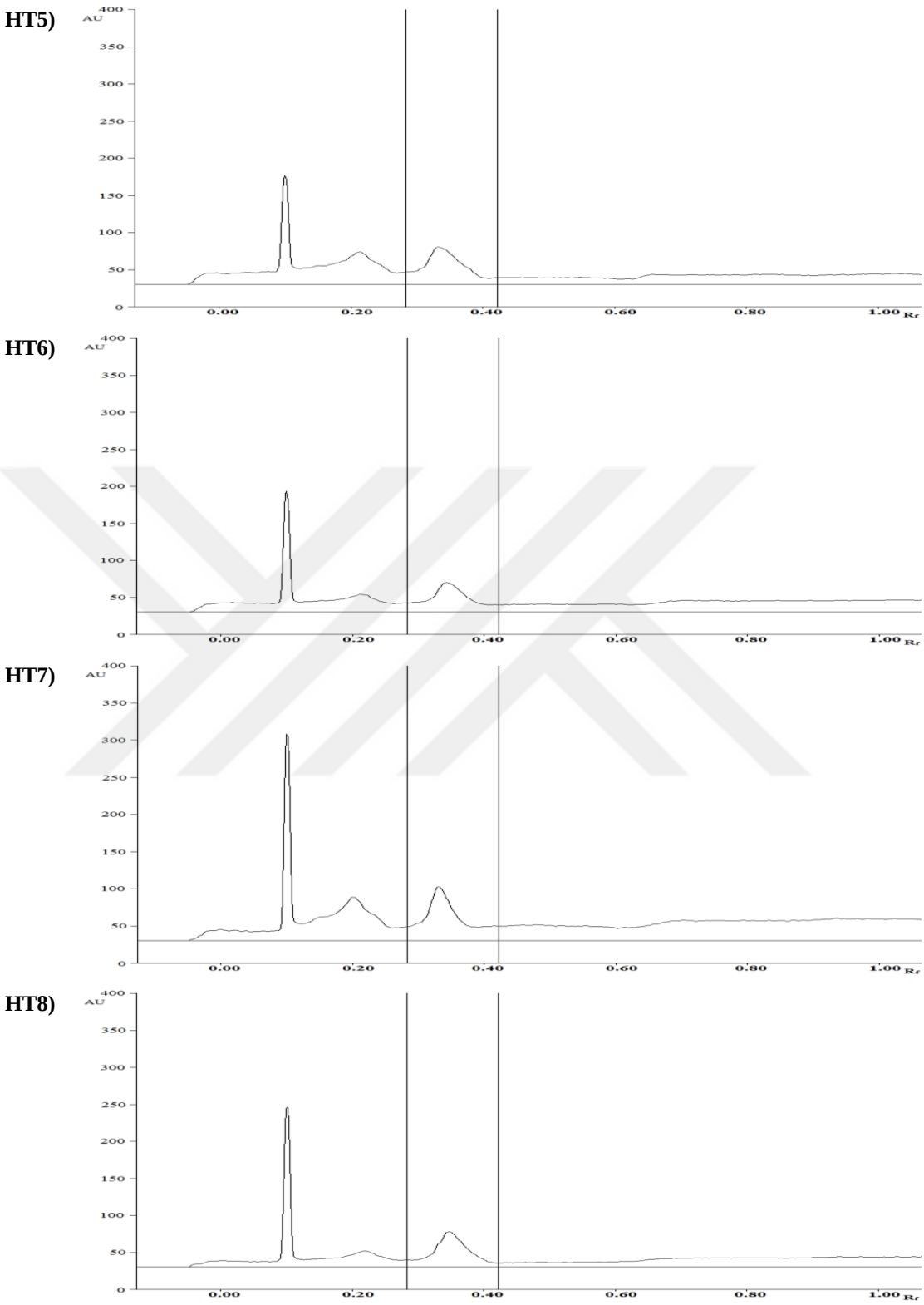


Figure 4.29. HPTLC densitograms from *H. perforatum* (SJW) tea products at 590 nm (continued). Test solutions of tea products: 40 µg/band.

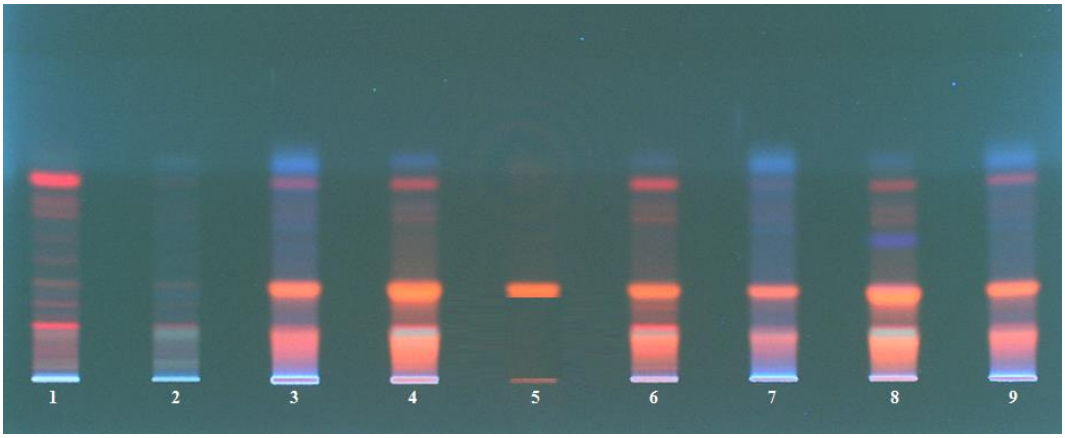
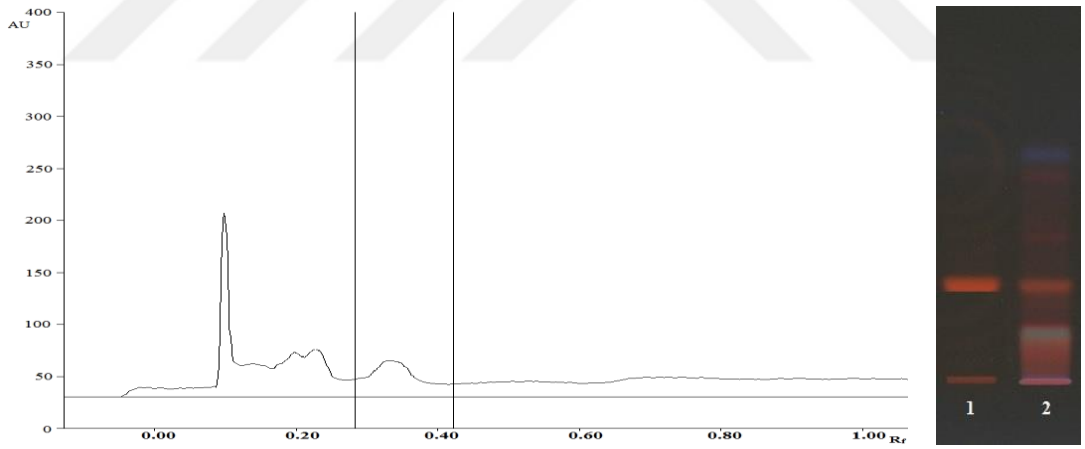


Figure 4.30. HPTLC chromatogram from *H. perforatum* (SJW) tea products at 366 nm. Tracks: (1) HT1; (2) HT2; (3) HT3; (4) HT4; (5) HyP reference (20 ng/band); (6) HT5; (7) HT6; (8) HT7; (9) HT8; applied volume of *H. perforatum* (SJW) tea products: 40 µg/band.



(a) **(b)**

Figure 4.31. (a) HPTLC densitogram obtained from *H. perforatum* (SJW) capsule formulation at 590 nm (20 µg/band) and (b) HPTLC chromatogram of *H. perforatum* (SJW) capsule formulation at 366 nm. Tracks: (1) HyP reference (20 ng/band); (2) HC (20 µg/band).

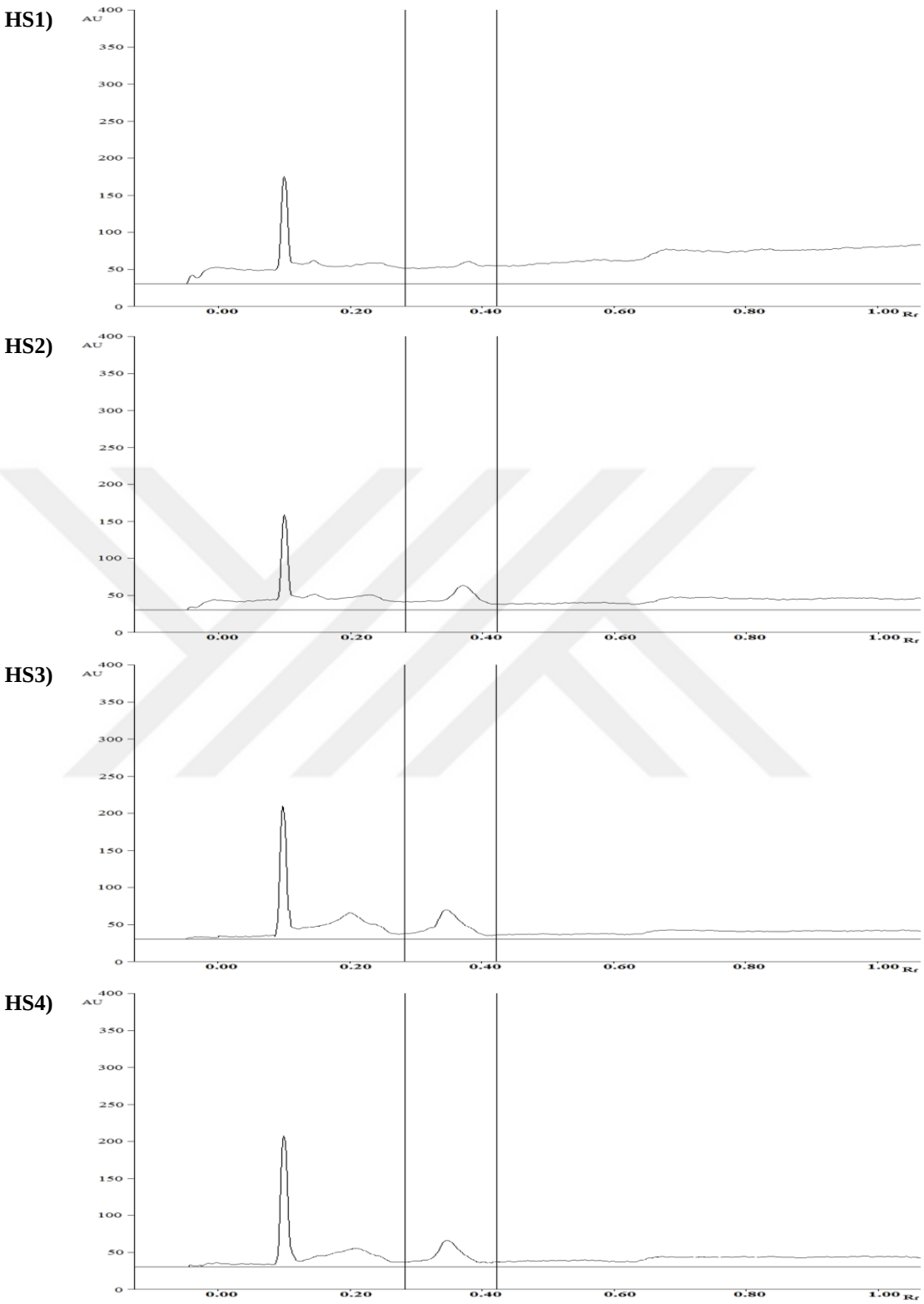


Figure 4.32. HPTLC densitograms from the marketed *H. perforatum* (SJW) herbal materials at 590 nm. Sample test solutions: 40 µg/band.

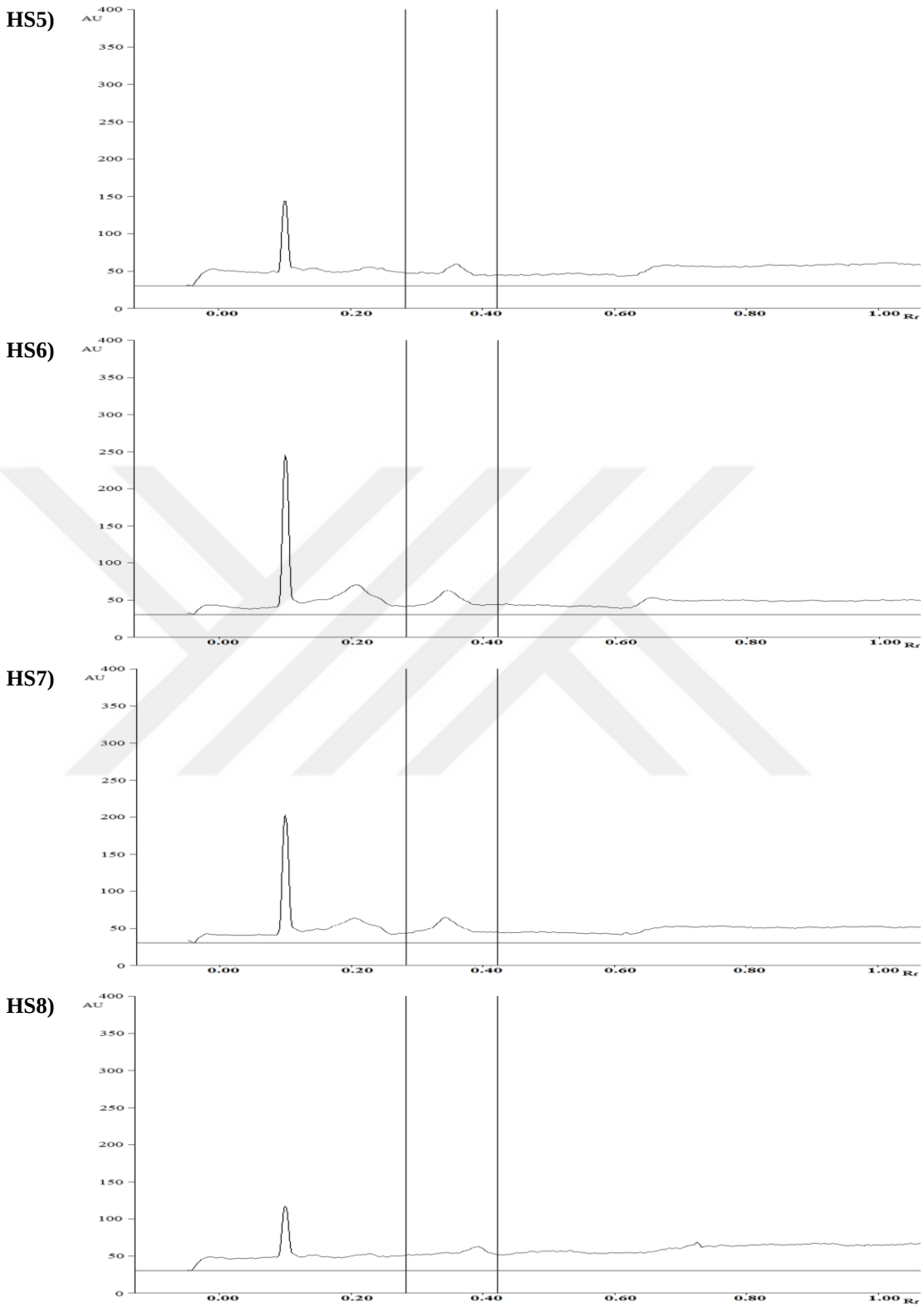


Figure 4.32. HPTLC densitograms from the marketed *H. perforatum* (SJW) herbal materials at 590 nm (continued). Sample test solutions: 40 µg/band.

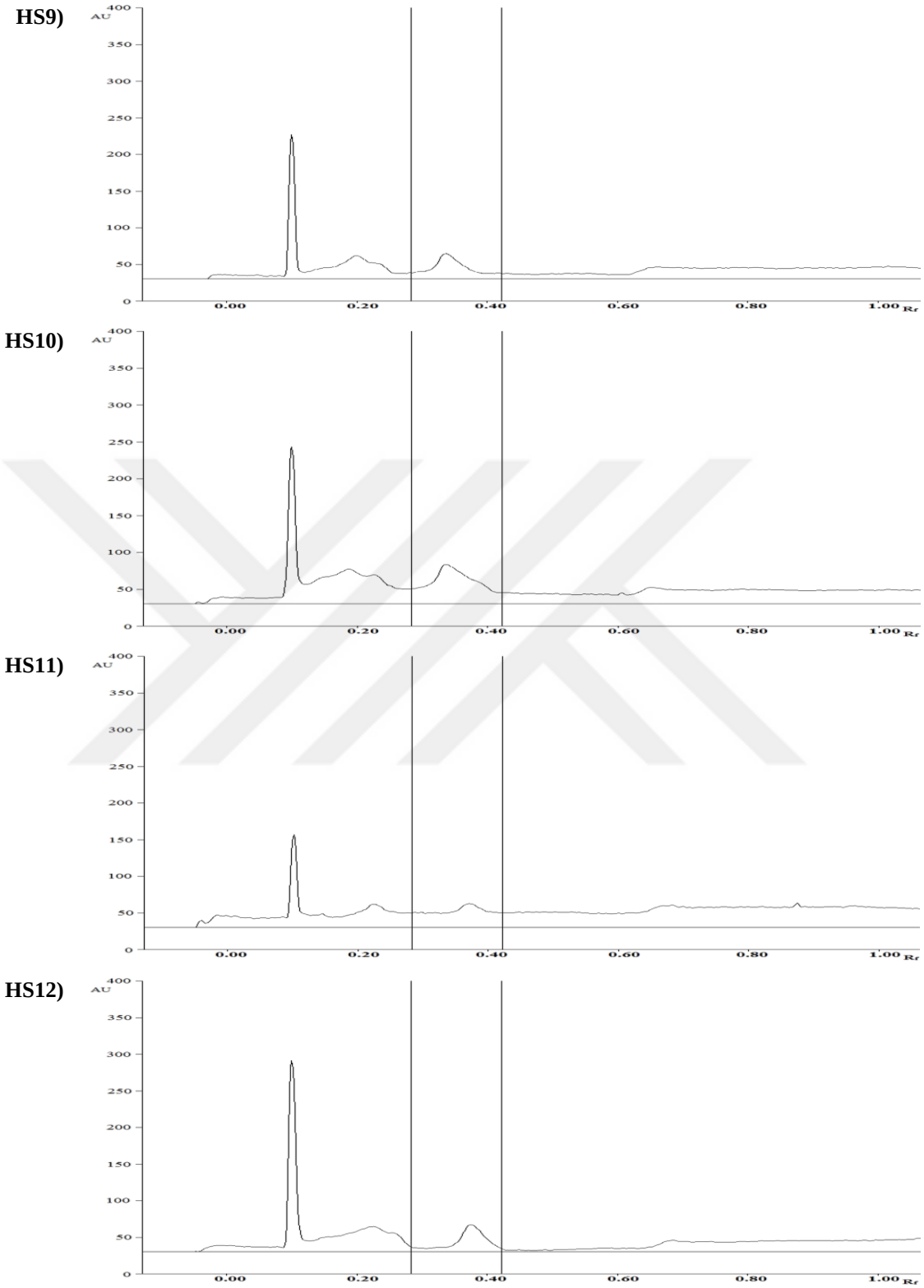


Figure 4.32. HPTLC densitograms from the marketed *H. perforatum* (SJW) herbal materials at 590 nm (continued). Sample test solutions: 40 µg/band.

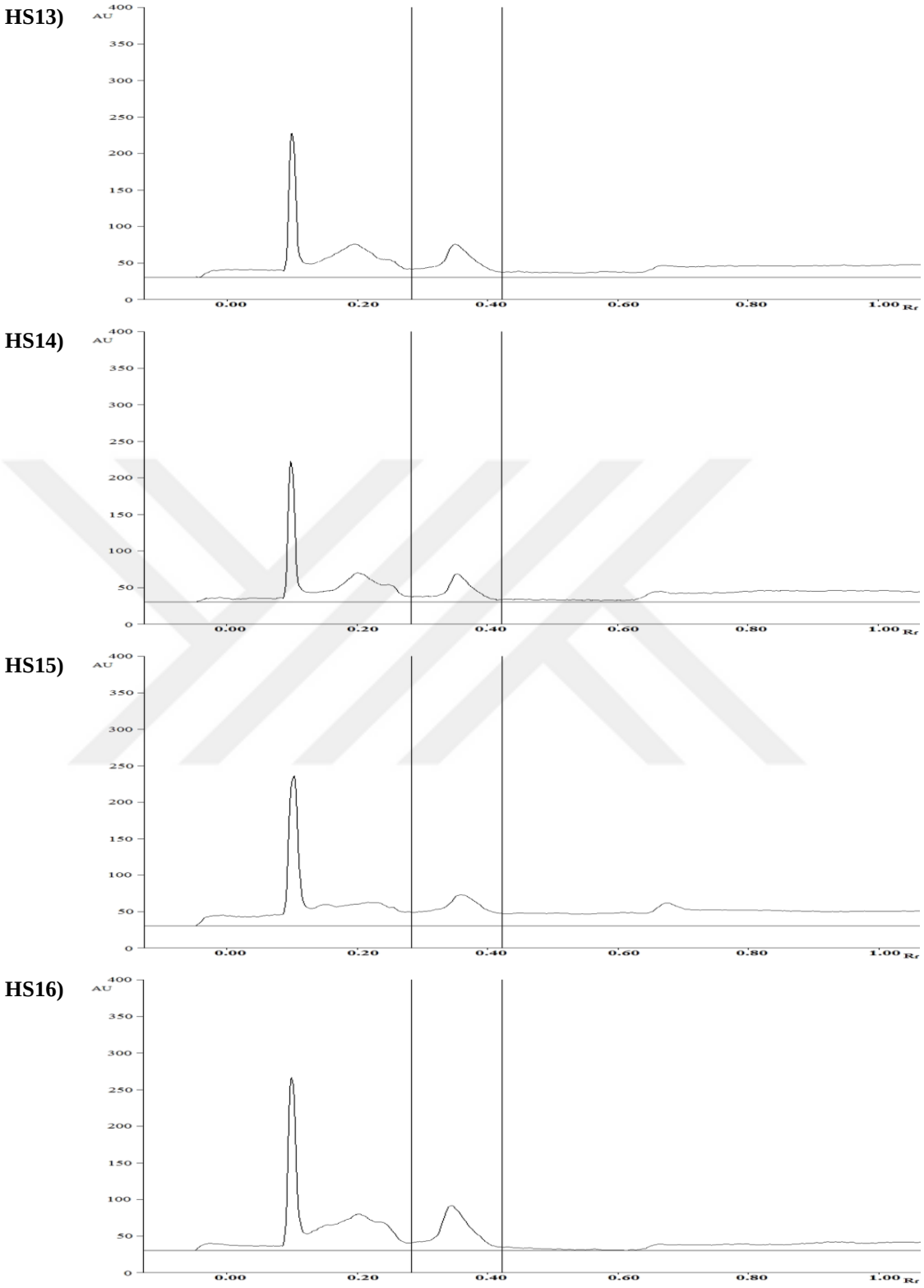


Figure 4.32. HPTLC densitograms from the marketed *H. perforatum* (SJW) herbal materials at 590 nm (continued). Sample test solutions: 40 µg/band.

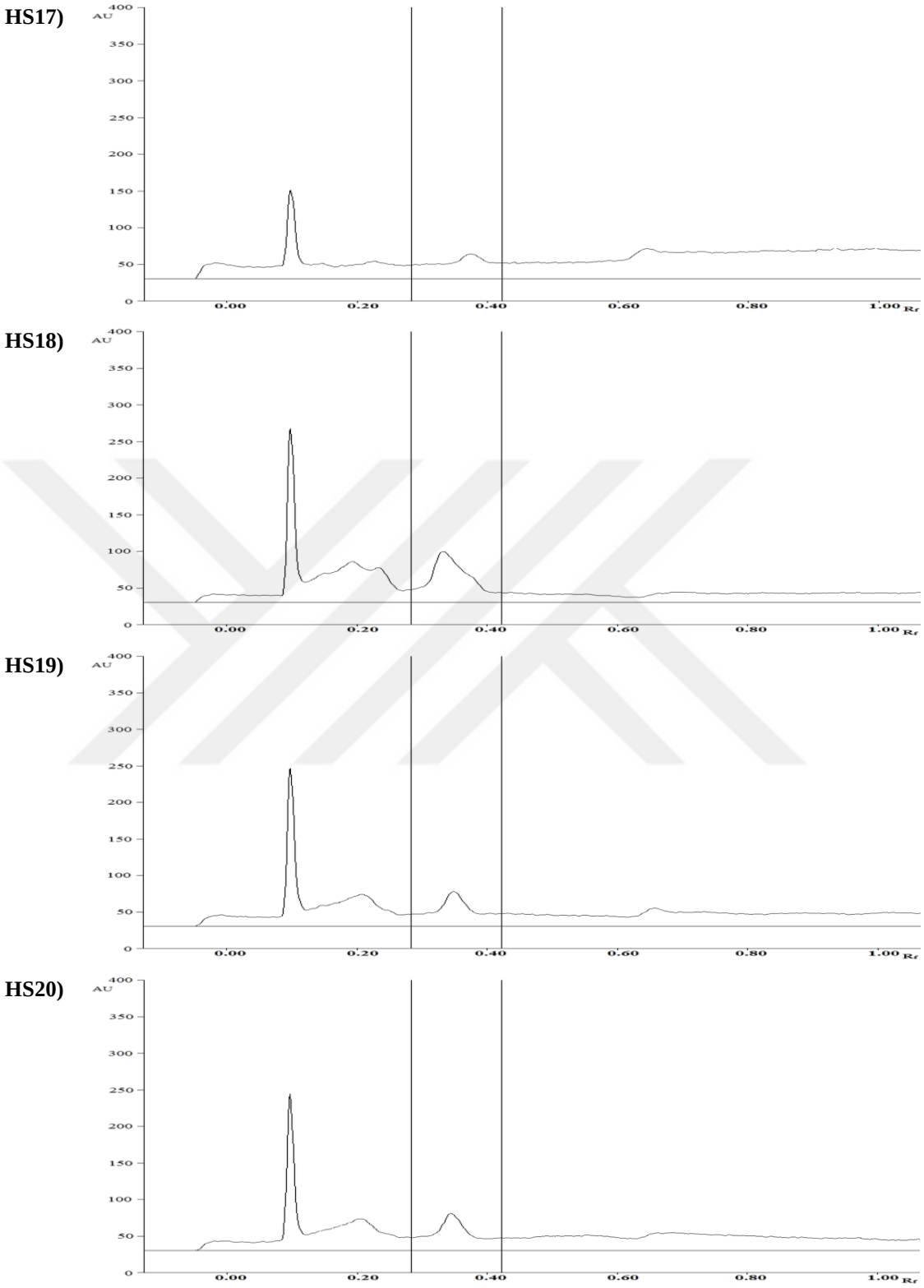


Figure 4.32. HPTLC densitograms from the marketed *H. perforatum* (SJW) herbal materials at 590 nm (continued). Sample test solutions: 40 µg/band.

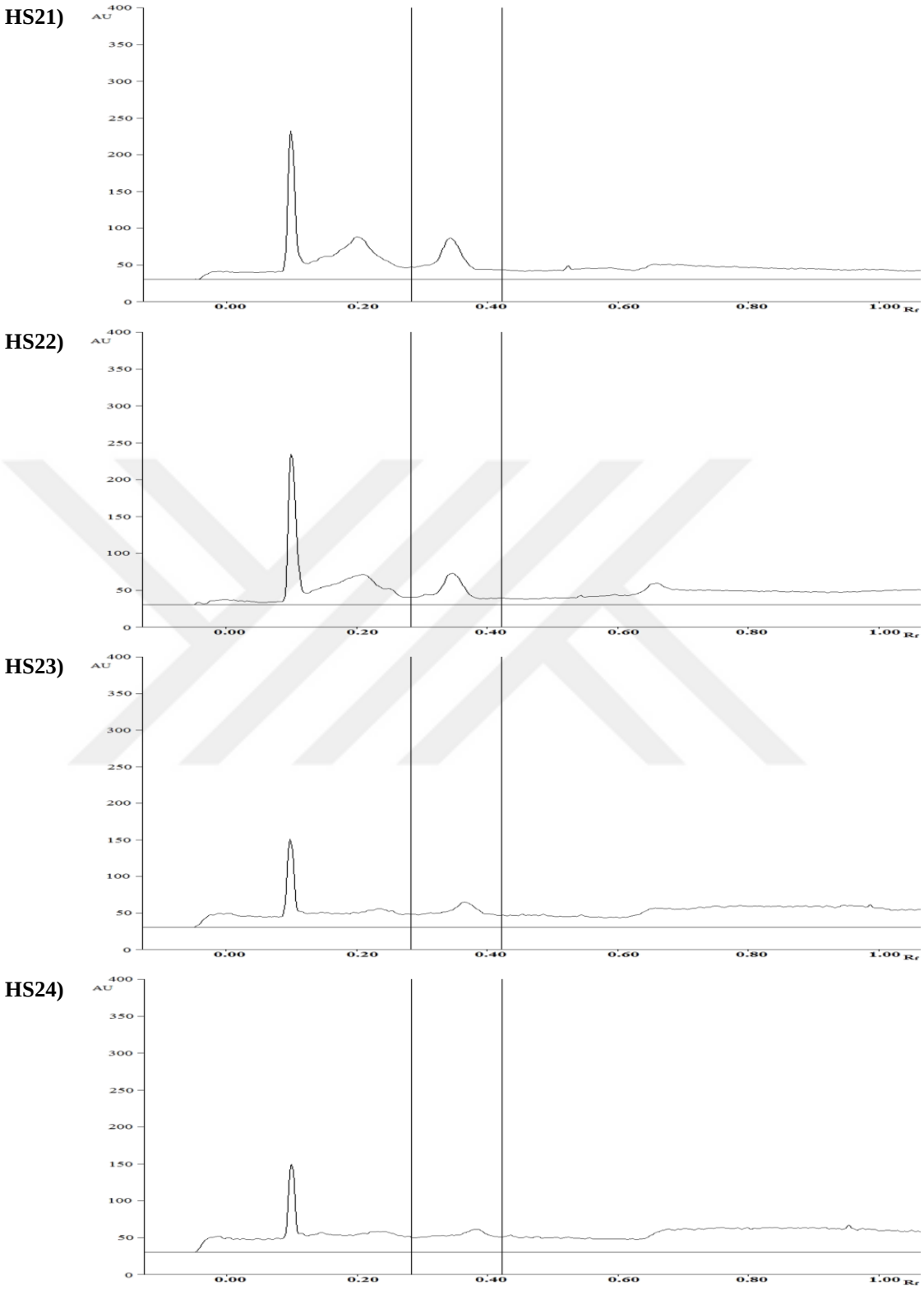


Figure 4.32. HPTLC densitograms from the marketed *H. perforatum* (SJW) herbal materials at 590 nm (continued). Sample test solutions: 40 µg/band.

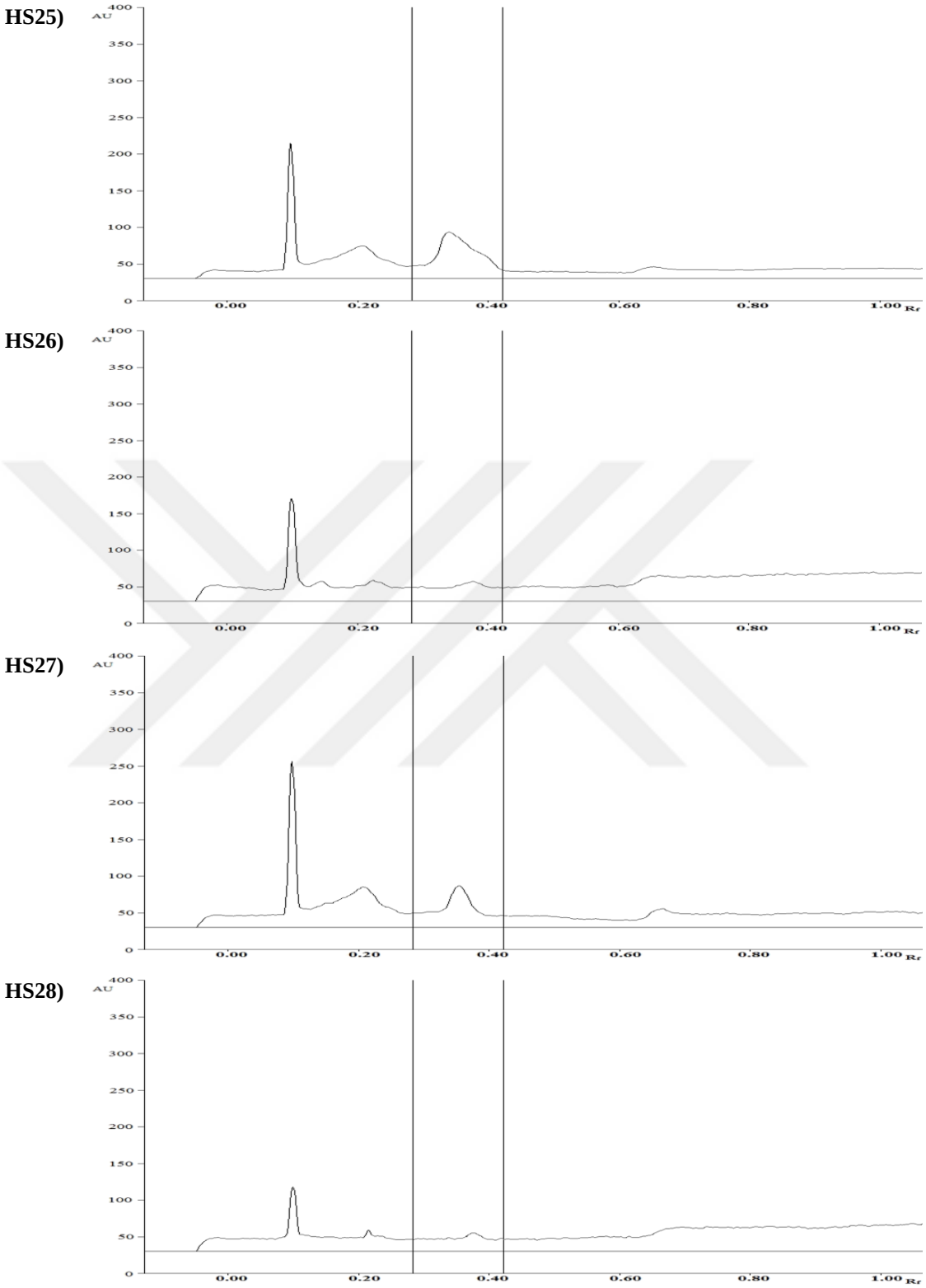


Figure 4.32. HPTLC densitograms from the marketed *H. perforatum* (SJW) herbal materials at 590 nm (continued). Sample test solutions: 40 µg/band.

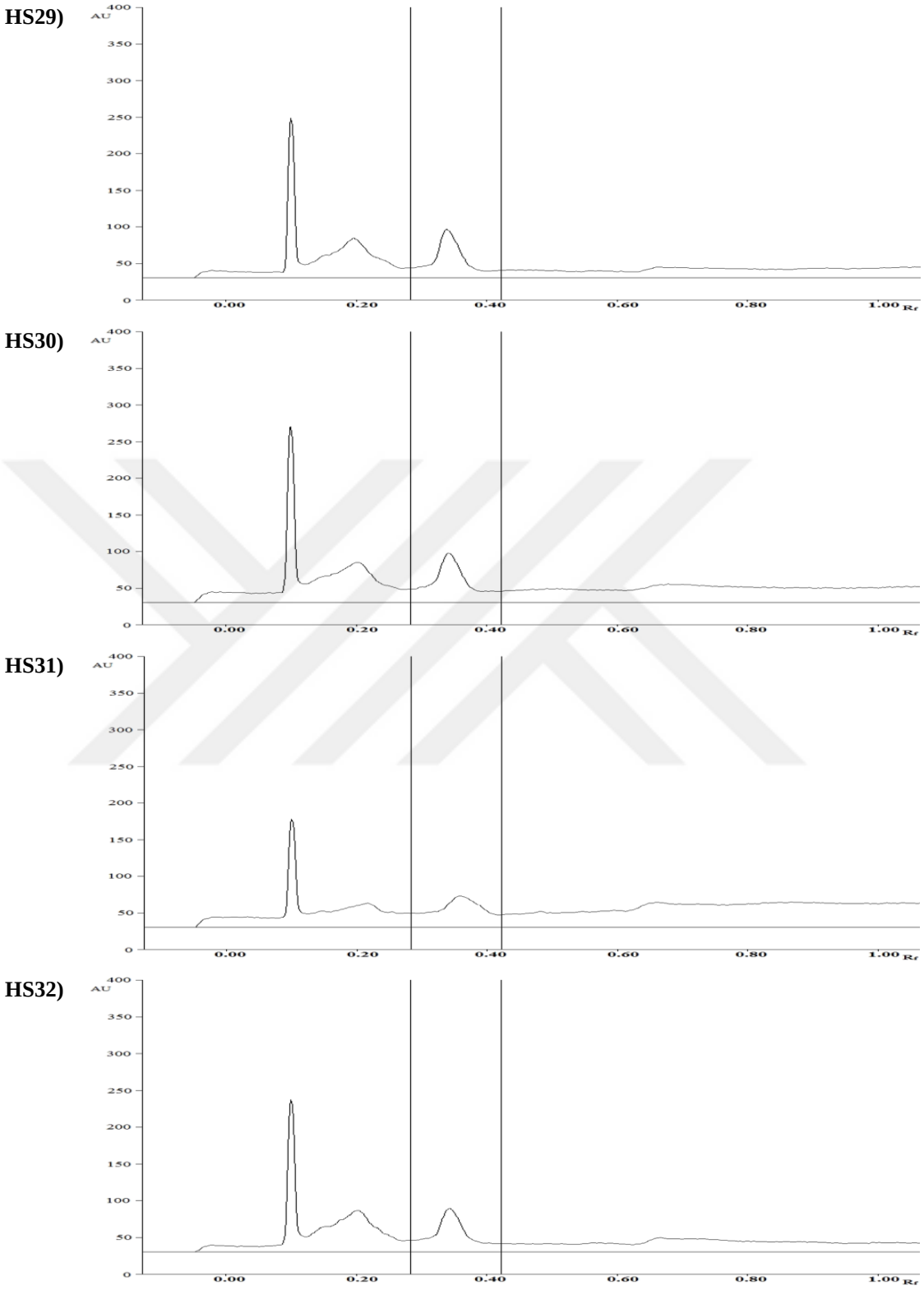


Figure 4.32. HPTLC densitograms from the marketed *H. perforatum* (SJW) herbal materials at 590 nm (continued). Sample test solutions: 40 µg/band.

HS33)

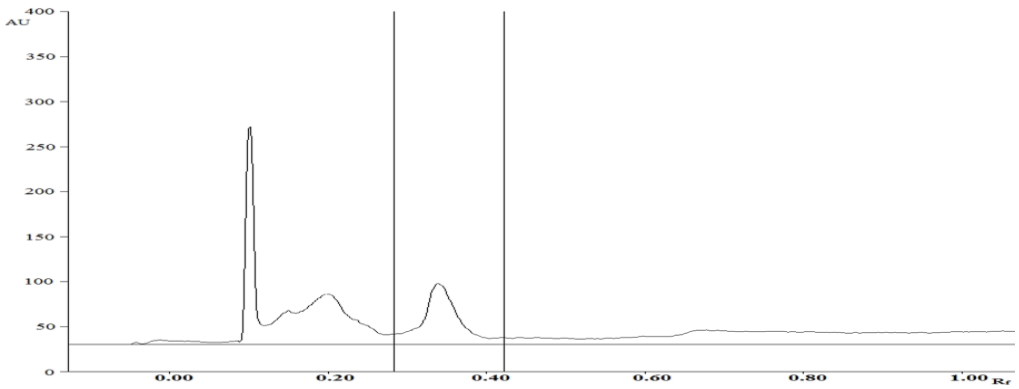


Figure 4.32. HPTLC densitograms from the marketed *H. perforatum* (SJW) herbal materials at 590 nm (continued). Sample test solutions: 40 µg/band.

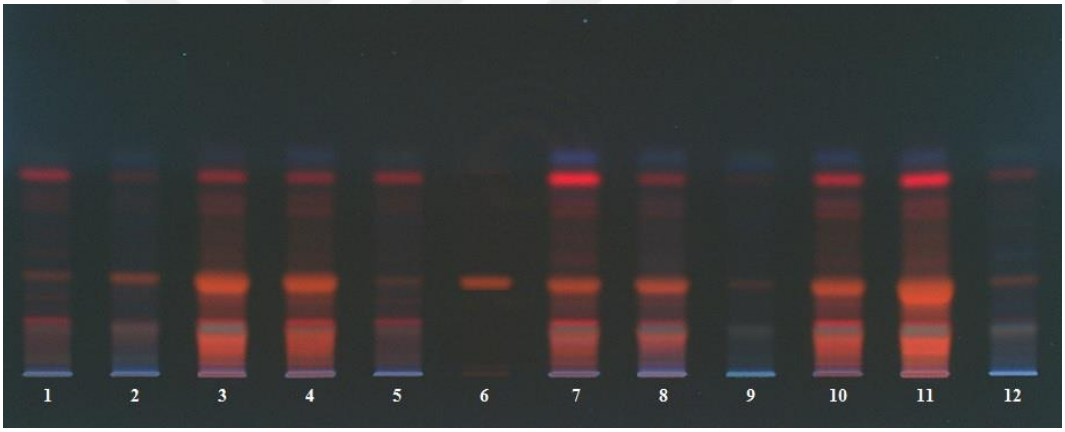


Figure 4.33. HPTLC chromatogram of the marketed *H. perforatum* (SJW) herbal materials at 366 nm. Tracks: (1) HS1; (2) HS2; (3) HS3; (4) HS4; (5) HS5; (6) HyP reference (20 ng/band); (7) HS6; (8) HS7; (9) HS8; (10) HS9; (11) HS10; (12) HS11; applied volume of *H. perforatum* (SJW) herbal samples: 40 µg/band.

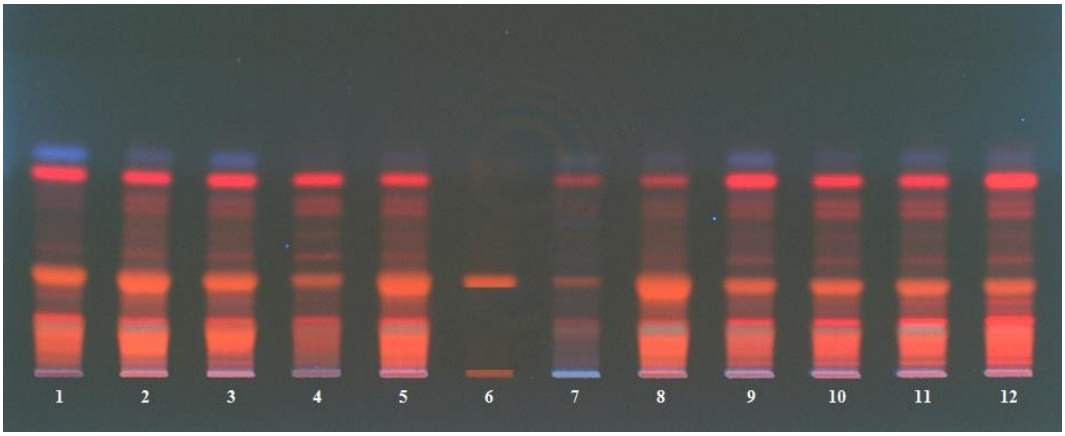


Figure 4.33. HPTLC chromatogram of the marketed *H. perforatum* (SJW) herbal materials at 366 nm (continued). Tracks: (1) HS12; (2) HS13; (3) HS14; (4) HS15; (5) HS16; (6) HyP reference (20 ng/band); (7) HS17; (8) HS18; (9) HS19; (10) HS20; (11) HS21; (12) HS22; applied volume of *H. perforatum* (SJW) herbal samples: 40 µg/band.

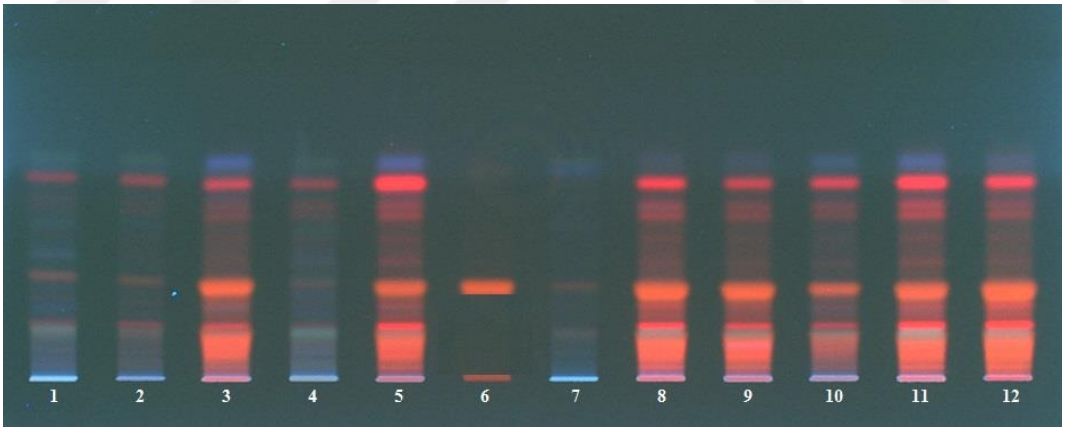


Figure 4.33. HPTLC chromatogram of the marketed *H. perforatum* (SJW) herbal materials at 366 nm (continued). Tracks: (1) HS23; (2) HS24; (3) HS25; (4) HS26; (5) HS27; (6) HyP reference (20 ng/band); (7) HS28; (8) HS29; (9) HS30; (10) HS31; (11) HS32; (12) HS33; applied volume of *H. perforatum* (SJW) herbal samples: 40 µg/band.

As shown in Figure 4.29 to Figure 4.33, HyP was detected in HT1-8, HC and HS1-33. Furthermore, HS14, HS15 and HS22 displayed similar HPTLC densitograms to those of the reference *H. perforatum* and wild *H. perforatum* samples under 590 nm-light. Additionally, HT1-8 and the remaining 30 *Hypericum* samples exhibited different densitograms with those of the reference and wild *H. perforatum* samples. Besides, the chromatograms and densitograms of each group of HS1, HS24 and HS26; HS7 and HS9; HS19 and HS22; HS21, HS29, HS30, HS32 and HS33 were found to be almost identical, respectively.

As shown in Table 4.17, in nine of the commercial *Hypericum* (SJW) products [HT1-2, HS1, HS5, HS8, HS11, HS24, HS26 and HS28] the amount of HyP detected was less than the LOQ. In addition, HyP content determined by the developed HPTLC system in six SJW tea products and SJW capsule formulation was between 0.50 to 1.49 mg/g. Besides, HyP contents in the marketed dried herbal materials of *H. perforatum* (SJW) purchased from the herb dealers were ranging from 0.10 to 1.49 mg/g.

4.3.5. Evaluation of Hypericin Content in Aqueous Extracts from *H. perforatum*

The presence of HyP in aqueous extracts from the flowering aerial parts of *H. perforatum* L. cultivar was detected by comparison of their R_f values and relevant peaks with that of HyP reference. The analysis was performed in three replicates. The quantities of HyP detected in aqueous extracts of *H. perforatum* L. reference sample were expressed as mean±SD in Table 4.18, while related HPTLC densitograms and fingerprints were also demonstrated below (Figure 4.34).

Table 4.18. HyP contents in aqueous extracts of *H. perforatum* L. cultivar

Aqueous extract	**HyP (mg/g)**
HP-W5	0.24±0.04
HP-W10	0.42±0.07

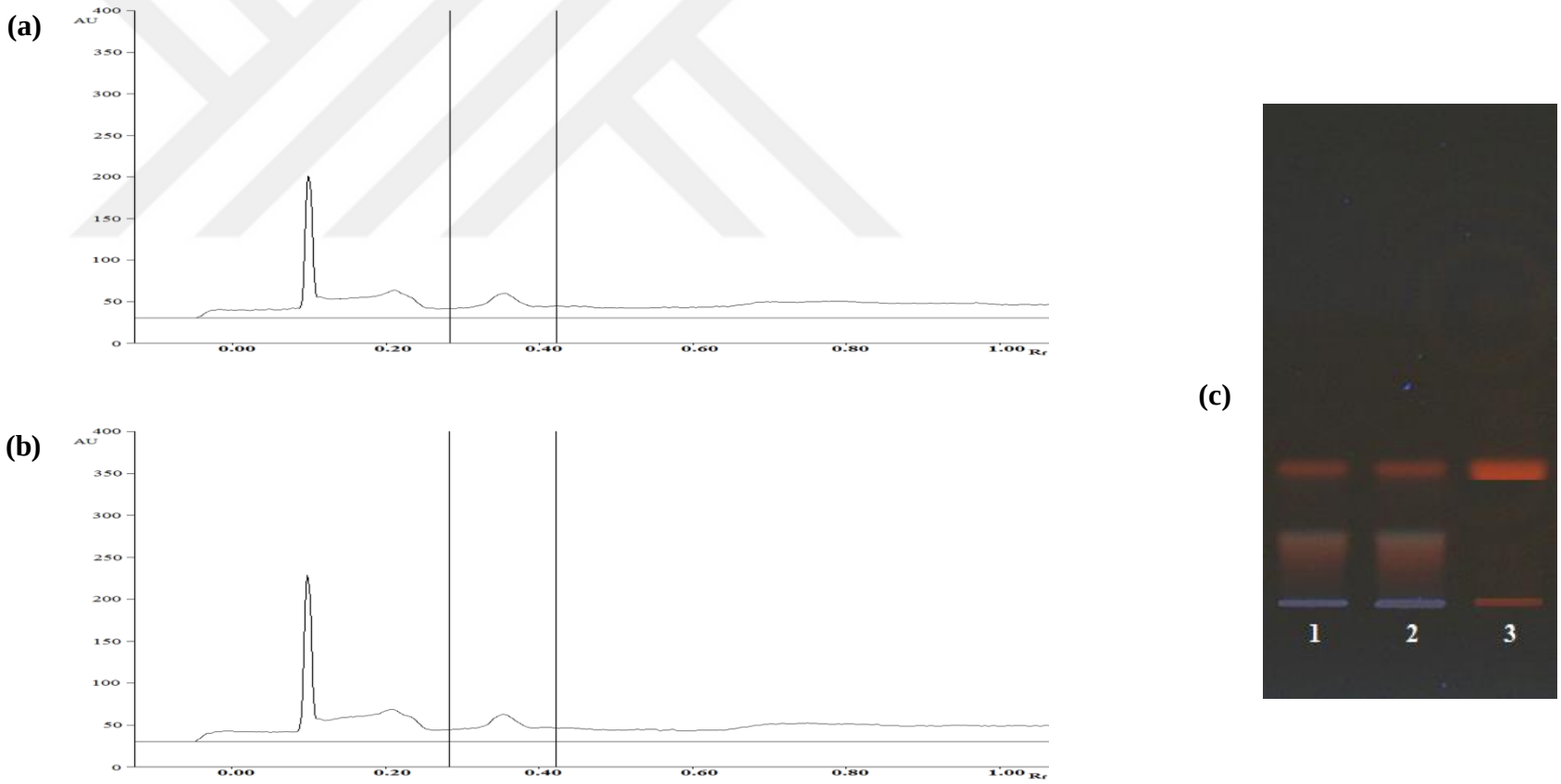


Figure 4.34. HPTLC densitograms obtained from the aqueous extracts of *H. perforatum* L. cultivar: (a) HP-W5 and (b) HP-W10 at 590 nm (40 µg/band); (c) HPTLC chromatogram of the aqueous extracts of *H. perforatum* L. cultivar at 366 nm.
Tracks: (1) HP-W5 (40 µg/band); (2) HP-W10 (40 µg/band); (3) HyP reference (20 ng/band).

As shown in Figure 4.34, HyP was detected in the aqueous extracts from *H. perforatum* L. cultivar HP-W5 and HP-W10 and the amount of HyP was found 0.24 mg/g for HP-W5 and 0.42 mg/g for HP-W10 by HPTLC. However, these values remained far below the HyP content of the methanolic extract of *H. perforatum* L. reference sample [1.60 mg/g].

4.3.6. Evaluation of Hypericin Content in Solvent Extracts from Oily Preparation of *H. perforatum*

Quantification of HyP contents in acetonitrile, methanol-hexane, acetone, and methanol extracts from the oily preparation of *H. perforatum* were examined by the developed HPTLC method. The amounts of HyP detected in these four different solvent extracts were given as mean±SD as the average of triplicate analysis in Table 4.19.

Table 4.19. HyP contents in different solvent extracts from the oily preparation of *H. perforatum*

Solvent extract	**HyP (µg/100 mg oil)**
Acetonitrile	0.41±0.04
Methanol-hexane	0.28±0.07
Acetone	0.25±0.05
Methanol	0.53±0.15

The HPTLC densitograms (Figure 4.35) and chromatographic image (Figure 4.36) of these solvent extracts from the oily preparation of *H. perforatum* were demonstrated below.

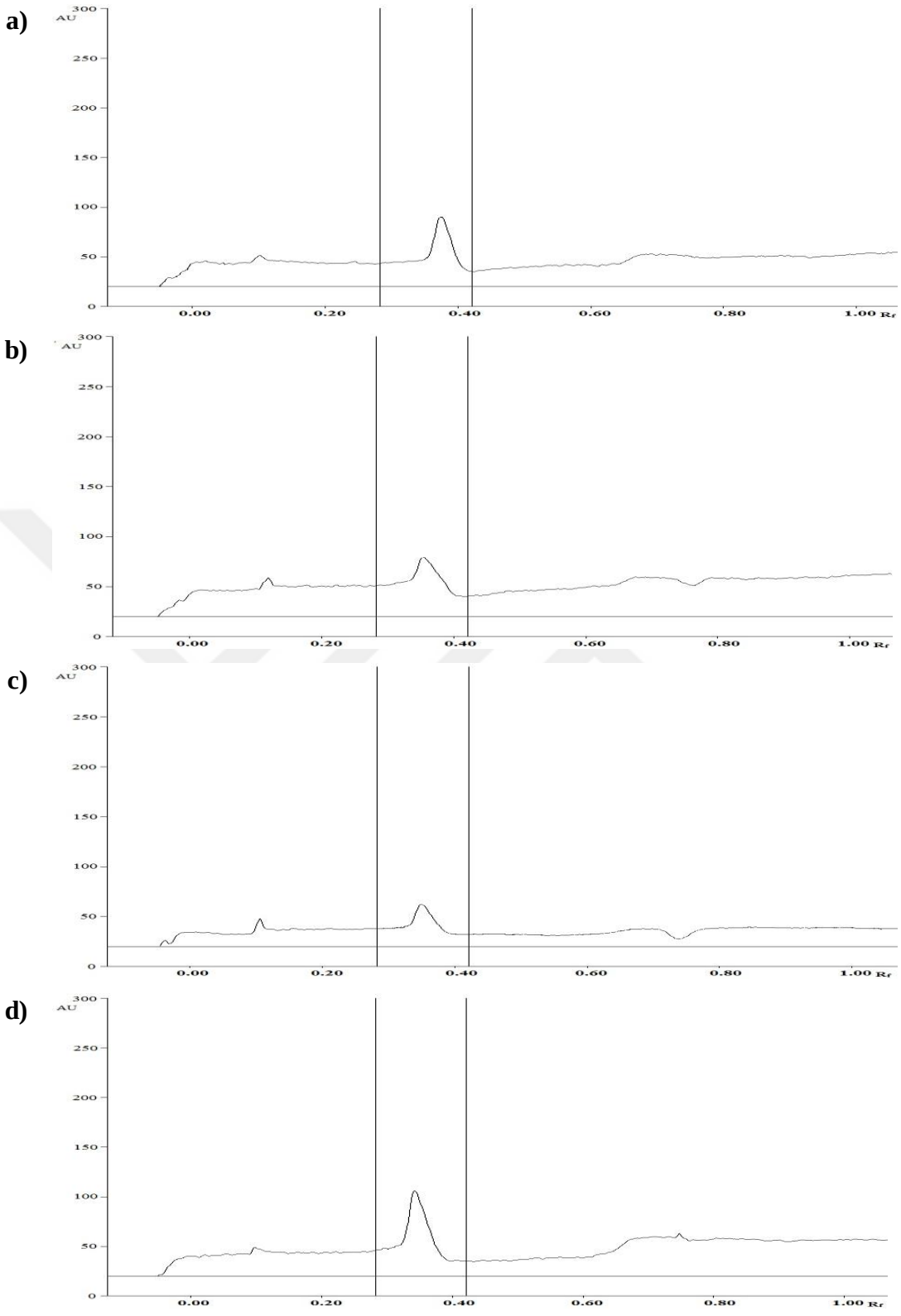


Figure 4.35. HPTLC densitograms obtained with the developed method for HyP at 590 nm extracted with various solvents from the oily preparation of *H. perforatum* (0.4 mg/band): (a) acetonitrile extract; (b) methanol-hexane extract; (c) acetone extract; (d) methanol extract.

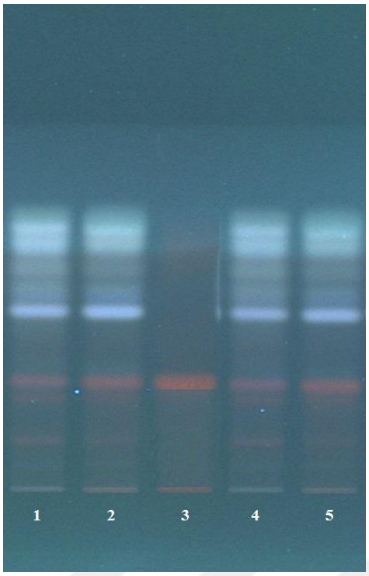


Figure 4.36. HPTLC chromatogram obtained with the developed method for HyP at 366 nm extracted with various solvents from the oily preparation of *H. perforatum*. Tracks: (1) acetonitrile extract; (2) methanol-hexane extract; (3) HyP (reference); (4) acetone extract; (5) methanol extract; derivatization: H_2SO_4 reagent.

The presence of HyP in the oily preparation of *H. perforatum* was examined by the comparison of their R_f values and peaks with that of HyP reference. According to Figure 4.36, when the HPTLC chromatogram was examined, it was seen that all the solvent extracts from the oily preparation of *H. perforatum* were found to contain HyP in varied concentrations (Table 4.19). Additionally, the highest HyP content was determined in methanol extract (Figure 4.35). Therefore, methanol was employed for further studies to extract HyP from the oily preparations of *H. perforatum* for quantitative assays.

4.3.7. Evaluation of Hypericin Content in Commercial *H. perforatum* (SJW) Oily Products

The presence of HyP in the methanolic extracts obtained from the commercial *Hypericum* oily products was identified by the comparison of their R_f values and peaks with that of HyP reference. The HPTLC analysis was carried out in three replicates and the amounts of HyP determined in these products were expressed as mean±SD in Table 4.20. HPTLC densitograms (Figure 4.37) and the related chromatographic image (Figure 4.38) were given below.

Table 4.20. HyP contents in the methanolic extracts from marketed SJW oily products

Oily product	HyP (µg/100 mg oil)
OE-1	0.05±0.03
OE-2	0.07±0.05
OE-3	0.06±0.04
OE-4	0.02±0.02
OE-5	0.04±0.03
OE-6	0.03±0.04
OE-7	ndt

ndt: HyP not detected

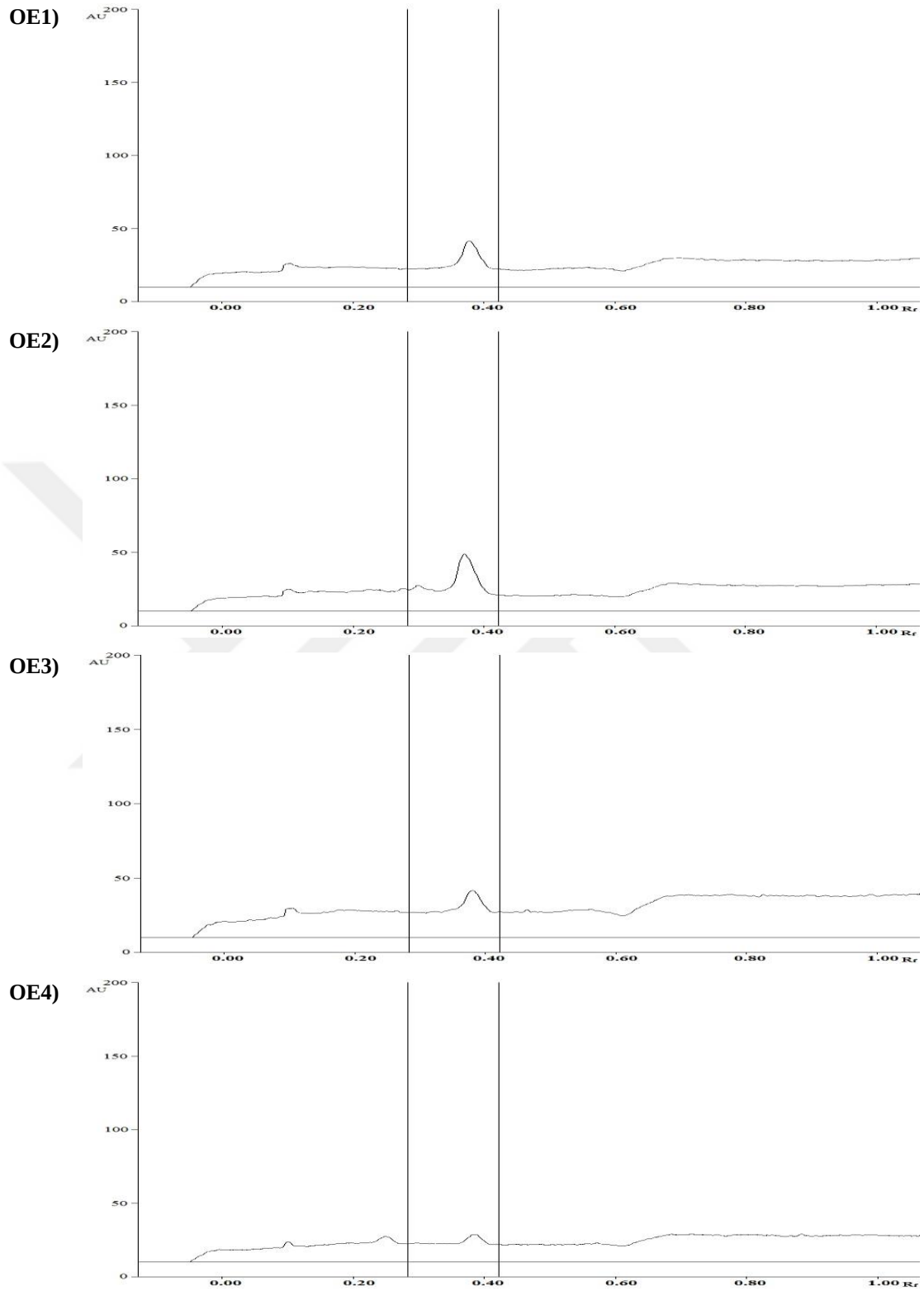


Figure 4.37. HPTLC densitograms from *H. perforatum* (SJW) oily products at 590 nm. Test solutions of oily products: 0.8 mg/band.

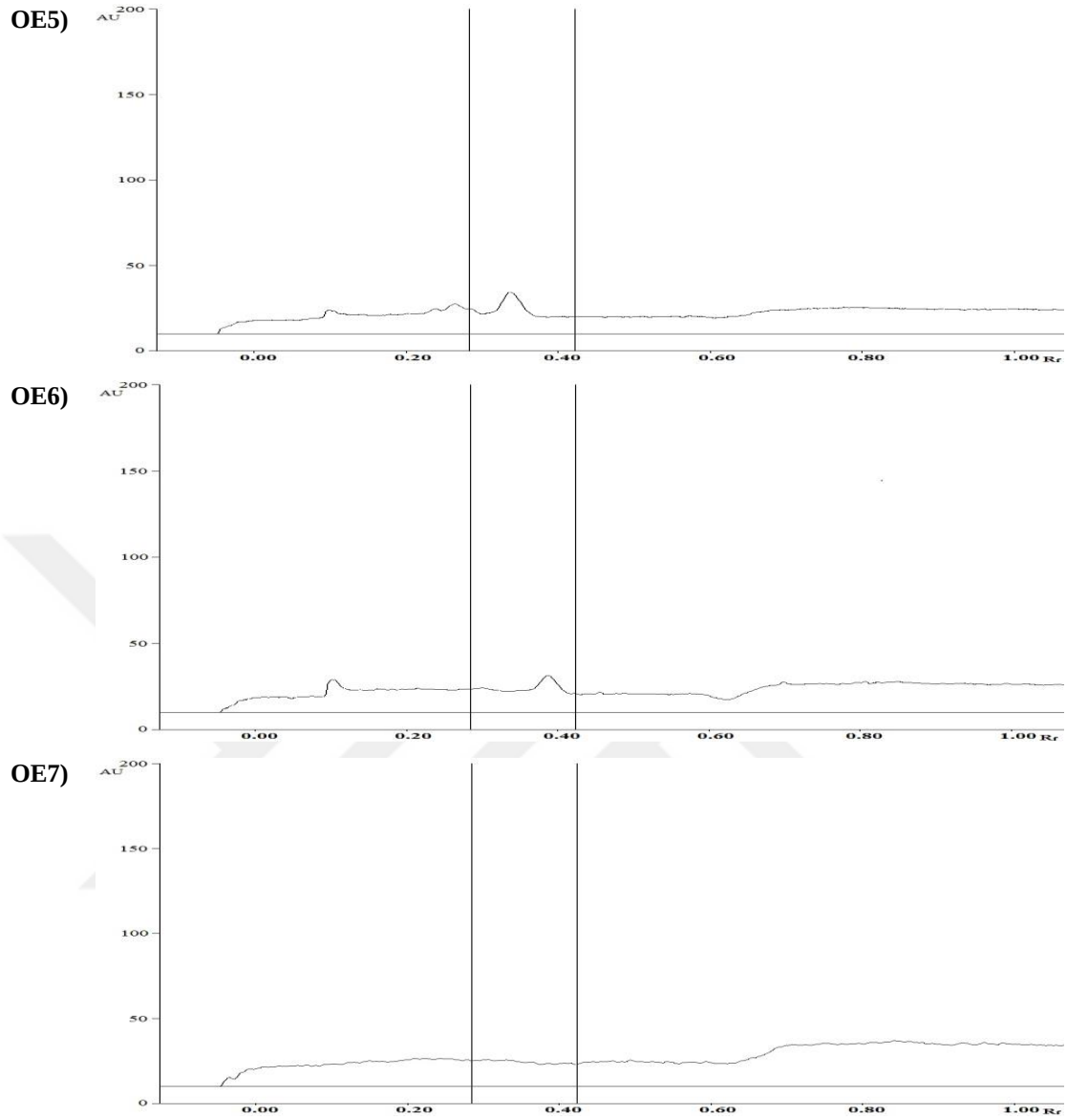


Figure 4.37. HPTLC densitograms from *H. perforatum* (SJW) oily products at 590 nm (continued). Test solutions of oily products: 0.8 mg/band.

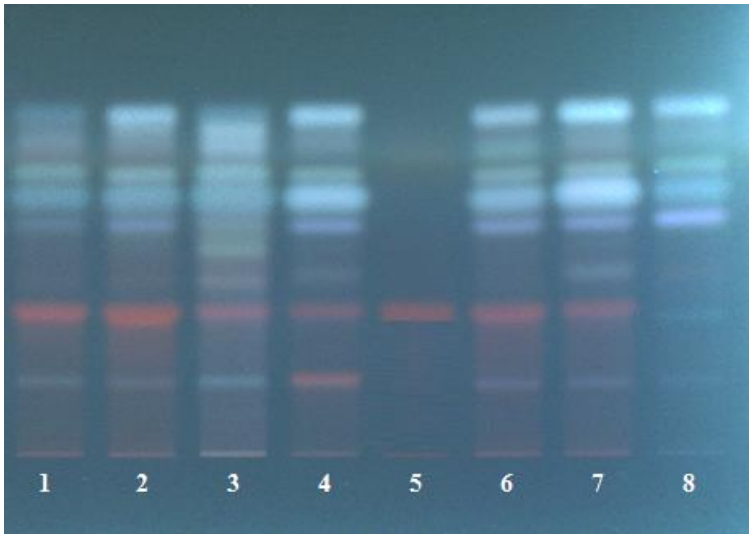


Figure 4.38. HPTLC chromatogram of the marketed *H. perforatum* (SJW) oily products at 366 nm. Tracks: (1) OE1; (2) OE2; (3) OE3; (4) OE4; (5) HyP (reference); (6) OE5; (7) OE6; (8) OE7; derivatization: H_2SO_4 reagent.

The existence of HyP in commercial *H. perforatum* (SJW) oily products was investigated by comparison of their R_f values and peaks with that of HyP reference. As shown in Figure 4.38, OE1-6 extracts were found to contain HyP, whereas HyP was not detected in the HPTLC fingerprint of OE7 at 366 nm. The highest HyP content was determined in OE2 (Figure 4.37 and Table 4.20).

4.4. Analysis of Hyperoside Content in Samples

4.4.1. HPLC Analysis of Hyperoside

The system suitability was confirmed for HPLC method of hyperoside standard according to the Ph. Eur. (234). In this study, retention time (t_R) of hyperoside in the reference solution was determined to be 4.99±0.07 (n=3) (Figure 4.39). The identity of hyperoside in the sample solution was approved by competing t_R with that of the standard solution, and it was found as 5.00±0.01 (n=3) (Figure 4.40). Additionally, the percentage content of hyperoside in *H. perforatum* L. cultivar was determined as 0.54% and thus, *H. perforatum* cultivar could be used as a reference material for hyperoside analyses.

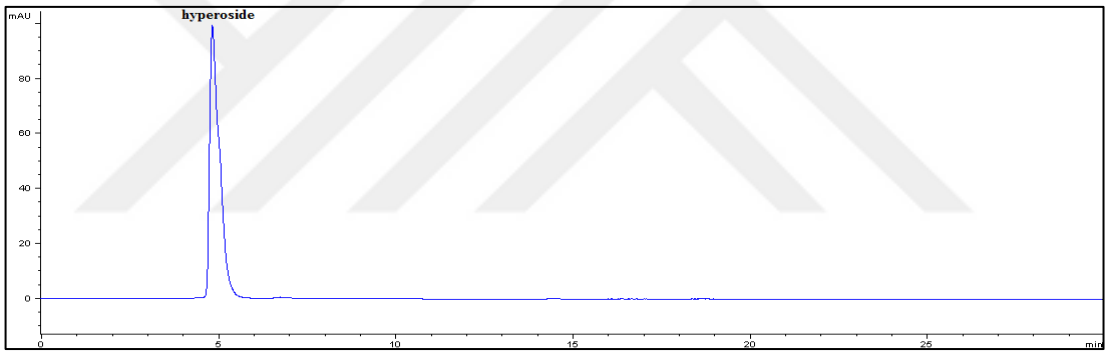


Figure 4.39. HPLC chromatogram of hyperoside reference solution at 360 nm

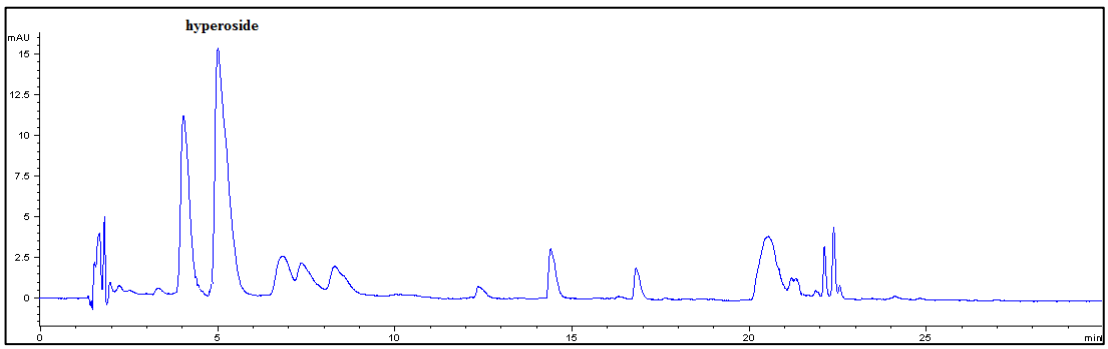


Figure 4.40. HPLC chromatogram of hyperoside in *H. perforatum* L. cultivar test solution at 360 nm

4.4.2. HPTLC Analysis of Hyperoside Content in Samples

4.4.2.1. Method development for hyperoside

While testing the solvent systems indicated in the preceding studies, 20 min saturated twin-through chamber was used. The outcomes of the literature investigations which include the mobile phases used in TLC and HPTLC methods for detection of hyperoside (MP-HyS) were listed in Table 4.21 and HPTLC plates after derivatization obtained from sixteen mobile phases were exhibited in Figure 4.41.

Table 4.21. Solvent systems tested in HPTLC analysis of hyperoside

Mobile phase	Solvent system	Reference
MP-HyS-1	ethyl acetate-dichloromethane-formic acid-acetic acid-water (100:25:10:10:11)	(116, 237)
MP-HyS-2	ethyl acetate-acetic acid-formic acid-water (100:11:11:27)	(238, 239)
MP-HyS-3	ethyl acetate-acetic acid-formic acid-water (100:11:11:26)	(109, 240)
MP-HyS-4	ethyl acetate-acetic acid-formic acid-water (100:11:11:23)	(241)
MP-HyS-5	ethyl acetate-acetic acid-formic acid-water (100:10:10:5)	(242)
MP-HyS-6	ethyl acetate-formic acid-water (15:1.5:1)	(111, 112, 114)
MP-HyS-7	toluene-ethyl formate-formic acid (5:4:1)	(115)
MP-HyS-8	ethyl acetate-methanol-water-formic acid (25:1:1.5:3)	(120)
MP-HyS-9	ethyl acetate-water-methanol (100:16.5:13.5)	(118)
MP-HyS-10	chloroform-water-methanol (61:7:32)	(118)
MP-HyS-11	chloroform-water-methanol (7:0.1:3)	(118)

Table 4.21. Solvent systems used in HPTLC method of hyperoside (continued)

Mobile phase	Solvent system	Reference
MP-HyS-12	chloroform-methanol (8:2)	(118)
MP-HyS-13	chloroform-methanol (9:1)	(118)
MP-HyS-14	toluene-ethyl acetate-formic acid (5:4:1)	(118)
MP-HyS-15	ethyl acetate-ethyl methyl ketone-formic acid-water (5:3:1:1)	(243)
MP-HyS-16	ethyl acetate-formic acid-methanol-water (50:3:3:6)	(116)

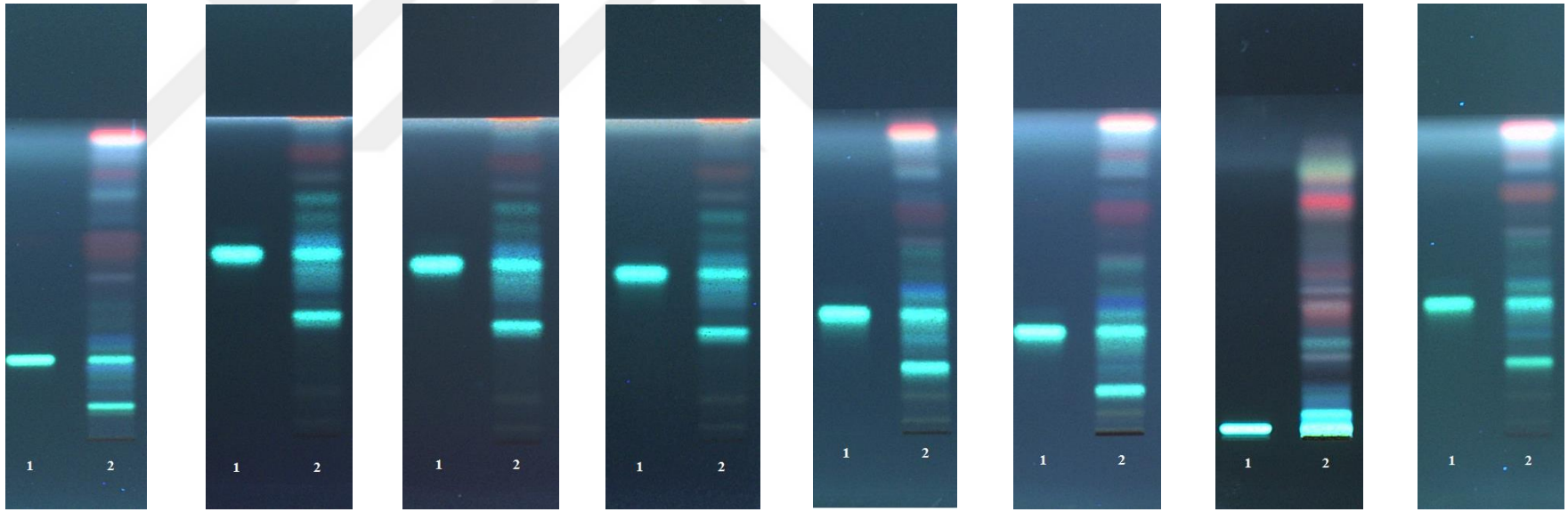


Figure 4.41. HPTLC chromatograms of HyS (reference) and the extract from the flowering aerial parts of *H. perforatum* L. cultivar obtained with various mobile systems at 366 nm.

HPTLC plates were developed by (a) MP-HyS-1; (b) MP-HyS-2; (c) MP-HyS-3; (d) MP-HyS-4; (e) MP-HyS-5; (f) MP-HyS-6; (g) MP-HyS-7; (h) MP-HyS-8. Tracks: (1) HyS (reference); (2) *H. perforatum* extract; derivatization: H_2SO_4 reagent.

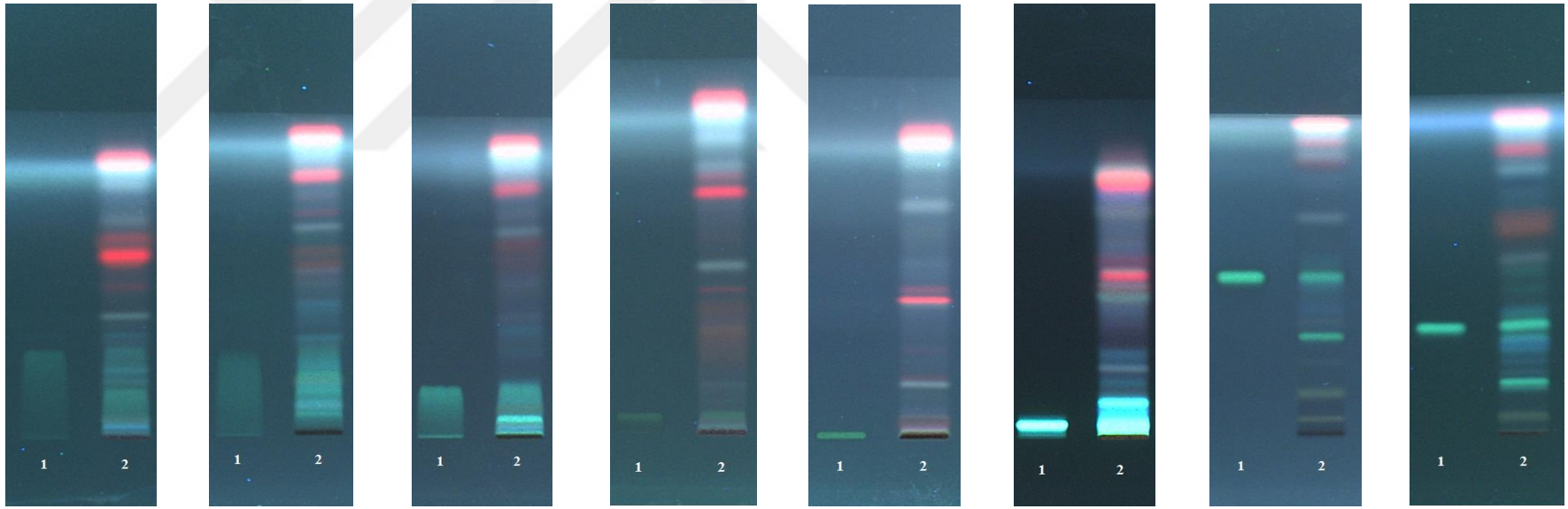


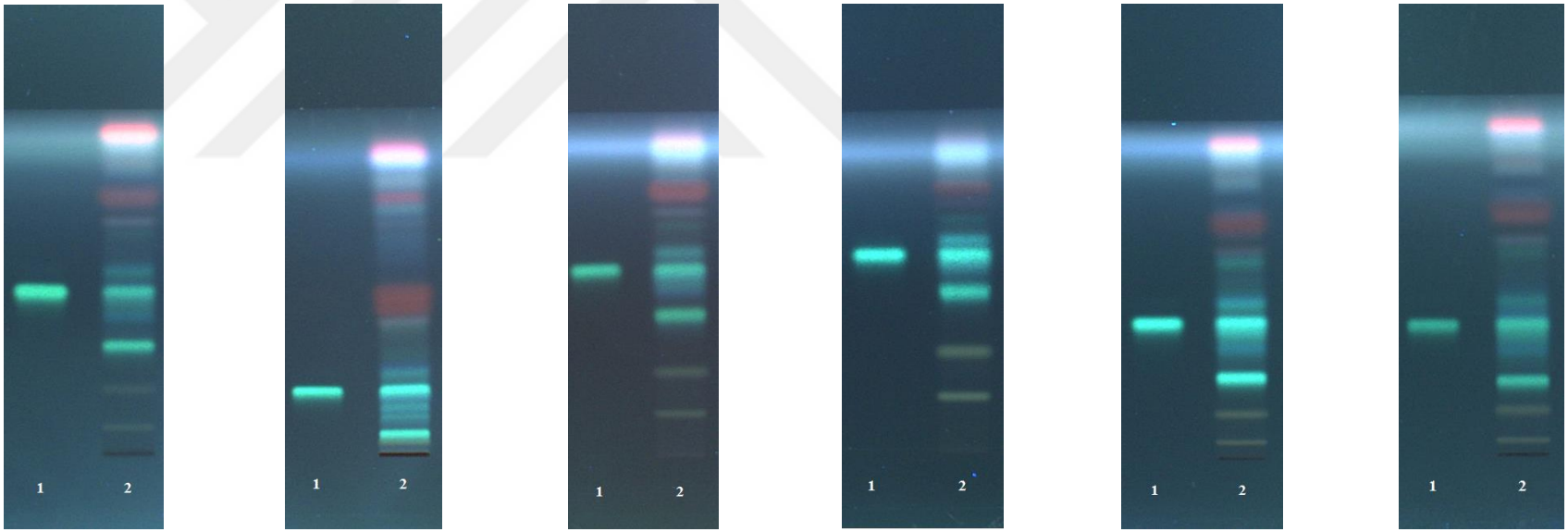
Figure 4.41. HPTLC chromatograms of HyS (reference) and the extract from the flowering aerial parts of *H. perforatum* L. cultivar obtained with various mobile systems at 366 nm (continued).

HPTLC plates were developed by (i) MP-HyS-9; (j) MP-HyS-10; (k) MP-HyS-11; (l) MP-HyS-12; (m) MP-HyS-13; (n) MP-HyS-14; (o) MP-HyS-15; (p) MP-HyS-16. Tracks: (1) HyS (reference); (2) *H. perforatum* extract; derivatization: H_2SO_4 reagent.

Several mobile systems reported in the previously published studies were also tested for suitability (Table 4.21) and MP-HyS-5 showed the best hyperoside resolution in HPTLC analysis. However, hyperoside peak images could not be obtained satisfactorily with this mobile phase. Based on these results, further attempts have been made to develop new solvent mixtures in different ratios to analyse hyperoside and to get the finest peak images. The tested solvent systems were given in Table 4.22 and HPTLC plates after derivatization were displayed in Figure 4.42. The best result was established by MP-HyS-17.

Table 4.22. New mobile systems developed for HPTLC analysis of hyperoside

Mobile phase	**Solvent system**
MP-HyS-17	ethyl acetate-formic acid-acetic acid-water (15:2:2:1)
MP-HyS-18	ethyl acetate-formic acid-acetic acid-water (45:2:2:1)
MP-HyS-19	ethyl acetate-formic acid-acetic acid-water (15:3:3:1)
MP-HyS-20	ethyl acetate-formic acid-acetic acid-water (15:4:4:1)
MP-HyS-21	ethyl acetate-formic acid-acetic acid-water (15:1.6:1.6:1)
MP-HyS-22	ethyl acetate-formic acid-acetic acid-water (15:1.6:1.6:0.6)



(a) **(b)** **(c)** **(d)** **(e)** **(f)**

Figure 4.42. HPTLC chromatograms of HyS (reference) and the extract from the flowering aerial parts of *H. perforatum* L. cultivar obtained with various new mobile systems at 366 nm.

HPTLC plates were developed by (a) MP-HyS-17; (b) MP-HyS-18; (c) MP-HyS-19; (d) MP-HyS-20; (e) MP-HyS-21; (f) MP-HyS-22. Tracks: (1) HyS (reference); (2) *H. perforatum* extract; derivatization: H_2SO_4 reagent.

4.4.2.2. Method validation of hyperoside

The developed HPTLC process was verified following the ICH guidelines for the points of stability, robustness, specificity, detection and quantitation limits, linearity, intra- and interday precisions as well as recovery (223).

4.4.2.2.1. Stability

The stability of HyS in methanol at several days and different temperatures, stability of HyS reference solutions adsorbed to the plate prior to chromatography, and stability of sample test solution through development were explored to assess the stability of the components during the chromatographic processing.

4.4.2.2.1.1. Stability of hyperoside solution during storage period

The stability of HyS standard in methanol (0.02 mg/mL) was tested to verify whether spontaneous degradation occurred within 21 days after preparation and during storage at -20°C and 4°C. All experiments were iterated three times. Each peak area of the working solutions was compared with that of the fresh preparation of HyS standard in methanol at all time intervals. No chromatographically detectable differences and degradations were observed. Thus, each stock solution prepared in methanol was found to be stable during storage at -20°C as well as 4°C for 3-week period, $F_{(3,20)}$=0.646, p=0.595 [$F_{crit\,(3,20)}$=3.098].

4.4.2.2.1.2. Stability of hyperoside solution on the stationary phase

The freshly prepared HyS reference solution (0.02 mg/mL) was applied on plate every 30 minutes up to 2 hours prior the development. The plate was left uncovered, subjected to daylight and air throughout these periods. This procedure was repeated in triplicate. One-way ANOVA was employed to assure the interruptions in time intervals. No important differences were seen amongst the mean areas of HyS reference solution, $F_{(4,10)}$=2.641, p=0.097 [$F_{crit\,(4,10)}$=3.478].

4.4.2.2.1.3. Stability of sample test solution during migration with the mobile phase

Stability of the components in *H. perforatum* sample solution on the plate during the migration was investigated by two-dimensional separation process. After first migration of the test solution, the plate was turned 90° and developed in the same mobile system at diagonal direction. All the components in sample were situated on the diagonal of the chromatogram demonstrating their stability during the migration (Figure 4.43).

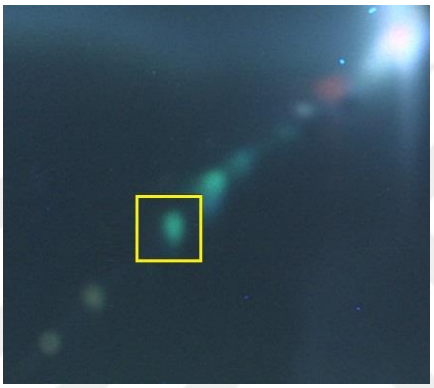


Figure 4.43. 2D-development of *H. perforatum* sample test solution for stability testing of components in ethyl acetate-formic acid-acetic acid-water (15:2:2:1, v/v/v/v) at 366 nm. Derivatization: H_2SO_4 reagent.

4.4.2.2.2. Robustness

Some modifications in system conditions were made to evaluate the robustness of the method. In this regard, time of chamber saturation and humidity control, plate preconditioning time as well as time from development to scanning were slightly modified and then, the proportions of the peak area of HyP reference (0.02 mg/mL) and sample solutions (5 mg/mL) were examined for the possible changes. One-way ANOVA was used to compare the results. Accordingly, no significant differences were detected in the mean rates across the conditional changes, including saturation ($F_{(6,14)}$=1.786, p=0.174), humidity control ($F_{(6,14)}$=1.409, p=0.279), preconditioning

($F_{(6,14)}$=1.948, p=0.143), and time from chromatography to scan ($F_{(6,14)}$=2.041, p=0.127) [($F_{crit\,(6,\,14)}$=2.848)].

4.4.2.2.3. Specificity

Specificity of the solvent system was assessed by examining HyS reference (0.02 mg/mL) and sample solution (5 mg/mL). Within this context, R_f value of HyS in *H. perforatum* test solution was compared with that of HyS in reference solution and the R_f value was found to be 0.49±0.04 (Figure 4.44). Additionally, HPTLC chromatogram visualized under 366 nm light and the densitograms of reference and sample solution containing HyS were employed for the approval.

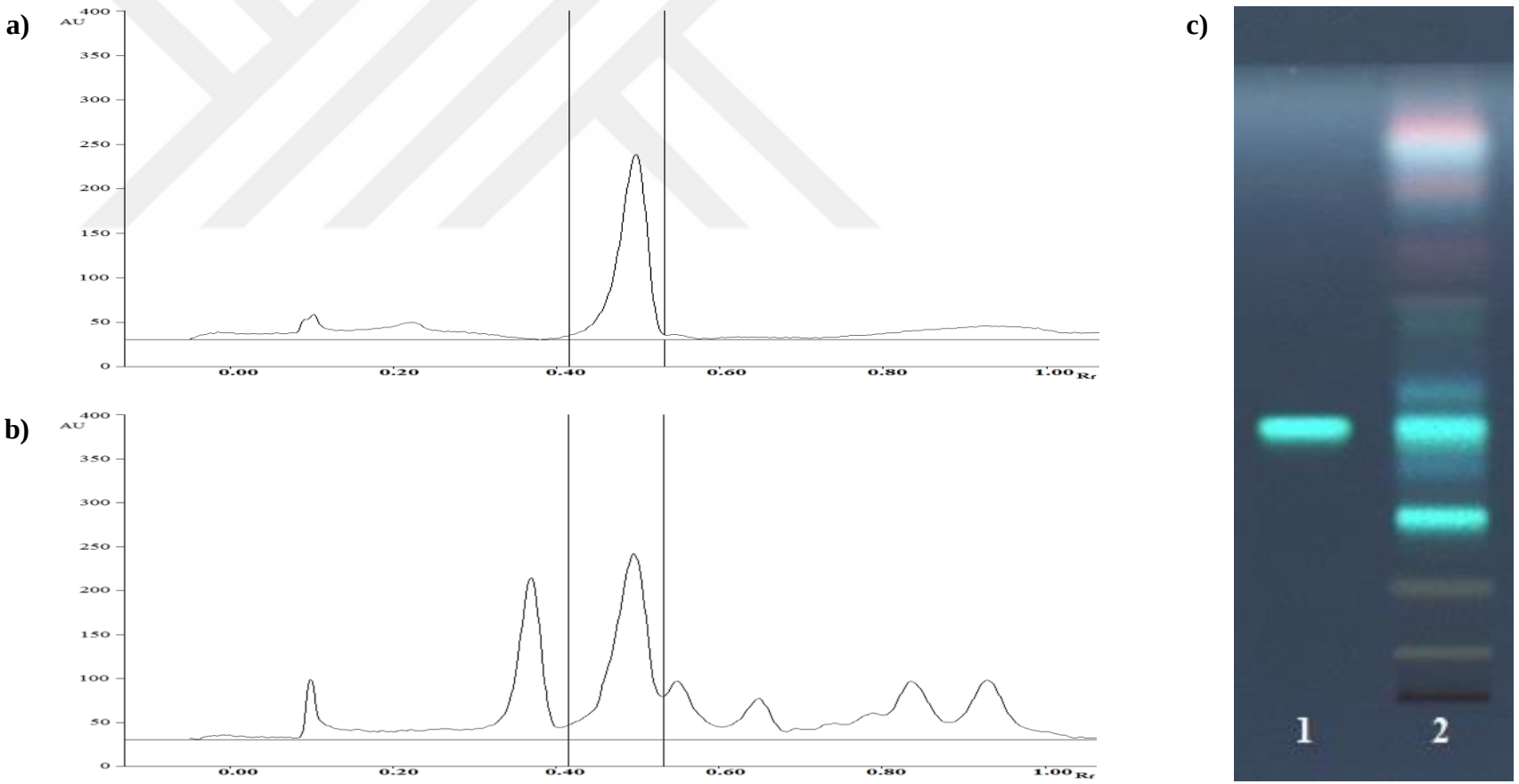


Figure 4.44. For specificity of the developed HPTLC process, HPTLC densitograms from (a) HyS reference solution (2.5 µL); (b) *H. perforatum* L. sample solution (1 µL) at 363 nm, and HPTLC fingerprints at 366 nm.

Tracks: (1) HyS reference solution (2.5 µL); (2) *H. perforatum* L. sample solution (1 µL); derivatization: H_2SO_4 reagent.

4.4.2.2.4. Limits of detection (LOD) and quantitation (LOQ)

HyS reference solutions in different concentrations (0.001 mg/mL, 0.01 mg/mL, and 0.05 mg/mL) and in increasing volumes (1-5 µL) were applied on several plates to estimate the detection limit and quantitation limit. Then the values of LOD and LOQ were found to be 3 and 10 ng/band, respectively.

4.4.2.2.5. Linearity and calibration curve

Linearity of the developed HPTLC system was evaluated at the applied values within the range of 10-100 ng/band of reference solutions and calibration curves were constructed with three independent injections. The quadratic correlation between the injected volumes and the peak area were obtained from the applied concentrations of HyS solution and the correlation coefficient was estimated to be 0.999±0.001. The regression equation was y=-636.60+128.97x-0.31x^2 [y=peak area; x=concentration in ng/band].

4.4.2.2.6. Precision

4.4.2.2.6.1. Repeatability

The intra-assay precision of the developed HPTLC process was conducted in three replicates of the experiment with *H. perforatum* samples prepared three time intervals on the same day. The quantities of HyS in samples expressed as mean± SD as well as the RSD values were given in Table 4.23.

4.4.2.2.6.2. Intermediate precision

The interday precision of the newly developed HPTLC system was carried out with the *H. perforatum* samples prepared on three different days. Analysis of each sample was repeated thrice and the amount of HyS in each sample was stated as mean± SD. The results of interday precision were similar to those of intraday precision and the findings were given in Table 4.23. Accordingly, the outcomes presented that the values

of RSD for intra- and interday precisions were within the expected range of assessment criteria (225).

Table 4.23. Intra- and interday precision results of developed HPTLC method for HyS

Precision analysis		**HyS (mg/g)** (*n*=3)		
		sample-I	**sample-II**	**sample-III**
Within-day	analysis-I	5.40±0.05	5.39±0.04	5.37±0.08
	analysis-II	5.34±0.01	5.35±0.03	5.41±0.10
	analysis-III	5.42±0.07	5.32±0.05	5.33±0.06
	RSD (%)	1.05	0.87	1.47
Between-day	analysis-I	5.34±0.12	5.31±0.09	5.29±0.08
	analysis-II	5.29±0.08	5.38±0.07	5.35±0.15
	analysis-III	5.37±0.09	5.34±0.04	5.34±0.11
	RSD (%)	1.72	1.27	1.97

4.4.2.2.7. Recovery

The accuracy of the developed HPTLC system was validated by standard addition method. The known quantities of HyS reference (10, 20 and 50 ng/band) were added to pre-analyzed sample solutions. Three replicates of each concentration were tested. The amount of HyS detected in mixed solution was expressed as mean±SD as well as the mean recovery and RSD were calculated as per cent (Table 4.24). The results presented that the means of percentage recoveries were within the expected range of assessment criteria (225).

Table 4.24. Recovery findings of HPTLC system of HyS

HyS added (ng/band)	**HyS found (ng/band)**	**RSD (%)**	**Recovery (%)**
10	10.14±0.18	1.81	101.37
20	20.08±0.20	1.01	100.42
50	50.39±0.38	0.76	100.78

4.4.3. Hyperoside Assay in Various *Hypericum* Species

The presence of HyS in the extracts from the flowering aerial parts of different *Hypericum* species was detected by the comparison of their R_f values and corresponding peaks related to retention spots with those of HyS reference. The analysis was performed in triplicate. The HyS concentrations in various *Hypericum* specimens were given as mean±SD in Table 4.25.

Table 4.25. HyS contents in various *Hypericum* species

***Hypericum* specimen**	**HyS (mg/g)**
H. perforatum (İstanbul)	4.62±0.03
H. perforatum (Tekirdağ)	5.27±0.06
H. bithynicum	9.73±0.05
H. calycinum	4.07±0.06
H. cerastoides	1.01±0.07
H. perfoliatum	9.07±0.08
H. triquetrifolium	3.29±0.02
H. kotschyanum	1.67±0.01
H. scabrum	2.51±0.05
H. thymopsis	2.21±0.02

Furthermore, the explanations of these findings as well as the designation of differentiation between these samples were supported by HPTLC densitograms (Figure 4.45) and fingerprint (Figure 4.46) of *Hypericum* species.

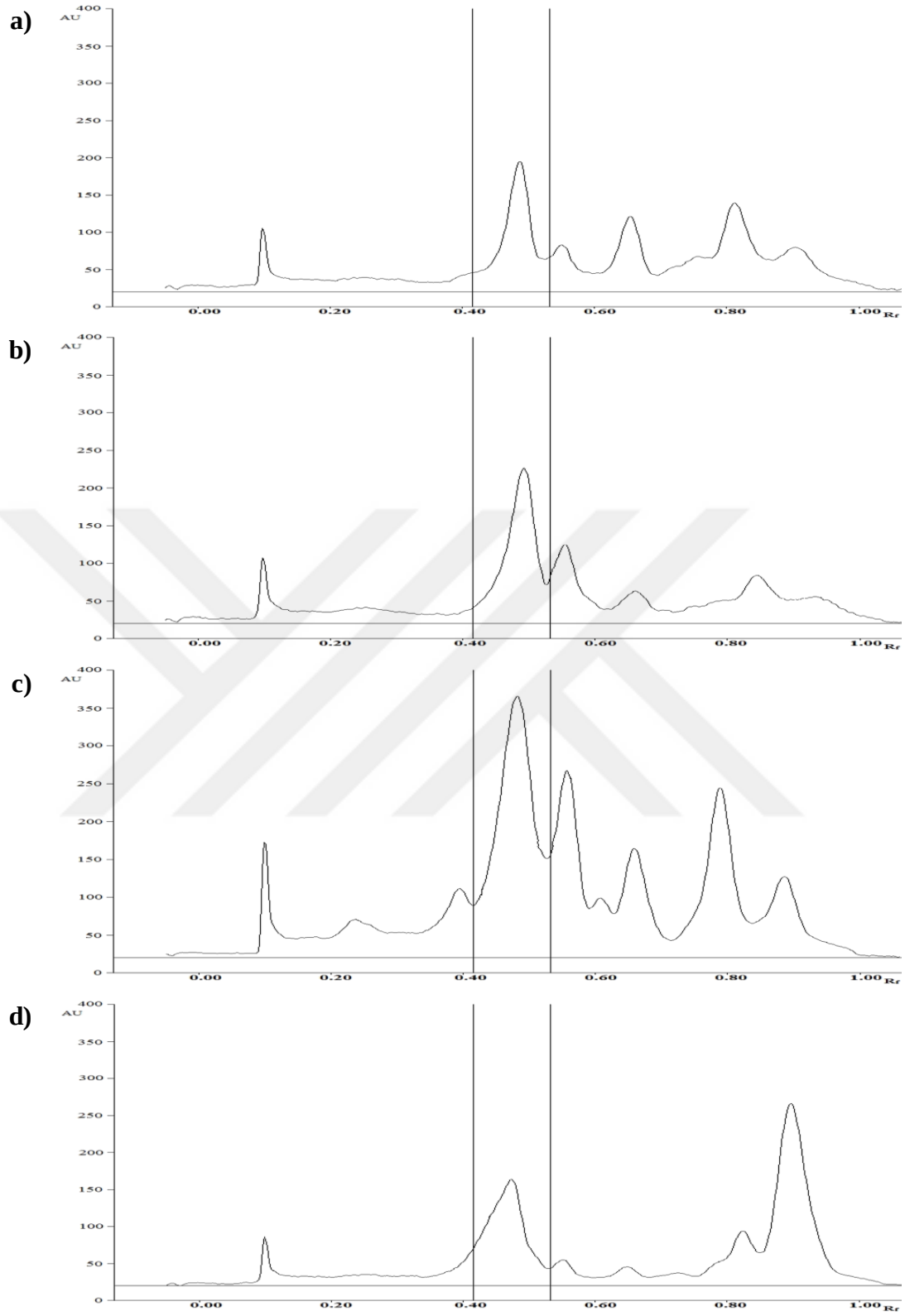


Figure 4.45. HPTLC densitograms of the extracts from different *Hypericum* species at 363 nm. (a) *H. perforatum* (İstanbul); (b) *H. perforatum* (Tekirdağ); (c) *H. bithynicum*; (d) *H. calycinum* (5 µg/band).

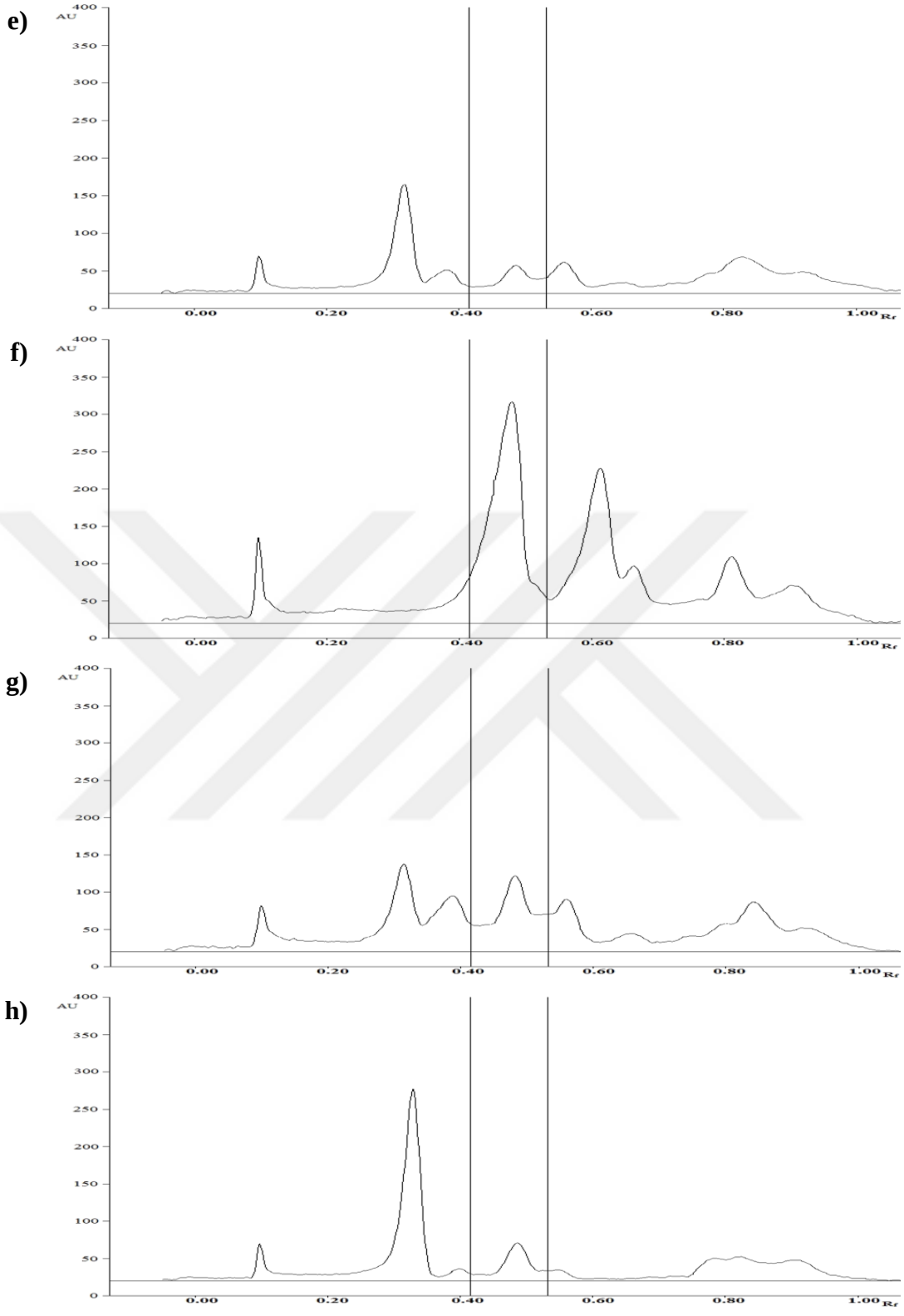


Figure 4.45. HPTLC densitograms of the extracts from different *Hypericum* species at 363 nm (continued). (e) *H. cerastoides*; (f) *H. perfoliatum*; (g) *H. triquetrifolium*; (h) *H. kotschyanum* (5 µg/band).

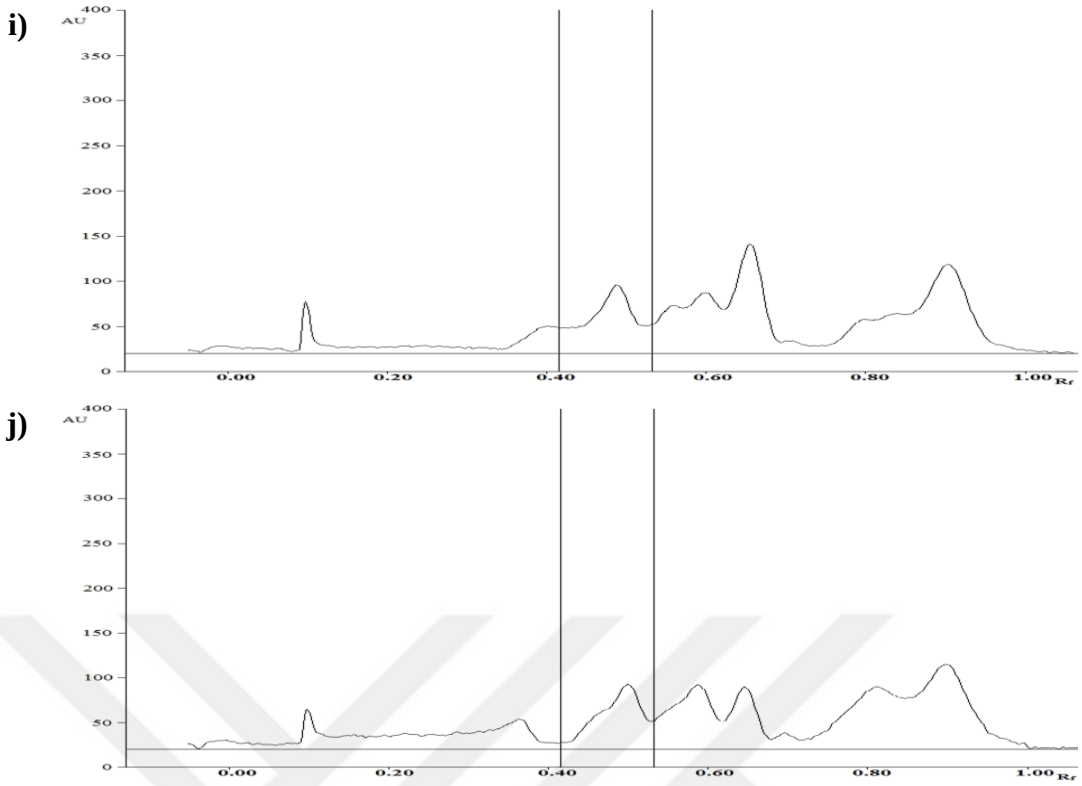


Figure 4.45. HPTLC densitograms of the extracts from different *Hypericum* species at 363 nm (continued). (i) *H. scabrum*; (j) *H. thymopsis* (5 µg/band).

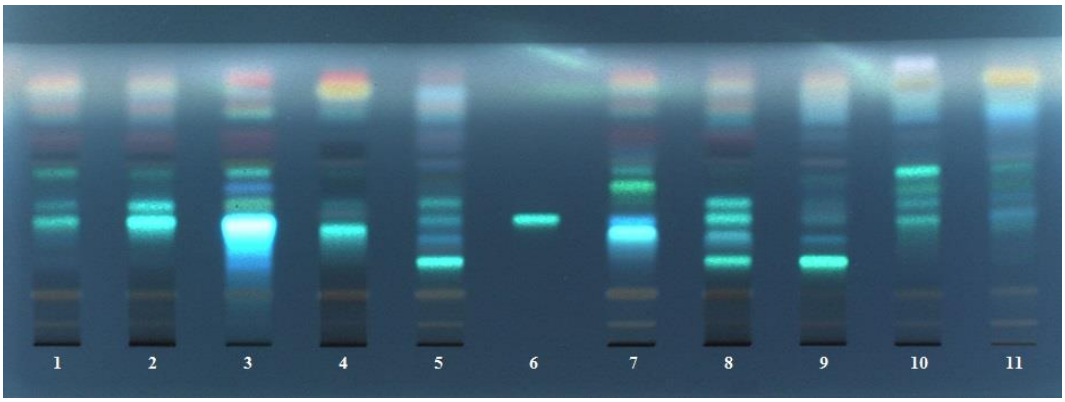


Figure 4.46. HPTLC chromatogram of the extracts from *Hypericum* species at 366 nm. Tracks: (1) *H. perforatum* (İstanbul); (2) *H. perforatum* (Tekirdağ); (3) *H. bithynicum*; (4) *H. calycinum*; (5) *H. cerastoides*; (6) HyS (reference) (50 ng/band); (7) *H. perfoliatum*; (8) *H. triquetrifolium*; (9) *H. kotschyanum*; (10) *H. scabrum*; (11) *H. thymopsis*; applied volume of *Hypericum* species: 5 µg/band; derivatization: H_2SO_4 reagent.

According to HPTLC densitograms and fingerprints, HyS was identified in *H. perforatum* obtained from two different locations and in other eight *Hypericum* specimens between the ranges from 1.01 to 9.73 mg/g (Table 4.25). HyS contents in the two *H. perforatum* specimens collected from İstanbul [4.62 mg/g] and Tekirdağ [5.27 mg/g] were lower than that of the *H. perforatum* L. reference sample [5.35 mg/g]. On the other hand, the quantities of HyS determined in *H. bithynicum* [9.73 mg/g] and *H. perfoliatum* [9.07 mg/g] were much higher than that of the *H. perforatum* L. reference sample.

4.4.4. Hyperoside Assay in the Marketed *H. perforatum* (SJW) Products

The presence of HyS in the *Hypericum* products marketed as tea, capsule or dried herbal material was detected by the comparison of their R_f values and relevant peaks with those of HyS reference. The analysis was conducted in triplicate. The amounts of HyS determined in these products were given as mean±SD in Table 4.26, while related HPTLC densitograms (Figure 4.47, Figure 4.49a, Figure 4.50) and fingerprints (Figure 4.48, Figure 4.49b, Figure 4.51) were also displayed below.

Table 4.26. HyS contents in marketed *H. perforatum* (SJW) products

Commercial product	HyS (mg/g)	Commercial product	HyS (mg/g)
HT1	1.17±0.03	HS13	2.08±0.04
HT2	0.72±0.05	HS14	2.25±0.02
HT3	1.45±0.03	HS15	3.31±0.12
HT4	3.14±0.04	HS16	3.63±0.12
HT5	3.20±0.09	HS17	1.72±0.05
HT6	1.39±0.06	HS18	4.10±0.08
HT7	2.81±0.07	HS19	3.92±0.19
HT8	1.45±0.02	HS20	3.87±0.16
HC	3.54±0.13	HS21	3.79±0.15
HS1	1.26±0.07	HS22	4.95±0.12
HS2	2.08±0.03	HS23	1.37±0.06
HS3	2.91±0.03	HS24	1.64±0.08
HS4	4.02±0.02	HS25	3.47±0.14
HS5	1.07±0.02	HS26	1.33±0.08
HS6	4.20±0.04	HS27	3.62±0.10
HS7	2.09±0.08	HS28	1.47±0.05
HS8	0.74±0.06	HS29	2.72±0.09
HS9	2.64±0.05	HS30	4.23±0.17
HS10	2.85±0.04	HS31	1.84±0.11
HS11	1.36±0.11	HS32	4.37±0.17
HS12	3.01±0.06	HS33	5.00±0.15

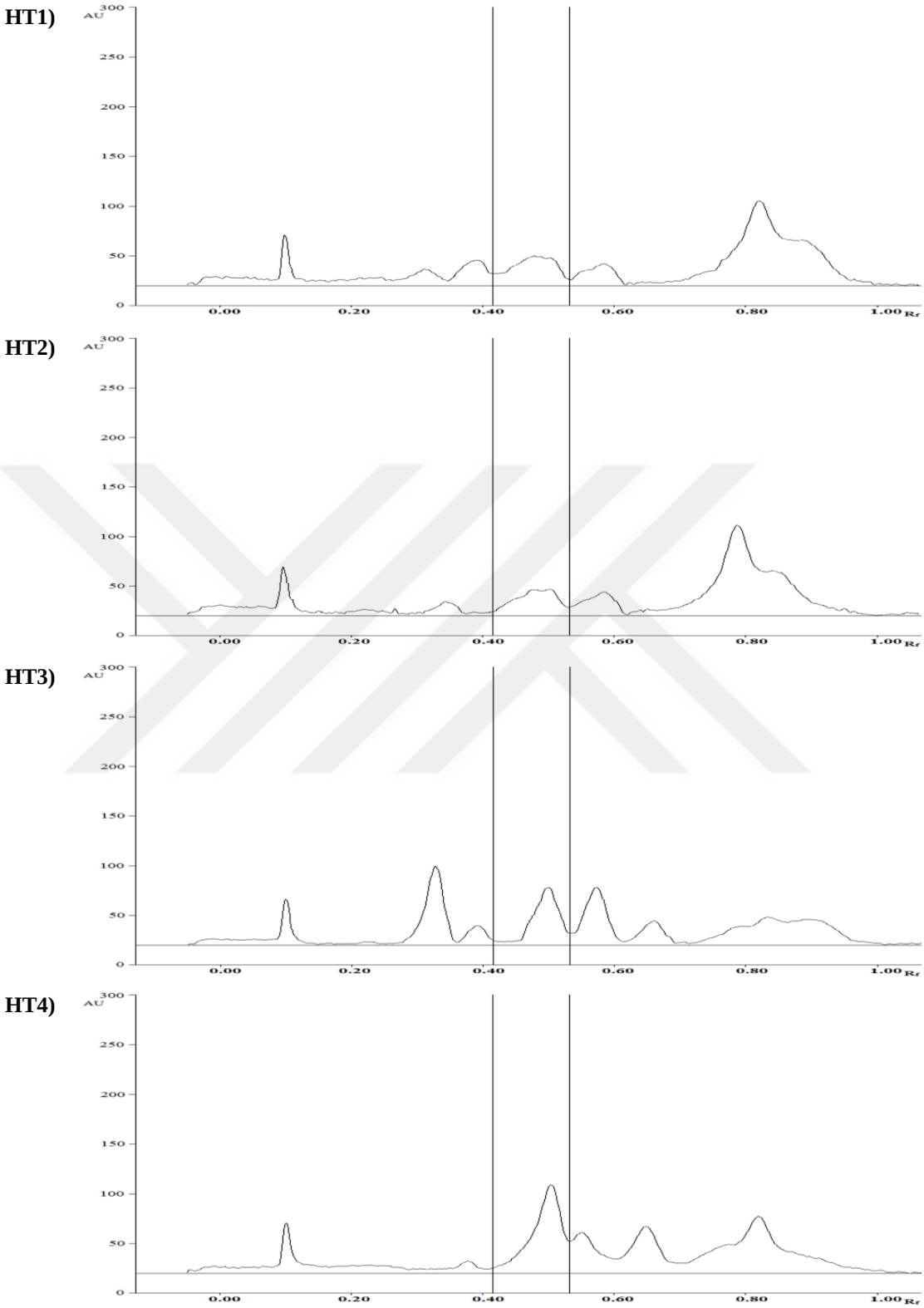


Figure 4.47. HPTLC densitograms from *H. perforatum* (SJW) tea products at 363 nm. Test solutions of tea products: 10 µg/band.

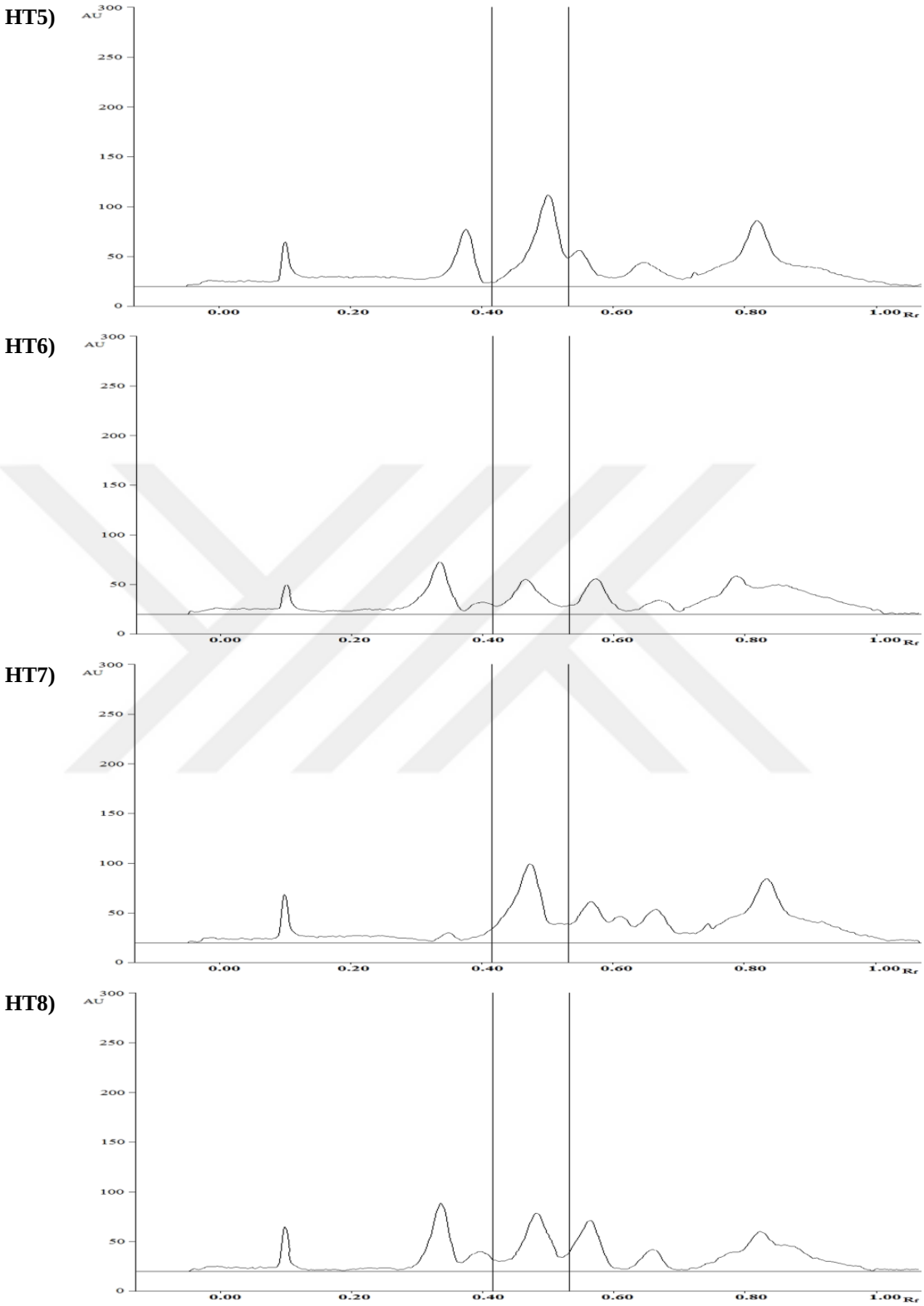


Figure 4.47. HPTLC densitograms from *H. perforatum* (SJW) tea products at 363 nm (continued). Test solutions of tea products: 10 µg/band.

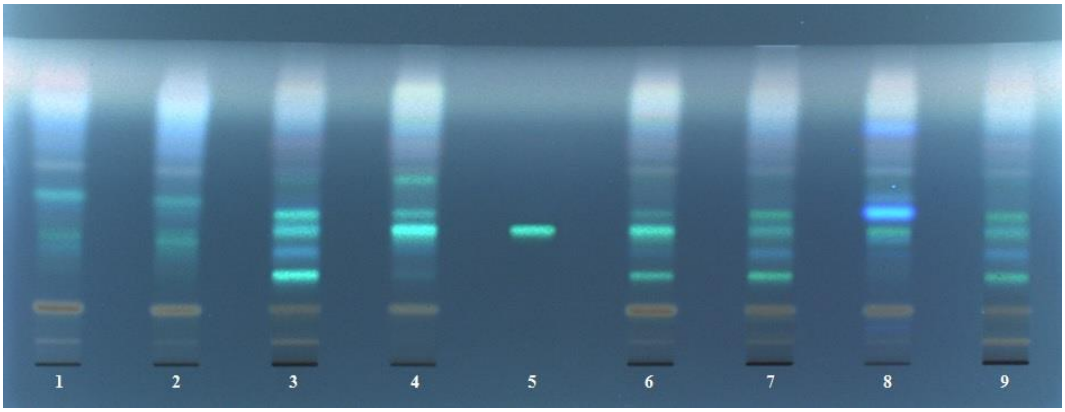
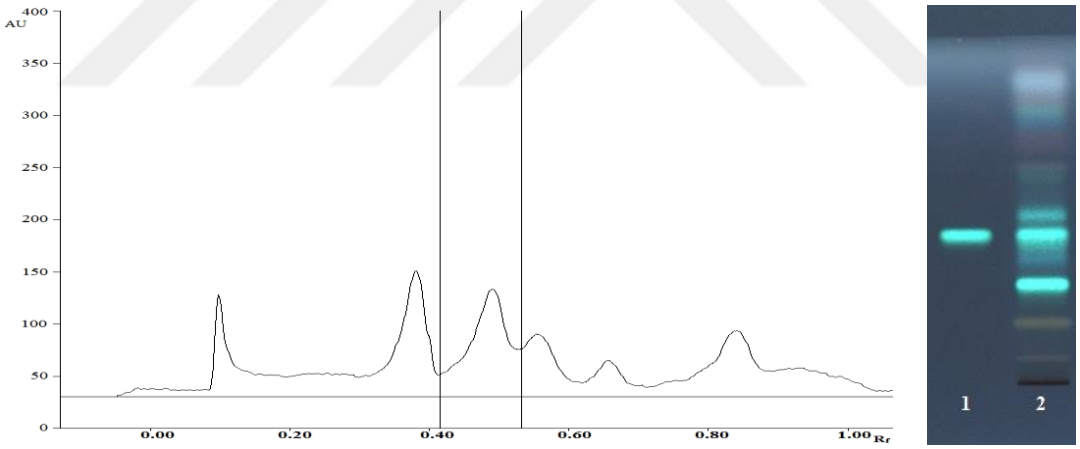


Figure 4.48. HPTLC chromatogram from *H. perforatum* (SJW) tea products at 366 nm. Tracks: (1) HT1; (2) HT2; (3) HT3; (4) HT4; (5) HyS reference (50 ng/band); (6) HT5; (7) HT6; (8) HT7; (9) HT8; applied volume of *H. perforatum* (SJW) tea products: 10 µg/band; derivatization: H_2SO_4 reagent.



(a) **(b)**

Figure 4.49. (a) HPTLC densitogram obtained from *H. perforatum* (SJW) capsule formulation at 363 nm (5 µg/band) and (b) HPTLC chromatogram of *H. perforatum* (SJW) capsule formulation at 366 nm. Tracks: (1) HyS reference (50 ng/band); (2) HC (5 µg/band); derivatization: H_2SO_4 reagent.

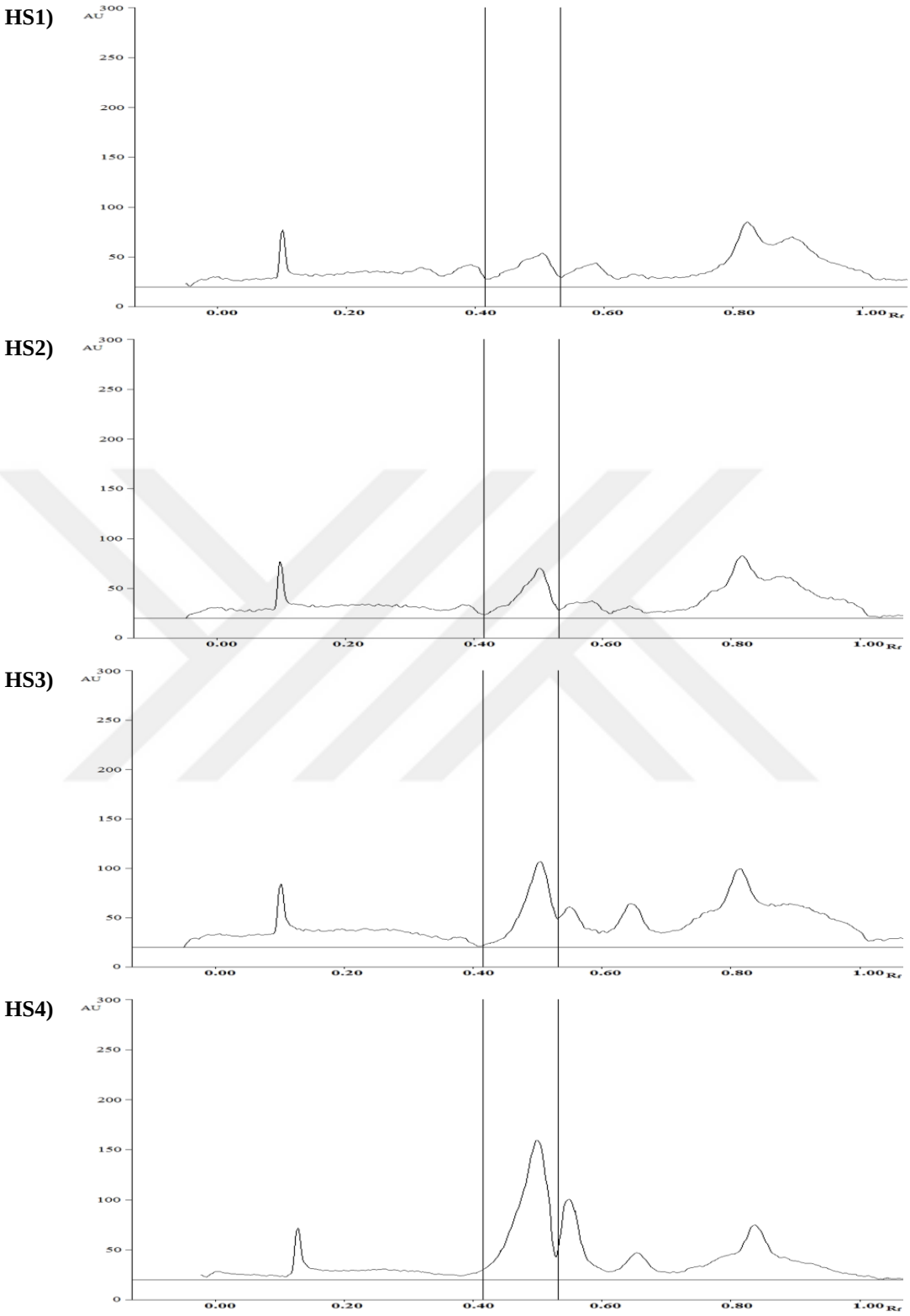


Figure 4.50. HPTLC densitograms from the marketed *H. perforatum* (SJW) herbal materials at 363 nm. Sample test solutions: 10 µg/band.

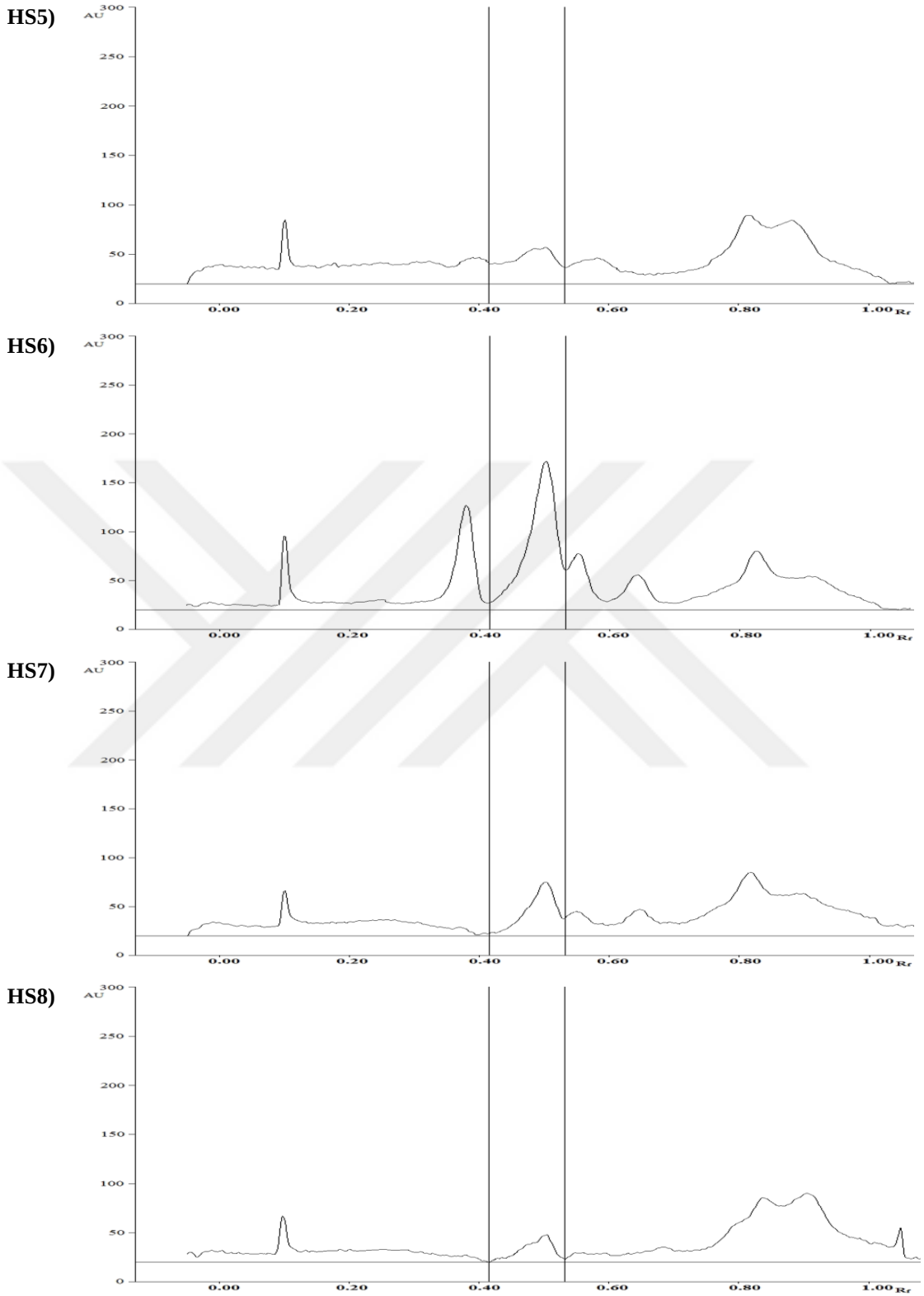


Figure 4.50. HPTLC densitograms from the marketed *H. perforatum* (SJW) herbal materials at 363 nm (continued). Sample test solutions: 10 µg/band.

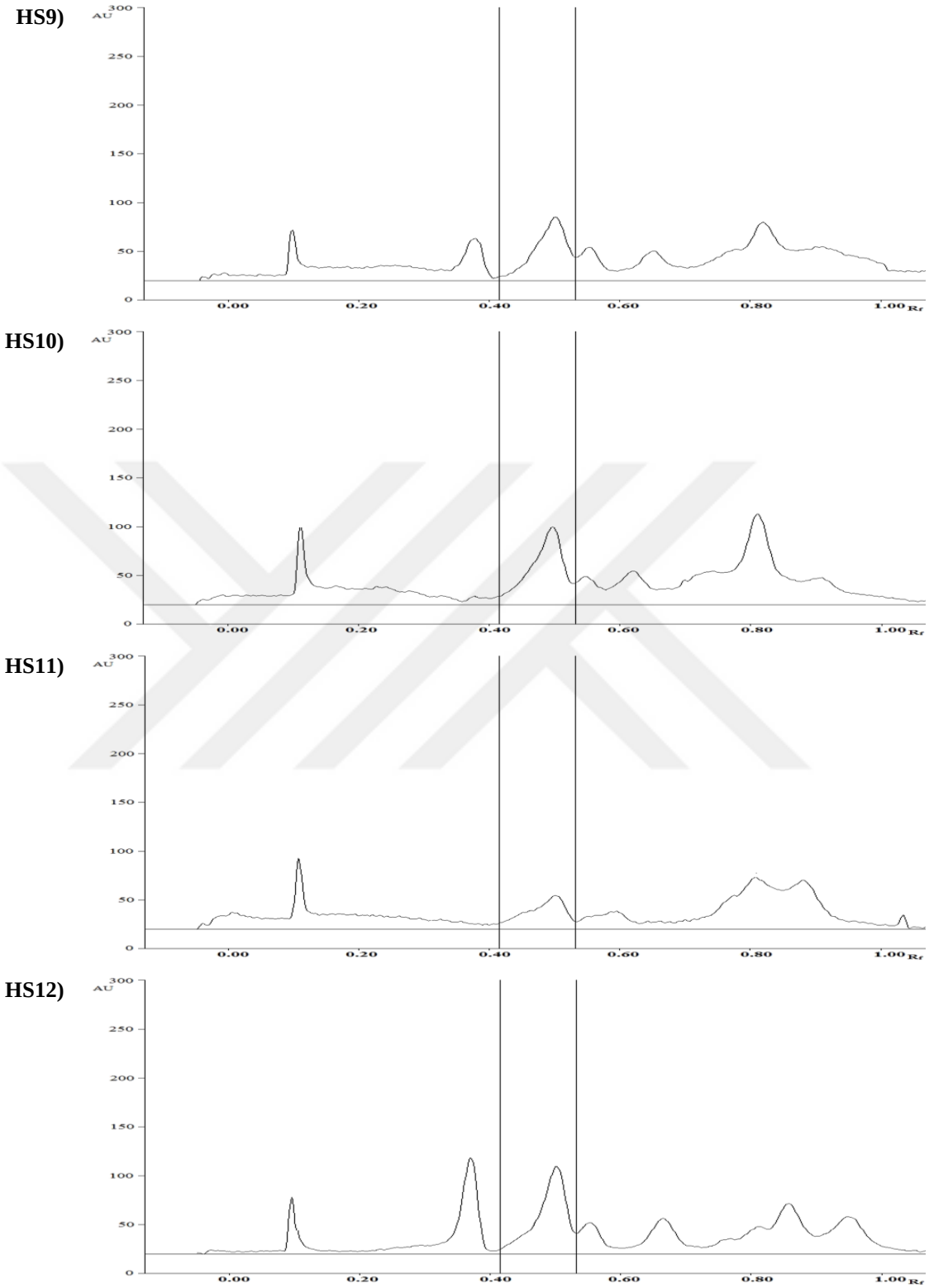


Figure 4.50. HPTLC densitograms from the marketed *H. perforatum* (SJW) herbal materials at 363 nm (continued). Sample test solutions: 10 µg/band.

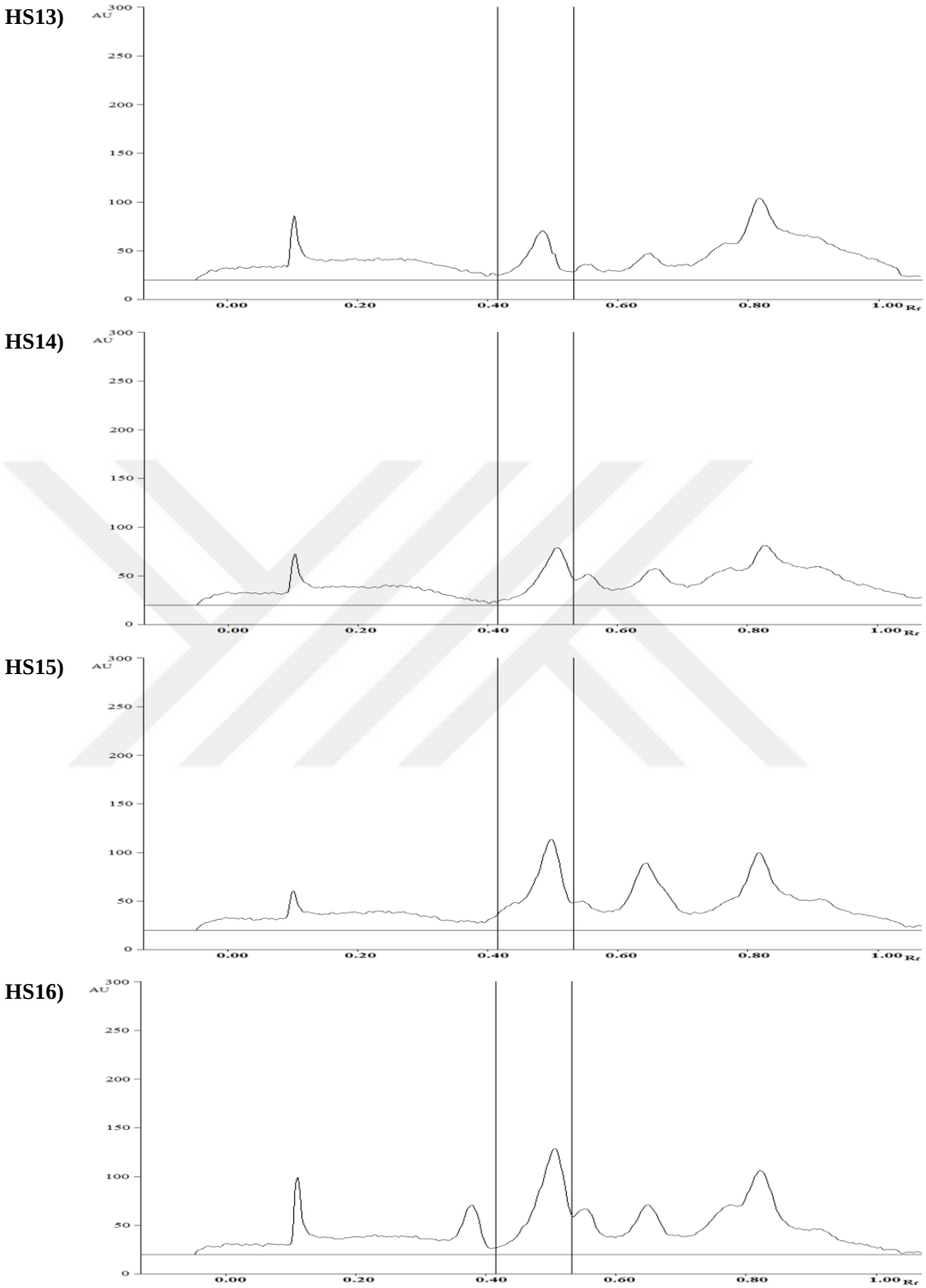


Figure 4.50. HPTLC densitograms from the marketed *H. perforatum* (SJW) herbal materials at 363 nm (continued). Sample test solutions: 10 µg/band.

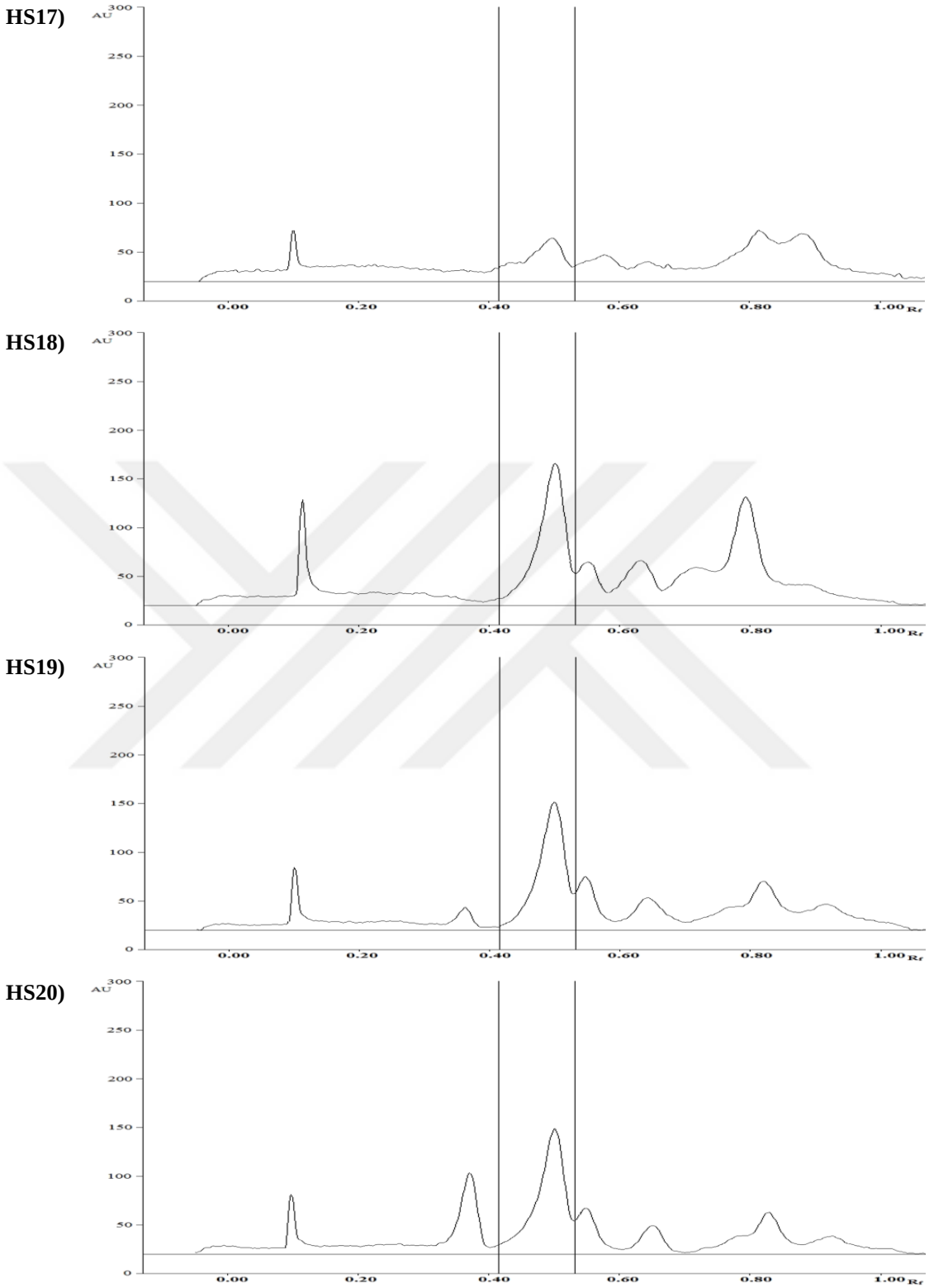


Figure 4.50. HPTLC densitograms from the marketed *H. perforatum* (SJW) herbal materials at 363 nm (continued). Sample test solutions: 10 µg/band.

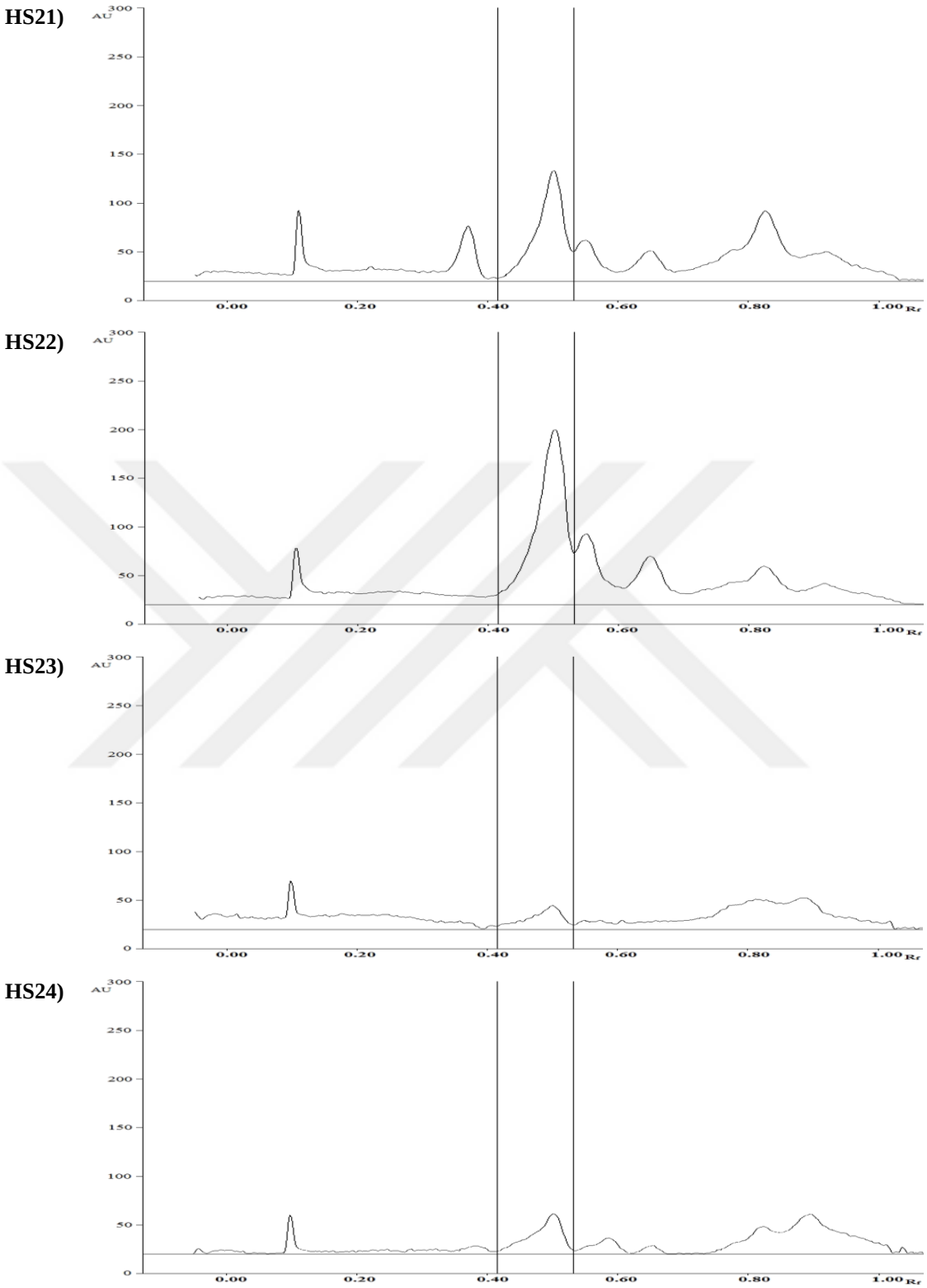


Figure 4.50. HPTLC densitograms from the marketed *H. perforatum* (SJW) herbal materials at 363 nm (continued). Sample test solutions: 10 µg/band.

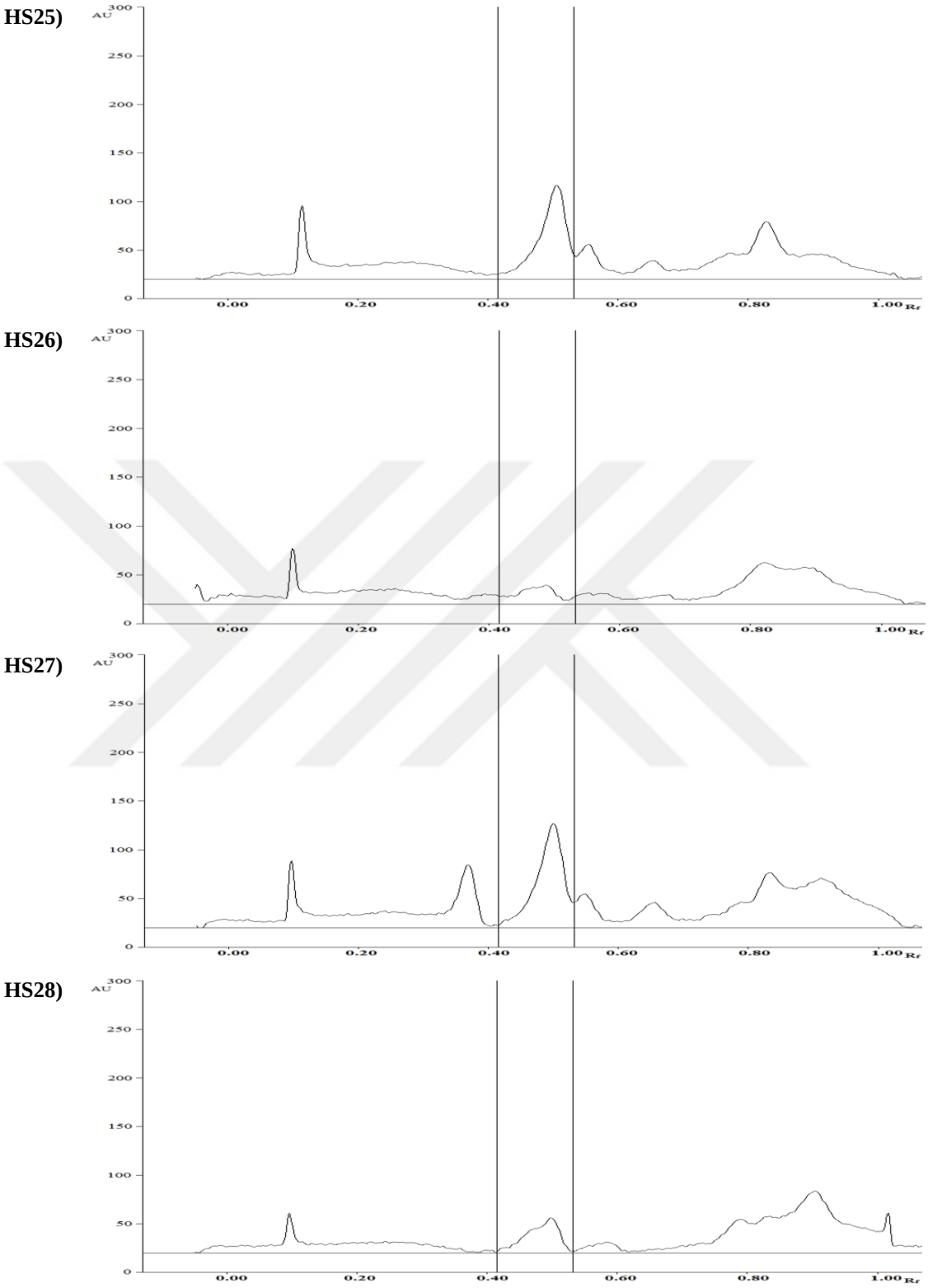


Figure 4.50. HPTLC densitograms from the marketed *H. perforatum* (SJW) herbal materials at 363 nm (continued). Sample test solutions: 10 µg/band.

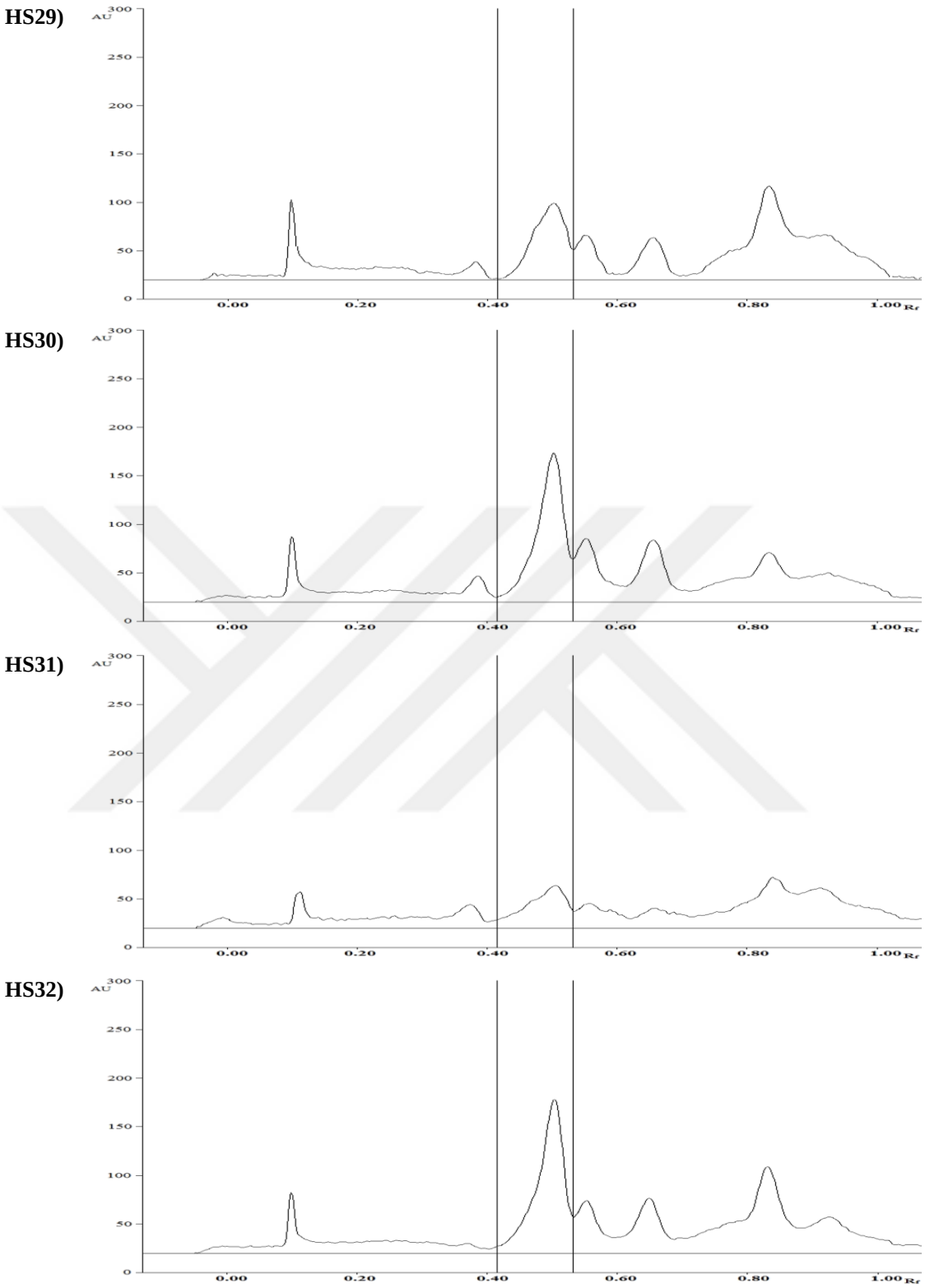


Figure 4.50. HPTLC densitograms from the marketed *H. perforatum* (SJW) herbal materials at 363 nm (continued). Sample test solutions: 10 µg/band.

HS33)

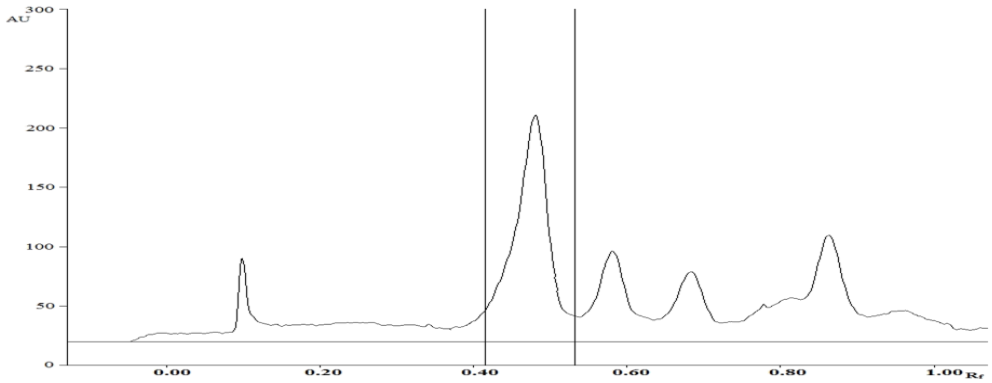


Figure 4.50. HPTLC densitograms from the marketed *H. perforatum* (SJW) herbal materials at 363 nm (continued). Sample test solutions: 10 µg/band.

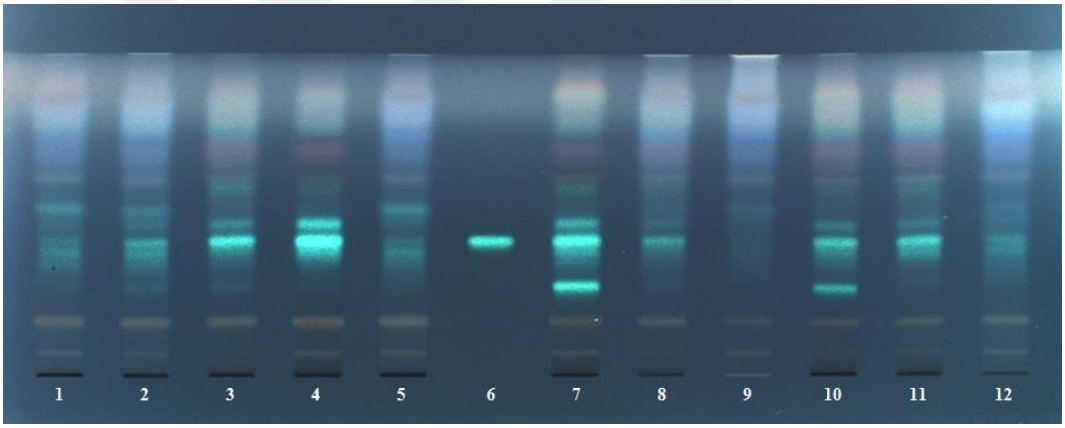


Figure 4.51. HPTLC chromatogram of the marketed *H. perforatum* (SJW) herbal materials at 366 nm. Tracks: (1) HS1; (2) HS2; (3) HS3; (4) HS4; (5) HS5; (6) HyS reference (50 ng/band); (7) HS6; (8) HS7; (9) HS8; (10) HS9; (11) HS10; (12) HS11; applied volume of *H. perforatum* (SJW) herbal samples: 10 µg/band; derivatization: H_2SO_4 reagent.

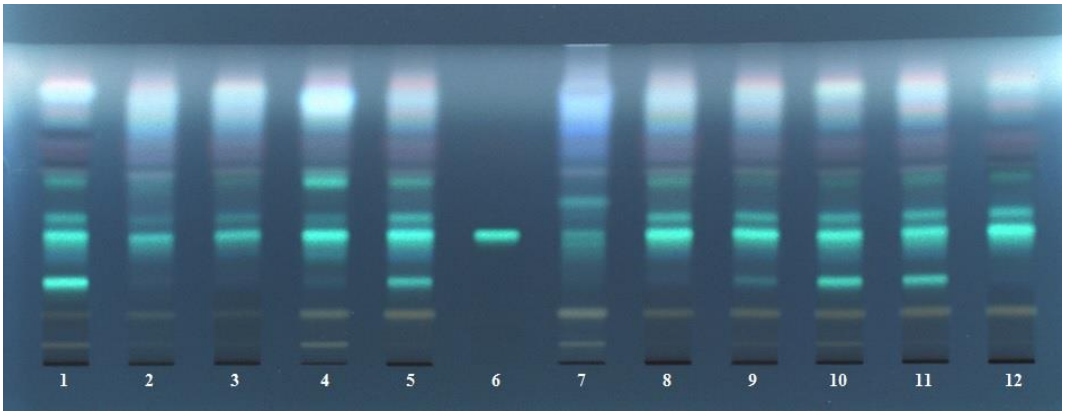


Figure 4.51. HPTLC chromatogram of the marketed *H. perforatum* (SJW) herbal materials at 366 nm (continued). Tracks: (1) HS12; (2) HS13; (3) HS14; (4) HS15; (5) HS16; (6) HyS reference (50 ng/band); (7) HS17; (8) HS18; (9) HS19; (10) HS20; (11) HS21; (12) HS22; applied volume of *H. perforatum* (SJW) herbal samples: 10 µg/band; derivatization: H_2SO_4 reagent.

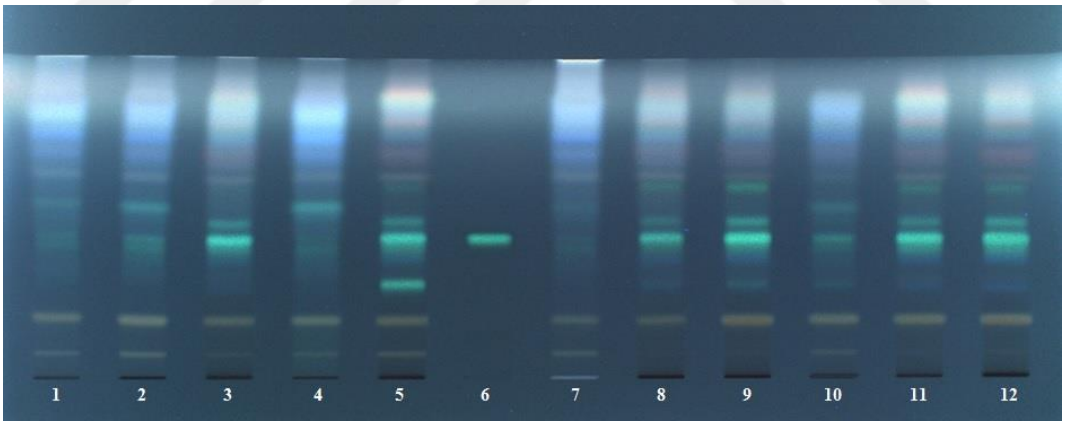


Figure 4.51. HPTLC chromatogram of the marketed *H. perforatum* (SJW) herbal materials at 366 nm (continued). Tracks: (1) HS23; (2) HS24; (3) HS25; (4) HS26; (5) HS27; (6) HyS reference (50 ng/band); (7) HS28; (8) HS29; (9) HS30; (10) HS31; (11) HS32; (12) HS33; applied volume of *H. perforatum* (SJW) herbal samples: 10 µg/band; derivatization: H_2SO_4 reagent.

As shown in Figure 4.47 to Figure 4.51, HyS was detected in all *Hypericum* products provided from the market as tea [HT1-8], capsule [HC] and dried herbal material [HS1-33]. Among these, HC, HS12, HS20 and HS27 displayed similar HPTLC densitograms to that of the reference *H. perforatum* under 363 nm-light whereas HT1-8 and the remaining 30 *Hypericum* samples exhibited different densitograms with that of the reference *H. perforatum* sample. On the other hand, quite similar fingerprint densitograms and chromatograms were observed for HS14, HS22, HS25 and HS32 with those of the wild *H. perforatum* samples obtained from İstanbul and Tekirdağ. Besides, the chromatograms and densitograms of each group of HS9, HS19 and HS21; HS11 and HS23; HS29 and HS30 were found to be almost identical, respectively.

The amount of HyS detected by the developed HPTLC system in eight *Hypericum* (SJW) tea products was between 0.72 to 3.20 mg/g, while a higher ratio was found in SJW capsule formulation [3.54 mg/g] (Table 4.26). On the other hand, HyS contents of marketed *H. perforatum* (SJW) herbal materials were ranging from 0.74 to 5.00 mg/g and the values in HS4, HS6, HS15-16, HS18-22, HS25, HS27, HS30 and HS32-33 were higher than that in tea products.

4.4.5. Evaluation of Hyperoside Content in Aqueous Extracts from *H. perforatum*

The presence of HyS in aqueous extracts from the flowering aerial parts of *H. perforatum* L. cultivar was detected by the comparison of their R_f values and relevant peaks with that of HyS reference. The analysis was performed in three replicates. The quantities of HyS detected in aqueous extracts of *H. perforatum* L. reference sample were expressed as mean±SD in Table 4.27, and related HPTLC densitograms and fingerprints were also demonstrated below (Figure 4.52).

Table 4.27. HyS contents in aqueous extracts of *H. perforatum* L. cultivar

Aqueous extract	**HyS (mg/g)**
HP-W5	4.43±0.04
HP-W10	4.95±0.10

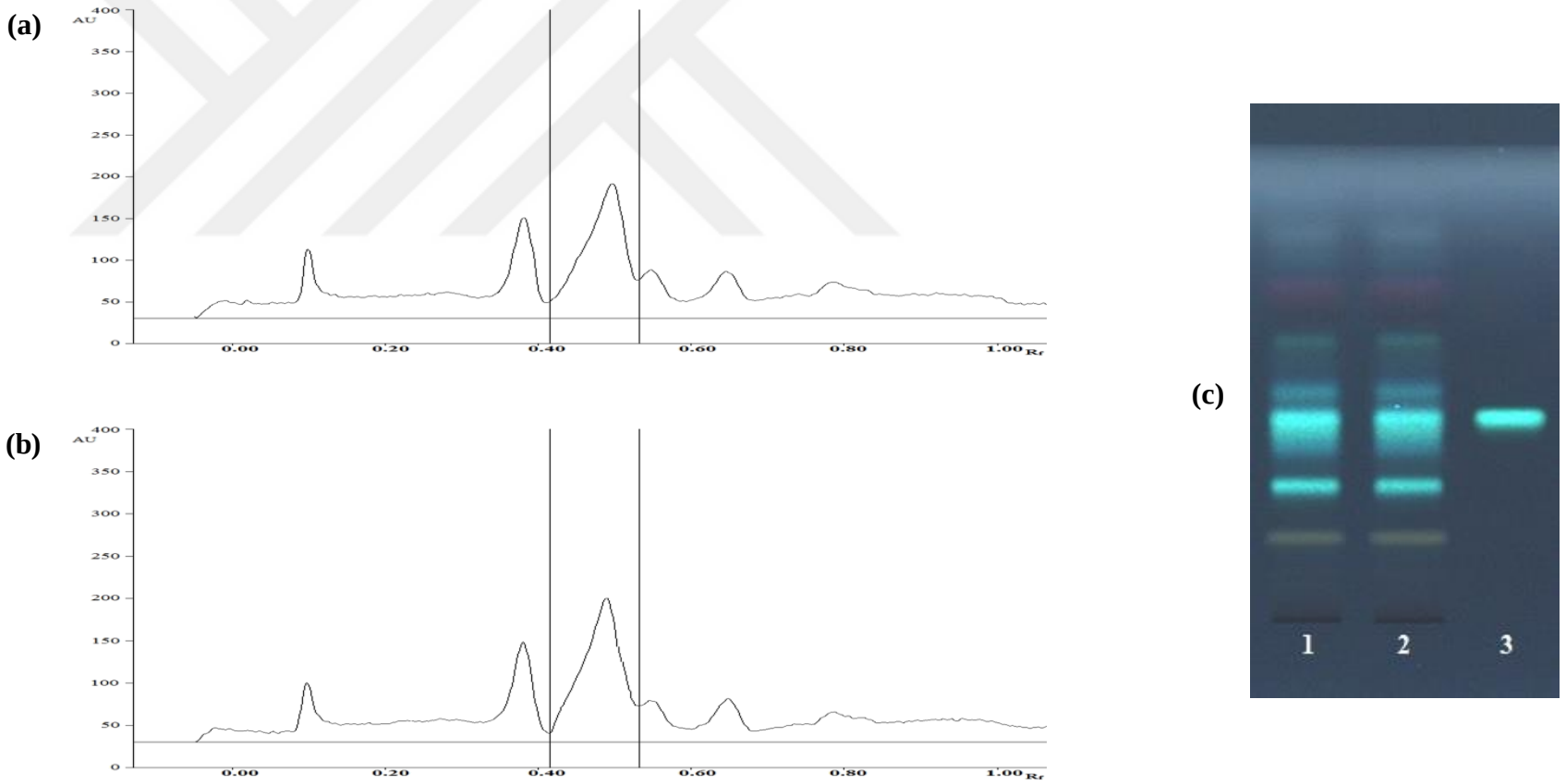


Figure 4.52. HPTLC densitograms obtained from the aqueous extracts of *H. perforatum* L. cultivar: (a) HP-W5 and (b) HP-W10 at 363 nm (10 µg/band); (c) HPTLC chromatogram of the aqueous extracts of *H. perforatum* L. cultivar at 366 nm.

Tracks: (1) HP-W5 (10 µg/band); (2) HP-W10 (10 µg/band); (3) HyS reference (50 ng/band); derivatization: H_2SO_4 reagent.

According to Figure 4.52, it was revealed that HyS was detected in HP-W5 and HP-W10. Regarding to Table 4.27, the amount of HyS determined by the developed HPTLC system in aqueous extracts from the flowering aerial parts of *H. perforatum* L. reference sample was found to be 4.43 mg/g for HP-W5 and 4.95 mg/g for HP-W10. However, HyS content determined in these aqueous extracts was lower than the amount in the methanolic extract of *H. perforatum* L. reference sample [5.35 mg/g].

4.4.6. Evaluation of Hyperoside Content in Solvent Extracts from Oily Preparation of *H. perforatum*

Quantification of HyS contents in acetonitrile, methanol-hexane, acetone, and methanol extracts from the oily preparation of *H. perforatum* were examined by the developed HPTLC method. The analysis was conducted in triplicate. The results were shown in Table 4.28 and the related chromatographic image (Figure 4.53) was displayed below. Consequently, there was no spot corresponding to HyS in all analyzed solvent extracts from the oily preparation of *H. perforatum* as predicted.

Table 4.28. HyS contents in different solvent extracts from the oily preparation of *H. perforatum*

Solvent extract	**HyS (µg/100 mg oil)**
Acetonitrile	ndt
Methanol-hexane	ndt
Acetone	ndt
Methanol	ndt

ndt: HyS not detected

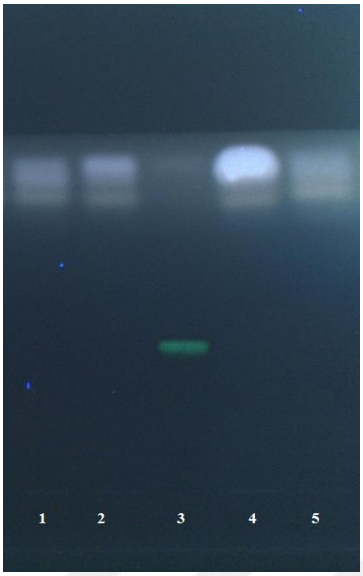


Figure 4.53. HPTLC chromatogram obtained with the developed method for HyS at 366 nm extracted with various solvents from the oily preparation of *H. perforatum*. Tracks: (1) acetonitrile extract; (2) methanol-hexane extract; (3) HyS (reference); (4) acetone extract; (5) methanol extract; derivatization: H_2SO_4 reagent.

5. DISCUSSION

DISCUSSION

A vast number of plant materials have been used worldwide either as food, in phytotherapy or in traditional medicine. Particularly, when the plant materials are used for health benefits or treatment purposes, quality control and standardization of these materials are extremely important in order to judge the therapeutic value. A large number of techniques are available which might be applicable to plant materials for such purposes, namely macroscopic, microscopic, biological and chemical approaches. Chemical examinations of plants have a variety of goals, such as detection of a particular group of substances or quantification of biologically active compounds, which might be beneficial or risky to health. Among the analytical methods, HPTLC technique has recently improved to serve as a useful tool for qualitative and quantitative analysis of compounds in plants.

The beneficial characteristics of HPTLC are ordered as follows: First it is a fast technique comparing to other analytical techniques. Multiple samples may be analyzed at the same time on one HPTLC plate. When compared to TLC, HPTLC allows a better separation owing to a smaller particle size of the stationary phase. Sample application for separation is done directly on the stationary phase and a solvent flows through the stationary phase achieved by capillary force forming the mobile and stationary phases (216). However, as a general approach in the Ph. Eur. for analyses of the chemical components in medicinal plants by TLC technique for qualification and HPLC technique for quantification are described. Nevertheless a number of studies recently describe suitability of the HPTLC techniques for such purposes. In this context, due to its importance and wide application in folk medicine, *H. perforatum* L. is selected to develop and validate HPTLC systems for qualitative and quantitative analysis of its main active components, namely hyperforin, hypericin and hyperoside.

Sample preparation and application, selection of correct stationary and mobile phases, as well as suitable mode of chromatogram development are the main elements that affect the resolution of mixture constituents. The HPTLC plate is chromatographed to evaluate the unknown substance(s) comparing with known standard(s). In addition, choice of appropriate densitometer, visual detection way, and derivatization reagent, reporting and storage of records are the other important steps while developing and

optimizing a HPTLC method. Method optimization should be performed effectively for the efficiency of qualitative and quantitative analysis. The optimization of chromatographic separation is facilitated by characteristic elements of HPTLC, including equipments, procedures, and software (116, 208).

Finding a suitable extraction procedure and the solvent to remove the ingredients from the plant matrix as much as possible for sample preparation is a challenging stage in medicinal plant analysis. In this study, the dried and powdered flowering aerial parts of *H. perforatum* L. cultivar was extracted either with 80% or 100% ethanol or methanol by rotation in a water bath (by rotary evaporator), or ultrasonic bath, or a shaker at room temperature in order to find the suitable solvent or by simmering in hot water to find the components extractable when used as tea. However, in the previous studies for the HPTLC analyses of *H. perforatum* samples alcoholic extracts were preferred for the quantification of the HyF content (119), HyP content (74, 114) or HyS content (114). The reasons might be the differences in solubility characteristics of these components; as a matter of fact our analyses have proven that; HyF was not present in water extracts (Figure 4.16) and the solubility of HyP was low in aqueous extracts (Figure 4.34). Among the analyzed extracts, HyF, HyP, and HyS contents were found to be different depending on the extraction process and solvents used. In this regard, extraction method VI was selected in the present study due to higher solubility of the investigated markers without applying heat.

During development of HPTLC methods, mobile phases used in the previous studies were tested to determine suitable solvent systems for the optimum separation of HyF (52, 75, 116, 119, 123-125); HyP (51, 74, 112-116, 118, 121, 122, 124, 125, 234); and HyS (109, 111, 112, 114-116, 118, 120, 237-243) in extracts from the flowering aerial parts of *H. perforatum* L. cultivar. Based on these preliminary test results, by changing the ratios of the solvents in the mixture new and suitable solvent systems were developed for the detection of HyF (Table 4.5), HyP (Table 4.13), and HyS (Table 4.22) in samples. Accordingly, the best results were provided by *n*-hexane-ethyl acetate (8:2, v/v) for HyF analysis; toluene-chloroform-ethyl acetate-formic acid (8:5:3.5:0.6, v/v/v/v) for HyP analysis; and ethyl acetate-formic acid-acetic acid-water (15:2:2:1, v/v/v/v) for HyS analysis.

Each developed HPTLC system was validated from the points of stability, robustness, specificity, detection and quantitation limits, linearity, intra- and interday precisions as well as recovery described in ICH guidelines (223) by repetitions using *H. perforatum* L. cultivar as a reference material. The developed and validated three individual HTPLC systems were applied to examine the content analysis of various *Hypericum* species, including two samples of *H. perforatum* L.(from İstanbul and Tekirdağ); *H. bithynicum* Boiss., *H. calycinum* L., *H. cerastoides* Spach, *H. perfoliatum* L. from İstanbul; *H. triquetrifolium* L. from Adana; *H. kotschyanum* Boiss. from Mersin; *H. thymopsis* Boiss. from Malatya; and *H. scabrum* L. from Sivas as well commercial *H. perforatum* (SJW) products, including different brands of *H. perforatum* tea bags, capsule containing *H. perforatum* extract, and packages of pulverized or bunches of dried aerial part of *H. perforatum*.

The suitability of the HyF, HyP and HyS composition in *H. perforatum* L. reference sample was confirmed by HPLC assay method described in the Ph. Eur. (234). In this study, each t_R of HyF, HyP, and HyS in each standard solution was determined to be 26.18±0.00 (Figure 4.4), 10.36±0.04 (Figure 4.21), and 4.99±0.07 (Figure 4.39), respectively. In addition, t_R of HyF, HyP, and HyS in the *H. perforatum* L. solution was found to be 26.19±0.01 (Figure 4.5), 10.28±0.02 (Figure 4.22), and 5.00±0.01 (Figure 4.40), respectively, approved by competing with each standard solution. Additionally, the quantities of HyF, HyP, and HyS in the *H. perforatum* L. methanolic extract were determined as 3.44%, 0.19%, and 0.54%, respectively.

HyF concentration in *H. perforatum* samples obtained from two different locations, *H. triquetrifolium*, and *H. scabrum* specimens (Table 4.8); 4 of 8 *Hypericum* tea products, a capsule formulation, and 14 of 33 *Hypericum* materials purchased from herbal shops (Table 4.9) were ranging from 6.20 to 35.36 mg/g. These values were lower than that of the *H. perforatum* L. reference sample [36.03 mg/g]. Additionally, HyF contents in 14 marketed *Hypericum* products were lower than LOQ. On the other hand, HyF was not found in *H. bithynicum*, *H. calycinum*, *H. cerastoides*, *H. perfoliatum*, *H. kotschyanum*, and *H. thymopsis* specimens. When comparing to the densitogram and chromatogram of the reference *H. perforatum* L. (Figure 4.8b), it was shown that the densitograms and chromatograms of two wild *H. perforatum* specimens (Figure 4.9a-b and Figure 4.10); HT 1-5 and 7 (Figure 4.11 and Figure 4.12); HS 3, 6,

12, 27 and 33 (Figure 4.14 and Figure 4.15) were overlapping with that of *H. perforatum* L. reference sample at 310 nm. Among the marketed samples, HS 28 demonstrated quite similar fingerprint densitogram and chromatogram with those of *H. thymopsis*.

Tewari et al. developed and standardized a HPTLC method to quantify only the hyperforin content in methanolic extracts from commercial extracts of *H. perforatum* L. In that study, the mobile phase used was petroleum ether-ethyl acetate (9:1, v/v); and the stationary phase used was silica gel 60 F_{254}. After spraying with 10% methanolic sulfuric acid reagent, hyperforin was observed at $R_f \approx 0.32$-0.35. The values of LOD and LOQ were found to be 100 ng/spot and 200 ng/spot at 290 nm, respectively. The calibration curve was obtained in the concentration range of 0.2-2.0 µg/spot and the correlation coefficients were found in the range of 0.9950-0.9972. The RSD results of precision were ranged from 2.54 to 3.95% for intraday and from 2.81 to 3.02% for interday. The mean recovery results were between 96.31 to 97.09%. The hyperforin content was determined to be between 2.06% and 27.04% in different samples of *H. perforatum* commercial extracts (119). On the other hand, in our study, the R_f value of HyF was found to be 0.49±0.01. Additionally, the values of LOD and LOQ were detected as 120 ng/band and 400 ng/ band at 310 nm, respectively. The calibration curve of HyF was obtained within the range of 0.4-1.4 µg/band and the coefficient of linear correlation was found to be 0.999±0.001. The RSD results of precision were ranged from 1.05 to 1.41% for intraday and from 1.71% to 1.88% for interday. The mean recovery results ranged from 100.28 to 101.22%. The variances in the findings of R_f, LOD, LOQ, precision and recovery might be occurred due to different extraction procedure, solvent system and wavelength.

HyP concentration in *H. perforatum* obtained from two different localities, *H. bithynicum*, *H. perfoliatum*, and *H. triquetrifolium* specimens (Table 4.16); 6 of 8 *Hypericum* tea products; a capsule sample, and 26 of 33 *Hypericum* materials purchased from herbal shops (Table 4.17) were ranging from 0.10 to 1.49 mg/g. These values were lower than the *H. perforatum* L. reference sample [1.60 mg/g]. Moreover, the HyP content quantified in *H. cerastoides* and 9 commercial *Hypericum* materials were lower than LOQ. On the other hand, HyP was not found in *H. calycinum*, *H. kotschyanum*, *H. scabrum*, and *H. thymopsis* specimens. While comparing to the densitogram and

chromatogram of the reference *H. perforatum* L (Figure 4.26), it was observed that the densitograms and chromatograms of two wild *H. perforatum* specimens (Figure 4.27a-b and Figure 4.28); HS 14, 15 and 22 (Figure 4.32 and Figure 4.33) were overlapping with those of *H. perforatum* L. reference sample at 590 nm.

Mulinacci et al. studied on the quantification of hypericin and pseudohypericin content in hydroalcoholic extracts (80% EtOH) prepared separately from the flowers and herbs of *H. perforatum* L. using TLC-densitometry with fluorescence detection and HPLC-DAD. In HPTLC procedure, toluene-ethyl acetate-formic acid (50:40:10, v/v/v) was utilized as the developing solvent on silica gel TLC plates. The hypericin was observed at R_f=0.611. The system was linear within the concentration range of 11.7-35.1 ng and the correlation coefficients were found within the ranges between 0.9825-0.9937 at 313 nm. The total amount of hypericin and pseudohypericin detected by HPTLC system was between 1.67 and 2.44 mg/g in different herbal and 1.92 to 2.79 mg/g in flower samples. In HPLC procedure, LiChrosorb RP18 column (5 μm, 250 x 4.6 mm) was employed for separation of hypericin and pseudohypericin. The mobile phase A was water (adjusted to pH 3.2 with phosphoric acid); the mobile phase B was methanol and the mobile phase C was acetonitrile. The gradient pattern was used as follows: 88% A and 12% C (0.1 min), 82% A and 18% C (10-15 min), 55% A and 45% C (30 min), 55% B and 45% C (35-42 min), 97-18% B (29-30 min). The injection volume was 25 μL, and the flow rate was 1.0 mL/min, as well as the detection was monitored at 590 nm for hypericin and pseudohypericin analysis. The total amount of hypericin and pseudohypericin determined by HPLC system was ranged from 1.72 to 2.52 mg/g in different herbal samples and 1.82 to 2.84 mg/g in flower samples. Based on the results, it was shown that the quantitative data of HPTLC were quite close to that of HPLC (74). However, the detection and quantitation limits, calibration curve, precision and recovery parameters were not practiced for the HPTLC method. On the other hand, in our study, the R_f value of HyP was found to be 0.35±0.03. In addition, the calibration curve of HyP was obtained within the range of 20-100 ng/band and the coefficient of linear correlation was found to be 0.999±0.001 at 590 nm. As mentioned before, the differences in the R_f and range values might be due to using different developing solvents and wavelength.

Nuevas-Paz et al. investigated the hypericin content in methanolic extracts prepared from the flowering aerial parts of *H. perforatum*, *H. tetrapetalum*, *H. nitidum*, and *H. styphelioides* comparatively by using HPTLC and HPLC techniques. In HPTLC procedure, three different mobile phases were used: MPA: ethyl acetate-glacial acetic acid-formic acid-water (100:11:11:27, v/v/v/v); MPB: toluene-ethyl formate-formic acid (5:4:1, v/v/v); and MPC: ethyl acetate-dichloromethane-acetic acid-formic acid-water (100:25:10:10:11, v/v/v/v/v). HPTLC silica gel 60 F_{254} plates were utilized as the stationary phase. The R_f values in these mobile systems for *H. perforatum* extract were found to be 0.50 for MPA, 0.95 for MPB, and 0.48 for MPC at UV 366 nm. The peaks of hypericin and pseudohypericin were only determined in *H. perforatum* extract at 590 nm, whereas the other extracts of three *Hypericum* species were devoid of these components. The detection limit was found to be 0.54 µg/spot for HPTLC analysis. In HPLC procedure, a Superspher RP 18 column (5 µm, 250 x 4 mm) was employed for the analyses of hypericin and pseudohypericin contents. The mobile phase A was the mix 5 volumes of methanol and 4 volumes of acetonitrile; and the mobile phase B was 0.1 M aqueous triethylammonium acetate. The gradient pattern was used as follows: 70% A (initial), 90% A (0-15 min), and 70% A (15-25 min). The injection volume was 20 µL, and the flow rate was 1.0 mL/min, as well as the detection was monitored at 590 nm for hypericin and pseudohypericin analysis. Again pseudohypericin and hypericin were determined only in *H. perforatum* extract at 3.86 min and 6.39 min, respectively, whereas they were not detected in other *Hypericum* species. The detection limit was found to be 18 ng (RSD=5.8%) for HPLC analysis (117). However, the quantitation limit, calibration curve, precision and recovery parameters were not evaluated in this HPTLC study. On the other hand, in our study, the R_f value of HyP was found to be 0.35±0.03 and the LOD value was detected as 6 ng/band at 590 nm. As previously indicated, using different mobile phases caused the differences in the values of R_f and LOD.

HyS concentration in all the investigated *Hypericum* specimens (Table 4.25) and marketed *Hypericum* (SJW) products (Table 4.26) were ranging from 0.72 to 9.73 mg/g. The HyS content quantified in *H. bithynicum* [9.73 mg/g] and *H. perfoliatum* [9.07 mg/g] were higher than that of *H. perforatum* L. reference sample [5.35 mg/g]. When comparing to the densitogram and chromatogram of the reference *H. perforatum* L. (Figure 4.44), it was demonstrated that the densitograms and chromatograms of HC

(Figure 4.49), HS 12, 20 and 27 (Figure 4.50 and Figure 4.51) were overlapping with those of *H. perforatum* L. reference sample under 363 nm.

Maffi et al. examined the contents of hypericin, pseudohypericin, hyperoside, isoquercitrin, quercetin, quercitrin and rutin in methanol-acetone extract from flower of *H. perforatum* collected from different localities in Italy. Ethyl acetate-water-formic acid (30:2:3, v/v/v) was used as the mobile phase and HPTLC silica gel F_{254} was utilized as the stationary phase. The amounts were ranged from 0.03 to 0.40% for hypericin, 0.10 to 0.34% for pseudohypericin at 590 nm; 0.14 to 0.86% for hyperoside, 0.05 to 0.36% for isoquercitrin, 0.10 to 0.66% for quercetin, 0.20 to 1.04% for quercitrin and 0.00 to 0.54% for rutin at 400 nm, respectively (114). However, validation parameters of the HPTLC method used in that study was not evaluated.

In the present study, the developed HPTLC methods was also practiced to determine the HyF, HyP and HyS contents in the olive oil macerate of *H. perforatum* prepared following the traditional recipes. Among the various solvents (acetonitrile, methanol-hexane, acetone, and methanol) were tested to extract these compounds from the oily macerate, the highest concentration of HyF was detected in acetonitrile extract, while the highest HyP content was found in methanol extract. In this regard, acetonitrile and methanol were employed for the extraction of HyF and HyP, respectively and their concentrations in the oily macerate were determined by the developed and validated HPTLC systems (Figure 4.17, Figure 4.18 and Table 4.10 for HyF; Figure 4.35, Figure 4.36 and Table 4.19 for HyP). On the other hand, as expected, HyS was not found in any of the solvent extracts (Figure 4.53).

HyF was found in 6 of 7 oily products by comparing the HPTLC densitograms (Figure 4.19) and chromatograms (Figure 4.20). Although the maximum quantity of HyF was detected in OE2, this value was lower than that of the oily macerate of *H. perforatum* L. reference sample. Additionally, HyF contents in 2 oily products [OE5-6] were remained below the LOQ and HyF was not determined in OE7 (Table 4.11). On the other hand, HyP was also found in 6 of 7 oily products by examining the HPTLC densitograms (Figure 4.37) and chromatogram (Figure 4.38) of methanol extracts from commercial *H. perforatum* (SJW) oily products. The highest HyP concentration was again determined in OE2, but this amount was lower than that of the oily preparation of

H. perforatum L. reference sample. Nonetheless, HyP was not determined in only OE7 (Table 4.20).

Southwell and Bourke studied the total hypericins concentration in the ethanolic extract from *H. perforatum* L. collected every 3 weeks in two consecutive seasons from Australia. The greater concentrations of hypericins were detected in the second year. They concluded that the rainfall in the second year was higher than that in the first year and accordingly the average daily sunlight hours and average daily maximum temperature in the second year were lower than in the first year. Therefore, they suggested that increased moisture may affect the hypericin concentration in *H. perforatum*. Moreover, they reported that the hypericin content in the narrow leaf biotype was roughly 75% more than that in the broadleaf biotype at the flowering stages (244).

Gray et al. investigated the effects of acute drought stress on the concentrations of compounds in *H. perforatum*. The contents of rutin, hyperoside, quercitrin, isoquercitrin, quercetin, chlorogenic acid, pseudohypericin and hypericin were increased by drought stress imposed at two times during the reproductive stage. In contrast, the contents of adhyperforin and hyperforin were reduced significantly. Then, it is evident that the environmental conditions would influence the proportions of these active components in *H. perforatum* samples (245). Furthermore, Gruszczyk and Kieltyka compared the hypericin content in *H. perforatum* L. from one-year and two-year old plantations. The hypericin content in the second year cultivation of *H. perforatum* was found to be higher than that in the first year (246). In essence, we also found that the HyF, HyP, and HyS contents were quite different in two *H. perforatum* samples collected from different locations. On the other hand, the concentrations of these marker components were found to be higher in the cultivated reference sample of *H. perforatum* L. than the two wild specimens. The reason for the difference between concentrations of the compounds in wild and cultivated grown plant might be related to the improved cultivation factors, including standardized production, elimination of environmental factors (biotic stress consisting of bacteria, virus, fungi and parasites; as well as abiotic stress comprising light exposure, water, soil, altitude, chemicals, temperature and geographical variations) as well as prevention of adulteration. The

content of these compounds as well as quality and efficacy of the medicinal plant can be increased by cultivation and standardization process (247, 248).

Table 5.1. Summary of the emphasized points

HYPERFORIN		**Present study**	**Literature (119)**
Mobile phase		*n*-hexane-ethyl acetate (8:2) 310 nm	petroleum ether-ethyl acetate (9:1) 290 nm
R_f value		0.49±0.01	0.32-0.35
LOD-LOQ		120 ng/band-400 ng/band	100 ng/spot-200 ng/spot
Coefficient		0.999±0.001	0.9950-0.9972
Precision (RSD, %)	**intraday**	1.05-1.41	2.54-3.95
	interday	1.71-1.88	2.81-3.02
Recovery (%)		100.28-101.22	96.31-97.09

Table 5.1. Summary of the emphasized points (continued)

HYPERICIN	**Present study**	**Literature (74)**	**Literature (117)**
Mobile phase	toluene-chloroform-ethyl acetate-formic acid (8:5:3.5:0.6) 590 nm	toluene-ethyl acetate-formic acid (50:40:10) 313 nm	A: ethyl acetate-glacial acetic acid-formic acid-water (100:11:11:27) B: toluene-ethyl formate-formic acid (5:4:1) C: ethyl acetate-dichloromethane-acetic acid-formic acid-water (100:25:10:10:11) 590 nm
R_f value	0.35±0.03	0.611	A: 0.50; B: 0.95; C: 0.48
LOD-LOQ	6 ng/band-20 ng/band	–	0.54 µg/spot - –
Coefficient	0.999±0.001	0.9825-0.9937	–
Precision (RSD, %)	1.26-2.79	–	–
Recovery (%)	98.84-100.06	–	–

Table 5.1. Summary of the emphasized points (continued)

HYPEROSIDE	**Present study**	**Literature (114)**
Mobile phase	ethyl acetate-formic acid-acetic acid-water (15:2:2:1) 363 nm	ethyl acetate-water-formic acid (30:2:3) 400 nm
R_f value	0.49±0.04	–
LOD-LOQ	3 ng/band-10 ng/band	–
Coefficient	0.999±0.001	–
Precision (RSD, %)	0.87-1.97	–
Recovery (%)	100.42-101.37	–

Table 5.1. Summary of the emphasized points (continued)

***Hypericum* specimen**	**HyF (mg/g)**	**HyP (mg/g)**	**HyS (mg/g)**
H. perforatum (İstanbul)	26.20±0.02	1.41±0.04	4.62±0.03
H. perforatum (Tekirdağ)	26.40±0.07	1.28±0.05	5.27±0.06
H. bithynicum	ndt	0.81±0.07	9.73±0.05
H. calycinum	ndt	ndt	4.07±0.06
H. cerastoides	ndt	<LOQ	1.01±0.07
H. perfoliatum	ndt	1.09±0.08	9.07±0.08
H. triquetrifolium	6.40±0.05	0.83±0.03	3.29±0.02
H. kotschyanum	ndt	ndt	1.67±0.01
H. scabrum	9.70±0.10	ndt	2.51±0.05
H. thymopsis	ndt	ndt	2.21±0.02

ndt: not detected; <LOQ: under the limit of quantitation

Table 5.1. Summary of the emphasized points (continued)

Commercial product		HyF (mg/g)	HyP (mg/g)	HyS (mg/g)
***H. perforatum* tea bags**	HT1	8.20±0.03	<LOQ	1.17±0.03
	HT2	8.90±0.01	<LOQ	0.72±0.05
	HT3	<LOQ	0.76±0.09	1.45±0.03
	HT4	14.30±0.01	1.40±0.03	3.14±0.04
	HT5	6.20±0.07	0.56±0.07	3.20±0.09
	HT6	ndt	0.50±0.04	1.39±0.06
	HT7	<LOQ	1.49±0.03	2.81±0.07
	HT8	ndt	0.85±0.01	1.45±0.02
***H. perforatum* formulation (capsule)**	HC	17.90±0.02	0.91±0.07	3.54±0.13
Commercially available *H. perforatum* materials from herbal shops	HS1	10.17±0.01	<LOQ	1.26±0.07
	HS2	ndt	0.35±0.09	2.08±0.03
	HS3	14.95±0.03	1.30±0.01	2.91±0.03
	HS4	<LOQ	0.92±0.04	4.02±0.02
	HS5	27.95±0.01	<LOQ	1.07±0.02
	HS6	30.57±0.05	0.73±0.07	4.20±0.04
	HS7	<LOQ	0.86±0.09	2.09±0.08
	HS8	ndt	<LOQ	0.74±0.06
	HS9	14.88±0.01	1.07±0.10	2.64±0.05
	HS10	<LOQ	1.36±0.06	2.85±0.04
	HS11	9.10±0.04	<LOQ	1.36±0.11
	HS12	26.39±0.05	1.29±0.08	3.01±0.06
	HS13	<LOQ	1.32±0.05	2.08±0.04
	HS14	20.77±0.04	1.28±0.04	2.25±0.02
	HS15	<LOQ	0.42±0.07	3.31±0.12
	HS16	<LOQ	1.39±0.05	3.63±0.12
	HS17	ndt	0.10±0.01	1.72±0.05
	HS18	<LOQ	1.49±0.09	4.10±0.08
	HS19	23.76±0.02	1.34±0.03	3.92±0.19
	HS20	<LOQ	1.35±0.06	3.87±0.16
	HS21	<LOQ	1.37±0.09	3.79±0.15
	HS22	26.52±0.07	1.31±0.06	4.95±0.12
	HS23	ndt	0.13±0.02	1.37±0.06
	HS24	ndt	<LOQ	1.64±0.08
	HS25	14.41±0.01	1.40±0.14	3.47±0.14
	HS26	ndt	<LOQ	1.33±0.08
	HS27	35.36±0.02	1.37±0.04	3.62±0.10
	HS28	ndt	<LOQ	1.47±0.05
	HS29	<LOQ	1.42±0.09	2.72±0.09
	HS30	<LOQ	1.44±0.03	4.23±0.17
	HS31	<LOQ	0.56±0.05	1.84±0.11
	HS32	16.50±0.01	1.38±0.08	4.37±0.17
	HS33	10.39±0.03	1.46±0.03	5.00±0.15

ndt: not detected; <LOQ: under the limit of quantitation

Table 5.1. Summary of the emphasized points (continued)

***H. perforatum* oily product**	**HyF (µg/100 mg oil)**	**HyP (µg/100 mg oil)**
OE1	1.17±0.19	0.05±0.03
OE2	1.52±0.41	0.07±0.05
OE3	1.23±0.29	0.06±0.04
OE4	0.74±0.16	0.02±0.02
OE5	<LOQ	0.04±0.03
OE6	<LOQ	0.03±0.04
OE7	ndt	ndt

ndt: not detected; <LOQ: under the limit of quantitation

6. CONCLUSION

CONCLUSION

The present study was targeted to develop and validate HPTLC methods for qualification and quantification of three compounds, namely HyF, HyP and HyS, in *H. perforatum* which has been extensively utilized as a natural remedy in worldwide traditional medicines for centuries as well as in phytotherapy practices. In addition, the new HPTLC methods were applied on different *Hypericum* species, oily macerate prepared by traditional technique, as well as employed on several marketed *H. perforatum* (SJW) products and SJW oily products purchased from different herbal markets.

Three different solvent systems were used during HPTLC method development for the methanolic extracts obtained from the flowering aerial parts of *H. perforatum* on preconditioned HPTLC plates coated with silica gel 60 F_{254} in saturated ADC2. The mobile phases *n*-hexane-ethyl acetate (8:2, v/v) for HyF analysis; toluene-chloroform-ethyl acetate-formic acid (8:5:3.5:0.6, v/v/v/v) for HyP analysis; and ethyl acetate-formic acid-acetic acid-water (15:2:2:1, v/v/v/v) for HyS analysis were found to be suitable and they were further validated by adequate repetition. The developed HPTLC systems were practiced for the qualitative and quantitative analysis of these active ingredients in various *Hypericum* species, the marketed *Hypericum* products as well as traditionally-prepared oil macerate and commercial oily products.

In terms of HyF concentration, although any limit value was not defined in the Ph. Eur., in various reliable sources the limit was set at minimum 2.0 per cent (13, 45, 249). The HyF content in our reference *H. perforatum* material was 3.60 per cent. However, only the contents of HyF in 7 marketed *H. perforatum* products [HS 5, 6, 12, 14, 19, 22 and 27] were found to be higher than this value, while the rest remained below.

In terms of HyP concentration, the official limit was set at minimum 0.08 per cent in the Ph. Eur. (234). Our reference *H. perforatum* sample was determined to contain 0.16 per cent. The HyP amounts in 24 marketed *H. perforatum* products [HT 4, 7, 8; HC; HS 3, 4, 7, 9, 10, 12-14, 16, 18-22, 25, 27, 29, 30, 32, 33] were also higher than this limit.

In terms of HyS concentration, there were no official limit described for the HyS content, but in Herbal Medicines (45) the HyS concentration was given as 0.9 per cent (9 mg/g) based on a previously published paper. Accordingly, HyS in all *H. perforatum* samples including our reference extract were found to be below the amount stated while the HyS content in *H. bithynicum* and *H. perfoliatum* were found to be higher. However, in a detailed review paper reporting the influence of various conditions including chemotyping, environmental and growing conditions, genotypes etc. in the chemical contents of *H. perforatum*, the authors found a value between 1-25 mg/g for HyS. If we accept these limits for the evaluation of HyS content in our samples, in 40 commercial products [HT 1, 3-8; HC; HS 1-7, 9-33] the level of HyS were within these limits (247). Since this later study reviewed the results of 173 papers, the limits given seem more reliable.

HPTLC method provides both qualitative and quantitative results of herbal components whereas TLC method is used for only qualification and HPLC method is used for identification and quantitation. In fact, these tests were given only for HyP analysis in the Ph. Eur. In order to find out the reliability of the developed HPTLC technique for quantitative analysis of HyP content we studied comparatively our reference *H. perforatum* sample by HPLC and HPTLC. According to this experimentation HyP content was determined to be 0.19 and 0.16 per cent, respectively.

Relying on the present study, the outcomes obtained from quantitative analysis should be evaluated with the consequences of the qualitative analysis for quality assessment of medicinal plants and preparations. The quality assurance ensures the safety and effectiveness of herbal products. Overall, three different HPTLC methods developed and validated in the present study may provide guidance for future researches.

7. REFERENCES

1) Gurib-Fakim A. Medicinal plants: traditions of yesterday and drugs of tomorrow. *Mol Aspects Med*. 2006; 27: 1-93.
2) Crockett SL, Robson NKB. Taxonomy and chemotaxonomy of the genus *Hypericum*. *Med Aromat Plant Sci Biotechnol*. 2011; 5 (Special Issue 1): 1-13.
3) Robson NKB. *Hypericum* L. In: Davis PH, ed. *Flora of Turkey and the East Aegean Islands*. Edinburgh: Edinburgh University Press; 1967: 355-401.
4) Akgöz Y. Türkiye florası'na ait *Hypericum* L. cinsinin tehlike kategorileri ve bulunan yeni türleri. *J of Ins Nat App Sci*. 2013; 18(1-(2)): 62-69.
5) Çırak C, Kurt D. Önemli tıbbi bitkiler olarak *Hypericum* türleri. *J of AARI*. 2014; 24(1): 42-58.
6) *PDR for Herbal Medicines*. 4th ed. Montvale: Medical Economics Company; 2000.
7) Bilia AR, Gallori S, Vincieri FF. St. John's wort and depression - efficacy, safety and tolerability - an update. *Life Sci*. 2002; 70: 3077-3096.
8) Lawvere S, Mahoney MC. St. John's wort. *Am Fam Physician*. 2005; 72(11): 2249-2254.
9) Kumar V, Singh PN, Muruganandam AV, Bhattacharya SK. *Hypericum perforatum*: nature's mood stabilizer. *Indian J Exp Biol*. 2000; 38: 1077-1085.
10) Heinrich M, Barnes J, Gibbons S, Williamson EM. *Fundamentals of Pharmacognosy and Phytotherapy*. Spain: Churchill Livingstone; 2004.
11) Barnes J, Anderson LA, Phillipson JD. St John's wort (*Hypericum perforatum* L.): a review of its chemistry, pharmacology and clinical properties. *J Pharm Pharmacol*. 2001; 53: 583-600.
12) Linde K. St. John's wort - an overview. *Forsch Komplementmed*. 2009; 16: 146-155.
13) Bruneton J. *Pharmacognosy, Phytochemistry, Medicinal Plants*. 2nd ed. Paris: Lavoisier Publishing; 1999.
14) Saki K, Bahmani M, Rafieian-Kopaei M. The effect of most important medicinal plants on two important psychiatric disorders (anxiety and depression) - a review. *Asian Pac J Trop Med*. 2014; 7(Suppl 1): S34-S42.
15) Crockett SL. Essential oil and volatile components of the genus *Hypericum* (Hypericaceae). *Nat Prod Commun*. 2010; 5(9): 1493-1506.

16) Silva BA, Ferreres F, Malva JO, Dias ACP. Phytochemical and antioxidant characterization of *Hypericum perforatum* alcoholic extracts. *Food Chem*. 2005; 90: 157-167.
17) Sagratini G, Ricciutelli M, Vittori S, Öztürk N, Öztürk Y, Maggi F. Phytochemical and antioxidant analysis of eight *Hypericum* taxa from Central Italy. *Fitoterapia*. 2008; 79: 210-213.
18) Stojanovic NM, Radulovic NS, Randjelovic PJ, Laketic D. Antinociceptive properties of St. John's wort (*Hypericum perforatum*) and other *Hypericum* species. *Nat Prod Commun*. 2016; 11(11): 1741-1747.
19) Borrelli F, Ernst E. Alternative and complementary therapies for the menopause. *Maturitas*. 2010; 66: 333-343.
20) Sosa S, Pace R, Bornancin A, Morazzoni P, Riva A, Tubaro A, Loggia RD. Topical anti-inflammatory activity of extracts and compounds from *Hypericum perforatum* L. *J Pharm Pharmacol*. 2007; 59: 703-709.
21) Wölfle U, Seelinger G, Schempp CM. Topical application of St. John's wort (*Hypericum perforatum*). *Planta Med*. 2014; 80: 109-120.
22) Altan A, Damlar İ, Aras MH, Alpaslan C. Sarı kantaronun (*Hypericum perforatum*) yara iyileşmesi üzerine etkisi. *Arch Med Rev J*. 2015; 24(4): 578-591.
23) Wurglics M, Schubert-Zsilavecz M. *Hypericum perforatum*: a modern herbal antidepressant. *Clin Pharmacokinet*. 2006; 45(5): 449-468.
24) Chatterjee SS, Bhattacharya SK, Wonnemann M, Singer A, Müller WE. Hyperforin as a possible antidepressant component of *Hypericum* extracts. *Life Sci*. 1998; 63(6): 490-510.
25) Beerhues L. Hyperforin. *Phytochemistry*. 2006; 67: 2201-2207.
26) Medina MA, Martinez-Poveda B, Amores-Sanchez MI, Quesada AR. Hyperforin: more than an antidepressant bioactive compound? *Life Sci*. 2006; 79: 105-111.
27) Quiney C, Billard C, Salanoubat C, Fourneron JD, Kolb JP. Hyperforin, a new lead compound against the progression of cancer and leukemia? *Leukemia*. 2006; 20: 1519-1525.
28) Agostinis P, Vantieghem A, Merlevede W, De Witte PAM. Hypericin in cancer treatment: more light on the way. *Int J Biochem Cell Biol*. 2002; 34: 221-241.
29) Hışıl Y, Şahin F, Omay SB. Kantaronun (*Hypericum perforatum* L.) bileşimi ve tıbbi önemi. *Int J Hematol Oncol*. 2005; 4(15): 212-218.

30) Kubin A, Wierrani F, Burner U, Alth G, Grünberger W. Hypericin - the facts about a controversial agent. *Curr Pharm Des*. 2005; 11: 233-253.

31) Davids LM, Kleemann B, Kacerovska D, Pizinger K, Kidson SH. Hypericin phototoxicity induces different modes of cell death in melanoma and human skin cells. *J Photochem Photobiol B: Biol*. 2008; 91: 67-76.

32) Karioti A, Bilia AR. Hypericins as potential leads for new therapeutics. *Int J Mol Sci*. 2010; 11: 562-594.

33) Liu Z, Tao X, Zhang C, Lu Y, Wei D. Protective effects of hyperoside (quercetin-3-*o*-galactoside) to PC12 cells against cytotoxicity induced by hydrogen peroxide and *tert*-butyl hydroperoxide. *Biomed Pharmacother*. 2005; 59: 481-490.

34) Gong J, Huang J, Ge Q, Chen F, Zhang Y. Advanced research on the antidepressant effect of flavonoids. *Curr Opin Complement Alternat Med*. 2014; 1(2): 1-6.

35) Baytop T. *Therapy with Medicinal Plants in Turkey*. 2nd ed. İstanbul: Nobel Tıp Kitabevleri; 1999.

36) Sargın SA, Akçiçek E, Satıl F. An ethnobotanical study of medicinal plants used by local people of Alaşehir (Manisa) in Turkey. *J Ethnopharmacol*. 2013; 150: 860-874.

37) Mills S, Bone K. *Principles and Practice of Phytotheraphy - Modern Herbal Medicine*. 1st ed. Edinburgh: Churchill Livingstone; 2000.

38) Patocka J. The chemistry, pharmacology, and toxicology of the biologically active constituents of the herb *Hypericum perforatum* L. *J App Biomed*. 2003; 1: 61-70.

39) Sleno L, Daneshfar R, Eckert GP, Müller WE, Volmer DA. Mass spectral characterization of phloroglucinol derivatives hyperforin and adhyperforin. *Rapid Commun Mass Spectrom*. 2006; 20: 2641-2648.

40) Verma V, Smelcerovic A, Zuehlke S, Hussain MA, Ahmad SM, Ziebach T, Qazi GN, Spiteller M. Phenolic constituents and genetic profile of *Hypericum perforatum* L. from India. *Biochem Syst Ecol*. 2008; 36: 201-206.

41) Russo E, Scicchitano F, Whalley BJ, Mazzitello C, Ciriaco M, Esposito S, Patane M, Upton R, Pugliese M, Chimirri S, Mammi M, Palleria C, De Sarro G. *Hypericum perforatum*: pharmacokinetic, mechanism of action, tolerability, and clinical drug-drug interactions. *Phytother Res*. 2014; 28: 643-655.

42) Richard J-A. Chemistry and biology of the polycyclic polyprenylated acylphloroglucinol hyperforin. *Eur J Org Chem*. 2014: 273-299.

43) Ciochina R, Grossman RB. Polycyclic polyprenylated acylphloroglucinols. *Chem Rev*. 2006; 106: 3963-3986.

44) Li Y, Cao X. Isolation and characterization of furohyperforin from *Hypericum perforatum* L. by high-speed countercurrent chromatography. *J Liq Chromatogr Related Technol*. 2012; 35: 2558-2566.

45) Barnes J, Anderson LA, Phillipson JD. *Herbal Medicines*. 3rd ed. London: Pharmaceutical Press; 2007: 549-569.

46) Saddiqe Z, Naeem I, Maimoona A. A review of the antibacterial activity of *Hypericum perforatum* L. *J Ethnopharmacol*. 2010; 131: 511-521.

47) Di YM, Li CG, Xue CC, Zhou S-F. Clinical drugs that interact with St. John's wort and implication in drug development. *Curr Pharm Des*. 2008; 14: 1723-1742.

48) Vazzana M, Macedo AS, Santini A, Faggio C, Souto EB. Novel neuroprotective formulations based on St. John's wort extract. *J Food Res*. 2014; 3(4): 3-17.

49) Kusari S, Sezgin S, Nigutova K, Cellarova E, Spiteller M. Spatial chemo-profiling hypericin and related phytochemicals in *Hypericum* species using MALDI-HRMS imaging. *Anal Bioanal Chem*. 2015; 407: 4779-4791.

50) Nahrstedt A, Butterweck V. Biologically active and other chemical constituents of the herb of *Hypericum perforatum*. *Pharmacopsychiat*. 1997; 30 (Suppl): 129-134.

51) Nicoletti M, Petitto V, Gallo FR, Multari G, Federici E, Palazzino G. The Modern Analytical Determination of Botanicals and Similar Novel Natural Products by the HPTLC Fingerprint Approach. In: Atta-ur-Rahman, ed. *Studies in Natural Product Chemistry*. Great Britain: Elsevier; 2012: 217-258.

52) Vattikuti UMR, Ciddi V. An overview on *Hypericum perforatum* Linn. *Nat Prod Rad*. 2005; 4(5): 368-381.

53) Sofic E, Copra-Janicijevic A, Salihovic M, Tahirovic I, Kroyer G. Screening of medicinal plant extracts for quercetin-3-rutinoside (rutin) in Bosnia and Herzegovina. *Med Plants*. 2010; 2(2): 97-102.

54) Butterweck V. St. John's wort: quality issues and active components. *Botanical Medicine: From Bench to Bedside*. New York: Mary Ann Liebert, Inc.; 2009.

55) Crockett SL, Poller B, Tabanca N, Pferschy-Wenzig E-M, Kunert O, Wedge DE, Bucar F. Bioactive xanthones from the roots of *Hypericum perforatum* (common St John's wort). *J Sci Food Agric*. 2011; 91(3): 428-434.

56) Smelcerovic A, Spiteller M, Ligon AP, Smelcerovic Z, Raabe N. Essential oil composition of *Hypericum* L. species from Southeastern Serbia and their chemotaxonomy. *Biochem Syst Ecol*. 2007; 35: 99-113.

57) Petrakis PV, Couladis M, Roussis V. A method for detecting the biosystematic significance of the essential oil composition: the case of five Hellenic *Hypericum* L. species. *Biochem Syst Ecol*. 2005; 33: 873-898.

58) Radusiene J, Judzentiene A, Bernotiene G. Essential oil composition and variability of *Hypericum perforatum* L. growing in Lithuania. *Biochem Syst Ecol*. 2005; 33: 113-124.

59) Erken S, Malyer H, Demirci F, Demirci B, Baser KHC. Chemical investigations on some *Hypericum* species growing in Turkey-I. *Chem Nat Compd*. 2001; 37(5): 434438.

60) Schwob I, Bessiere J-M, Viano J. Composition of the essential oils of *Hypericum perforatum* L. from southeastern France. *C R Biologies*. 2002; 325: 781-785.

61) Morshedloo MR, Ebadi A, Maggi F, Fattahi R, Yazdani D, Jafari M. Chemical characterization of the essential oil compositions from Iranian populations of *Hypericum perforatum* L. *Ind Crops Prod*. 2015; 76: 565-573.

62) Hajdari A, Mustafa B, Nebija D, Kashtanjeva A, Widelski J, Glowniak K, Novak J. Essential oil composition and variability of *Hypericum perforatum* L. from wild population in Kosovo. *Curr Issues Pharm Med Sci*. 2014; 27(1): 51-54.

63) Benna A, Goncearіuc M, Kulcitki V, Dragalin I, Nistreanu A. Essential oil chemical composition biodiversity in the *Hypericum* L. species from the spontaneous flora of the republic of Moldova. *Muzeul Olteniei Craiova*. 2013; 29(2): 47-52.

64) Saroglou V, Marin PD, Rancic A, Veljic M, Skaltsa H. Composition and antimicrobial activity of the essential oil of six *Hypericum* species from Serbia. *Biochem Syst Ecol*. 2007; 35: 146-152.

65) Yüce E. Analysis of the essential oils of two *Hypericum* species (*H. lanuginosum* var. *lanuginosum* Lam. and *H. perforatum* L.) from Turkey. *Hacettepe J Biol Chem*. 2016; 44(1): 29-34.

66) Çakir A, Duru ME, Harmandar M, Ciriminna R, Passannanti S, Piozzi F. Comparison of the volatile oils of *Hypericum scabrum* L. and *Hypericum perforatum* L. from Turkey. *Flavour Frag J*. 1997; 12: 285-287.

67) Moroydor-Derun E, Eslek Z, Piskin S. Extraction and analysis of *Hypericum perforatum* L. from Turkey. *Int J Chem Mol Nucl Mater Metall Eng*. 2013; 7(7): 495-499.

68) Bertoli A, Çırak C, Teixeira da Silva JA. *Hypericum* species as sources of valuable essential oils. *Med Aromat Plant Sci Biotechnol*. 2011; 5(1): 29-47.

69) Pirbalouti AG, Fatahi-Vanani M, Craker L, Shirmardi H. Chemical composition and bioactivity of essential oils of *Hypericum helianthemoides*, *Hypericum perforatum* and *Hypericum scabrum*. *Pharm Biol*. 2014; 52(2): 175-181.

70) Jaimand K, Rezaee MB, Naderi M, Mozaffrian V, Azadi R, Karimi S, Gholipoor M. Chemical composition of the essential oils of six *Hypericum* species (Hypericaceae) from Iran. *J Med Plant By-Prod*. 2012; 1: 7-11.

71) Hosni K, Msaada K, Taarit MB, Marzouk B. Fatty acid composition and tocopherol content in four Tunisian *Hypericum* species: *Hypericum perforatum*, *Hypericcum tomentosum*, *Hypericum perfoliatum* and *Hypericum ericoides* ssp. *Roberti Arab J Chem*. 2017; 10(S2736-S2741).

72) Piperopoulos G, Lotz R, Wixforth A, Schmierer T, Zeller K-P. Determination of naphthodianthrones in plant extracts from *Hypericum perforatum* L. by liquid chromatography-electrospray mass spectrometry. *J Chromatogr B*. 1997; 695: 309-316.

73) Brolis M, Gabetta B, Fuzzati N, Pace R, Panzeri F, Peterlongo F. Identification by high-performance liquid chromatography-diode array detection-mass spectrometry and quantification by high-performance liquid chromatography-UV absorbance detection of active constituents of *Hypericum perforatum*. *J Chromatogr A*. 1998; 825: 9-16.

74) Mulinacci N, Bardazzi C, Romani A, Pinelli P, Vincieri FF, Costantini A. HPLC-DAD and TLC-densitometry for quantification of hypericin in *Hypericum perforatum* L. extracts. *Chromatographia*. 1999; 49(3/4): 197-201.

75) Orth HCJ, Rentel C, Schmidt PC. Isolation, purity analysis and stability of hyperforin as a standard material from *Hypericum perforatum* L. *J Pharm Pharmacol*. 1999; 51(2): 193-200.

76) Draves AH, Walker SE. Determination of hypericin and pseudohypericin in pharmaceutical preparations by liquid chromatography with fluorescence detection. *J Chromatogr B*. 2000; 749: 57-66.

77) Liu FF, Ang CYW, Heinze TM, Rankin JD, Beger RD, Freeman JP, Lay JO. Evaluation of major active components in St. John's wort dietary supplements by high-performance liquid chromatography with photodiode array detection and electrospray mass spectrometric confirmation. *J Chromatogr A*. 2000; 888: 85-92.

78) Bergonzi MC, Bilia AR, Gallori S, Guerrini D, Vincieri FF. Variability in the content of the constituents of *Hypericum perforatum* L. and some commercial extracts. *Drug Dev Ind Pharm*. 2001; 27(6): 491-497.

79) Bilia AR, Bergonzi MC, Morgenni F, Mazzi G, Vincieri FF. Evaluation of chemical stability of St. John's wort commercial extract and some preparations. *Int J Pharm*. 2001; 213: 199-208.

80) Li W, Fitzloff JF. High performance liquid chromatography analysis of St. John's wort with photodiode array detection. *J Chromatogr B*. 2001; 765: 99-105.

81) Pietta P, Gardana C, Pietta A. Comparative evaluation of St. John's wort from different Italian regions. *Il Farmaco*. 2001; 56: 491-496.

82) Poutaraud A, Lobstein A, Girardin P, Weniger B. Improved procedure for the quality control of *Hypericum perforatum* L. *Phytochem Anal*. 2001; 12: 355-362.

83) Reyes GC, Koda RT. Development of a simple, rapid and reproducible HPLC assay for the simultaneous determination of hypericins and stabilized hyperforin in commercial St. John's wort preparations. *J Pharm Biomed Anal*. 2001; 26: 959-965.

84) Stefova M, Kulevanova S, Stafilov T. Assay of flavonols and quantification of quercetin in medicinal plants by HPLC with UV-diode array detection. *J Liq Chromatogr Rel Technol*. 2001; 24(15): 2283-2292.

85) Walker L, Sirvent T, Gibson D, Vance N. Regional differences in hypericin and pseudohypericin concentrations and five morphological traits among *Hypericum perforatum* plants in the northwestern United States. *Can J Bot*. 2001; 79: 1248-1255.

86) Ganzera M, Zhao J, Khan IA. *Hypericum perforatum*-chemical profiling and quantitative results of St. John's wort products by an improved high-performance liquid chromatography method. *J Pharm Sci*. 2002; 91(3): 623-630.

87) Reyes GC, Koda RT. Determining hyperforin and hypericin content in eight brands of St. John's wort. *Am J Health-Syst Pharm*. 2002; 59: 545-547.

88) Sirvent TM, Walker L, Vance N, Gibson DM. Variation in hypericins from wild populations of *Hypericum perforatum* L. in the Pacific Northwest of the USA. *Econ Bot*. 2002; 56(1): 41-48.

89) Tolonen A, Uusitalo J, Hohtola A, Jalonen J. Determination of naphthodianthrones and phloroglucinols from *Hypericum perforatum* extracts by liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom*. 2002; 16(396-402).

90) Fogliani C, Watts J, Khoury R. Detection of *Hypericum* derivatives by HPLC. *J Aust Tradit-Med Soc*. 2003; 9(4): 177-178.

91) De Jager LS, Perfetti GA, Diachenko GW. Liquid chromatographic determination of St. John's wort components in functional foods. *J AOAC Int*. 2004; 87(5): 1042-1048.

92) Maggi F, Ferretti G, Pocceschi N, Menghini L, Ricciutelli M. Morphological, histochemical and phytochemical investigation of the genus *Hypericum* of the Central Italy. *Fitoterapia*. 2004; 75: 702-711.

93) Wang Z-J, Lin S-M, Hu M-L. Contents of hypericin and pseudohypericin in five commercial products of St John's wort (*Hypericum perforatum*). *J Sci Food Agric*. 2004; 84: 395-397.

94) Shah AK, Avery BA, Wyandt CM. Content analysis and stability evaluation of selected commercial preparations of St. John's wort. *Drug Dev Ind Pharm*. 2005; 31: 907-916.

95) Williams FB, Sander LC, Wise SA, Girard J. Development and evaluation of methods for determination of naphthodianthrones and flavonoids in St. John's wort. *J Chromatogr A*. 2006; 1115: 93-102.

96) Biesaga M, Stafiej A, Pyrzynska K. Extraction and hydrolysis parameters for determination of quercetin in *Hypericum perforatum*. *Chromatographia*. 2007; 65(11/12): 701-706.

97) Çırak C, Radusiene J, Janulis V, Ivanauskas L, Arslan B. Chemical constituents of some *Hypericum* species growing in Turkey. *J Plant Biol*. 2007; 50(6): 632-635.

98) Çirak C, Radusiene J, Karabük B, Janulis V. Variation of bioactive substances and morphological traits in *Hypericum perforatum* populations from Northern Turkey. *Biochem Syst Ecol*. 2007; 35: 403-409.

99) Wojdylo A, Oszmianski J, Czemerys R. Antioxidant activity and phenolic compounds in 32 selected herbs. *Food Chem*. 2007; 105: 940-949.

100) Ayan AK, Çirak C. Hypericin and pseudohypericin contents in some *Hypericum* species growing in Turkey. *Pharm Biol*. 2008; 46(4): 288-291.
101) Tewari N, Menghani H, Hamarapurkar PD, Mane T, Suthar A, Chauhan VS. Development of a high-performance liquid chromatographic method for the quantitative determination of hyperforin. *Int J Appl Sci Eng*. 2008; 6(1): 39-46.
102) Anyzewska M, Kowalczuk A, Lozak A, Jablczynska R, Fijalek Z. Determination of total hypericins in St. John's wort and herbal medicinal products. *Acta Pol Pharm - Drug Res*. 2010; 67(6): 587-593.
103) Bagdonaite E, Martonfi P, Repcak M, Labokas J. Variation in the contents of pseudohypericin and hypericin in *Hypericum perforatum* from Lithuania. *Biochem Syst Ecol*. 2010; 38: 634-640.
104) Filippini R, Piovan A, Borsarini A, Caniato R. Study of dynamic accumulation of secondary metabolites in three subspecies of *Hypericum perforatum*. *Fitoterapia*. 2010; 81: 115-119.
105) Hosni K, Msaada K, Taarit MB, Hammami M, Marzouk B. Bioactive components of three *Hypericum* species from Tunisia: a comparative study. *Ind Crops Prod*. 2010; 31: 158-163.
106) Franchi GG, Nencini C, Collavoli E, Massarelli P. Composition and antioxidant activity *in vitro* of different St. John's wort (*Hypericum perforatum* L.) extracts. *J Med Plant Res*. 2011; 5(17): 4349-4353.
107) Bagdonaite E, Martonfi P, Repcak M, Labokas J. Variation in concentrations of major bioactive compounds in Hypericum perforatum L. from Lithuania. *Ind Crops Prod*. 2012; 35: 302-308.
108) Erdogan-Orhan I, Kartal M. LC-DAD-MS-assisted quantification of marker compounds in *Hypericum perforatum* L. (St. John's wort) and its antioxidant activity. *Turk J Pharm Sci*. 2015; 12(3): 279-286.
109) Jesionek W, Majer-Dziedzic B, Choma IM. Separation, identification, and investigation of antioxidant ability of plant extract components using TLC, LC-MS, and TLC-DPPH. *J Liq Chromatogr Rel Technol*. 2015; 38: 1147-1153.
110) Porzel A, Farag MA, Mülbradt J, Wessjohann LA. Metabolite profiling and fingerprinting of *Hypericum* species: a comparison of MS and NMR metabolomics. *Metabolomics*. 2014; 10: 574-588.

111) Wolfram E, Peter S, Könye R, Meier B, Danova K. Development of biotechnological protocols in medicinal plants research - contribution for safeguarding and standardization of raw material for phytotherapy using *Hypericum* species indigenous to the Balkan region. 2014; Available from: phytotherapie2014.smgp.ch/poster/QK11_small.pdf. [cited 2016 July, 9].

112) Fornasiero RB, Maffi L, Benvenuti S, Bianch A. Morphological and phytochemical features of secretory structures in *Hypericum richeri* (Clusiaceae). *Nord J Bot*. 2000; 20(4): 427-434.

113) Kitanov GM. Hypericin and pseudohypericin in some *Hypericum* species. *Biochem Syst Ecol*. 2001; 29: 171-178.

114) Maffi L, Benvenuti S, Fornasiero RB, Bianchi A, Melegari M. Inter-population variability of secondary metabolites in *Hypericum* ssp. (Hypericaceae) of the Northern Apennines, Italy. *Nord J Bot*. 2001; 21(6): 585-593.

115) Wagner H, Bladt S. *Plant Drug Analysis - A Thin Layer Chromatography Atlas*. 2nd ed. Germany: Springer; 2001.

116) Reich E, Schibli A. *High-Performance Thin Layer Chromatography for the Analysis of Medicinal Plants*. China: Thieme Medical Publishers; 2006.

117) Nuevas-Paz L, Molina-Torres J, Prieto-Gonzalez S. Determination of hypericin in *Hypericum* species grown in Cuba. *Acta Farm Bonaerense*. 2005; 24(1): 89-90.

118) Peşin İ. *Hypericum perforatum L. ve Hypericum scabrum L. bitkilerinin yara iyilestirici ve antienflamatuvar aktiviteleri üzerinde çalışmalar* [dissertation]. Ankara: Gazi Üniversitesi; 2007.

119) Tewari N, Menghani H, Hamarapurkar P, Suthar A, Chauhan VS. Developing a new high performance thin layer chromatography method for quantitative estimation of hyperforin in *Hypericum perforatum*. *Phcog Mag*. 2008; 4(16): 303-307.

120) Cretu R, Mihailescu R, Mitroi G, Iacob E, Verdeş R, Tebrencu C. Phytochemical investigation of Crataegi folium cum flos (Hawthorn leaves and flowers) and Hyperici herba (St. John's wort aerial parts) hydroalcoholic extracts. *Sectiunea Genet şi Biol Mol*. 2011; 12: 133-138.

121) Nicoletti M. HPTLC fingerprint: a modern approach for the analytical determination of botanicals. *Braz J Pharmacog*. 2011; 21(5): 818-823.

122) Nicoletti M, Toniolo C. Fingerprint HPTLC analysis of marketed botanicals. *Pharmacol Online*. 2012; 3: 30-36.

123) Nicoletti M. Special issue on role of HPTLC in analysis - Nutraceuticals and botanicals, towards the analytical validation. *Int J Pharm Chem Res*. 2013; 2(4): 1-12.

124) Marrelli M, Conforti F, Toniolo C, Nicoletti M, Statti G, Menichini F. *Hypericum perforatum*: influences of the habitat on chemical composition, photo-induced cytotoxicity, and antiradical activity. *Pharm Biol*. 2014; 52(7): 909-918.

125) Toniolo C, Nicoletti M, Maggi F, Venditti A. HPTLC determination of chemical composition variability in raw materials used in botanicals. *Nat Prod Res*. 2014; 28(5): 119-126.

126) Sharpley AL, McGavin CL, Whale R, Cowen PJ. Antidepressant-like effect of *Hypericum perforatum* (St John's wort) on the sleep polysomnogram. *Psychopharmacology*. 1998; 139: 286-287.

127) Behnke K, Jensen GS, Graubaum H, Gruenwald J. *Hypericum perforatum* versus fluoxetine in the treatment of mild to moderate depression. *Adv Ther*. 2002; 19(1): 43-52.

128) Husain GM, Chatterjee SS, Singh PN, Kumar V. Beneficial effect of *Hypericum perforatum* on depression and anxiety in a type 2 diabetic rat model. *Acta Pol Pharm - Drug Res*. 2011; 68(6): 913-918.

129) Bukhari IA, Dar A. Behavioral profile of Hypericum perforatum (St. John's wort) extract. A comparison with standard antidepressants in animal models of depression. *Eur Rev Med Pharmacol Sci*. 2013; 17: 1082-1089.

130) Peron AP, Mariucci RG, Almeida IV, Düsman E, Mantovani MS, Vicentini VEP. Evaluation of the cytotoxicity, mutagenicity and antimutagenicity of a natural antidepressant, *Hypericum perforatum* L. (St. John's wort), on vegetal and animal test systems. *BMC Complement Altern Med*. 2013; 13(97): 1-9.

131) Ng QX, Venkatanarayanan N, Ho CYX. Clinical use of Hypericum perforatum (St. John's wort) in depression: a meta-analysis. *J Affect Disord*. 2017; 210: 211-221.

132) Avato P, Raffo F, Guglielmi G, Vitali C, Rosato A. Extracts from St John's wort and their antimicrobial activity. *Phytother Res*. 2004; 18: 230-232.

133) Milosevic T, Solujic S, Sukdolak S. *In vitro* study of ethanolic extract of *Hypericum perforatum* L. on growth and sporulation of some bacteria and fungi. *Turk J Biol*. 2007; 31: 237-241.

134) Süntar I, Oyardı O, Küpeli-Akkol E, Ozçelik B. Antimicrobial effect of the extracts from *Hypericum perforatum* against oral bacteria and biofilm formation. *Pharm Biol*. 2016; 54(6): 1065-1070.

135) Erdogan-Orhan I, Kartal M, Gülpinar AR, Cos P, Matheeussen A, Maes L, Tasdemir D. Assessment of antimicrobial and antiprotozoal activity of the olive oil macerate samples of *Hypericum perforatum* and their LC-DAD-MS analyses. *Food Chem*. 2013; 138: 870-875.

136) Abdel-Salam OME. Anti-inflammatory, antinociceptive, and gastric effects of *Hypericum perforatum* in rats. *Sci World J*. 2005; 5: 585-596.

137) Hatanaka J, Shinme Y, Kuriyama K, Uchida A, Kou K, Uchida S, Yamada S, Onoue S. *In vitro* and *in vivo* characterization of new formulations of St. John's wort extract with improved pharmacokinetics and anti-nociceptive effect. *Drug Metab Pharmacokinet*. 2011; 26(6): 551-558.

138) Galeotti N, Ghelardini C. St. John's wort reversal of meningeal nociception: a natural therapeutic perspective for migraine pain. *Phytomedicine*. 2013; 20: 930-938.

139) Galeotti N, Ghelardini C. Reversal of NO-induced nociceptive hypersensitivty by St. John's wort and hypericin: NF-κB, CREB and STAT1 as molecular targets. *Psychopharmacology*. 2013; 227: 149-163.

140) Galeotti N, Bianchi E, Ghelardini C. *Hypericum perforatum* L. as adjuvant to opioid analgesia in an animal model of migraine. *Br J Med Med Res*. 2014; 4(7): 1591-1603.

141) Galeotti N, Maidecchi A, Mattoli L, Burico M, Ghelardini C. St. John's wort seed and feverfew flower extracts relieve painful diabetic neuropathy in a rat model of diabetes. *Fitoterapia*. 2014; 92: 23-33.

142) Ramos AA, Marques F, Fernandes-Ferreira M, Pereira-Wilson C. Water extracts of three *Hypericum* sps. protect DNA from oxidative and alkylating damage and enhance DNA repair in colon cells. *Food Chem Toxicol*. 2013; 51: 80-86.

143) Menichini G, Alfano C, Marrelli M, Toniolo C, Provenzano E, Statti GA, Nicoletti M, Menichini F, Conforti F. *Hypericum perforatum* L. subsp. *perforatum* induces inhibition of free radicals and enhanced phototoxicity in human melanoma cells under ultraviolet light. *Cell Prolif*. 2013; 46: 193-202.

144) Kıyan HT, Demirci B, Başer KHC, Demirci F. The *in vivo* evaluation of anti-angiogenic effects of *Hypericum* essential oils using the chorioallantoic membrane assay. *Pharm Biol*. 2014; 52(1): 44-50.

145) Zerrouki K, Djebli N, Eroglu-Ozkan E, Ozsoy N, Gul O, Mat A. *Hypericum perforatum* improve memory and learning in Alzheimer's model: (experimental study in mice). *Int J Pharm Pharm Sci*. 2016; 8(8): 49-57.

146) Altun ML, Yılmaz BS, Erdogan-Orhan I, Saltan-Citoglu G. Assessment of cholinesterase and tyrosinase inhibitory and antioxidant effects of *Hypericum perforatum* L. (St. John's wort). *Ind Crops Prod*. 2013; 43: 87-92.

147) Yeşilada E, Gürbüz İ, Shibata H. Screening of Turkish anti-ulcerogenic folk remedies for anti-*Helicobacter pylori* activity. *J Ethnopharmacol*. 1999; 66: 289-293.

148) Tanideh H, Nematollahi SL, Hosseini SV, Hosseinzadeh M, Mehrabani D, Safarpour A, Sepehrimanesh M, Koohi-Hosseinabadi O, Najibi A. The healing effect of *Hypericum perforatum* extract on acetic acid-induced ulcerative colitis in rat. *Ann Colorectal Res*. 2014; 2(4): 1-6.

149) Kurt H, Özbayer C, Değirmenci İ, Burukoğlu D, Saadat SM, Üstüner MC, Özdemir-Köroğlu Z, Tuncel T, Güneş HV. İndomethazine bağlı oluşan gastrik mukozal hasar üzerine *Hypericum perforatum* yağının koruyucu etkisi. *Bozok Med J*. 2016; 6(3): 46-52.

150) Eğilmez OK, Kökten N, Doğan-Ekici AI, Kalcıoğlu MT, Yesilada E, Tekin M. The effect of *Hypericum perforatum* L. (St. John's wort) on prevention of myringosclerosis after myringotomy in a rat model. *Int J Pediatr Otorhinolaryngol*. 2015; 79: 1128-1134.

151) İlhan M, Süntar İ, Demirel MA, Yeşilada E, Keleş H, Küpeli-Akkol E. A mixture of St. John's wort and sea buckthorn oils regresses endometriotic implants and affects the levels of inflammatory mediators in peritoneal fluid of the rat: a surgically induced endometriosis model. *Taiwan J Obstet Gynecol*. 2016; 55: 786-790.

152) Gioti EM, Fiamegos YC, Skalkos DC, Stalikas CD. Antioxidant activity and bioactive components of the aerial parts of *Hypericum perforatum* L. from Epirus, Greece. *Food Chem*. 2009; 117: 398-404.

153) Meinke MC, Schanzer S, Haag SF, Casetti F, Müller ML, Wölfle U, Kleemann A, Lademann J, Scempp CM. *In vivo* photoprotective and anti-inflammatory effect of hyperforin is associated with high antioxidant activity *in vitro* and *ex vivo*. *Eur J Pharm Biopharm*. 2012; (346-350).

154) Haag SF, Tscherch K, Arndt S, Kleemann A, Gersonde I, Lademann J, Rohn S, Meinke MC. Enhancement of skin radical scavenging activityand stratum corneum lipids after the application of a hyperforin-rich cream. *Eur J Pharm Biopharm*. 2014; 86: 227-233.

155) Li Y, Liu Y, Xiao B, Tang J, Huang R. Dynamic comparison of free radical scavenging abilities of *Hypericum perforatum* L., *Herba Verbenae officinalis*, and *Valeriana officinalis* L. extracts. *Phcog J*. 2015; 7(3): 198-204.

156) Uebelhack R, Blohmer J, Graubaum H, Busch R, Gruenwald J, Wernecke K. Black cohosh and St. John's wort for climateric complaints. *Obstet Gynecol*. 2006; 107(2): 247-255.

157) Al-Akoum M, Maunsell E, Verreault R, Provencher L, Otis H, Dodin S. Effects of *Hypericum perforatum* (St. John's wort) on hot flashes and quality of life in perimenopausal women: a randomized pilot trial. *Menopause*. 2009; 16(2): 307-314.

158) Van Die MD, Bone KM, Burger HG, Reece JE, Teede HJ. Effects of a combination of *Hypericum perforatum* and *Vitex agnus-castus* on PMS-like symptoms in late-perimenopausal women: findings from a subpopulation analysis. *J Altern Complement Med*. 2009; 15(9): 1045-1048.

159) Van Die MD, Burger HG, Bone KM, Cohen MM, Teede HJ. *Hypericum perforatum* with *Vitex agnus-castus* in menopausal symptoms: a randomized, controlled trial. *Menopause*. 2009; 16(1): 156-163.

160) Liu YR, Jiang YL, Huang RQ, Yang JY, Xiao BK, Dong JX. *Hypericum perforatum* L. preparations for menopause: a meta-analysis of efficacy and safety. *Climacteric*. 2014; 17: 325-335.

161) Kotsiou A, Seferos N, Hudu GM, Tesseromatis C. Pleiotropic activity of *Hypericum perforatum* L. *J Med Plants Stud*. 2016; 4(4): 256-258.

162) You M, Rhuy J, Jeong KS, Bang M, Kim M, Kim H. Effect of St. John's wort (*Hypericum perforatum*) on obesity, lipid metabolism and uterine epithelial proliferationin ovariectomized rats. *Nutr Res Pract*. 2014; 8(3): 292-296.

163) Öztürk N, Korkmaz S, Öztürk Y. Wound-healing activity of St. John's wort (*Hypericum perforatum* L.) on chicken embryonic fibroblasts. *J Ethnopharmacol*. 2007; 111: 33-39.

164) Süntar IP, Küpeli-Akkol E, Yılmazer D, Baykal T, Kırımızıbekmez H, Alper M, Yeşilada E. Investigations on the *in vivo* wound healing potential of *Hypericum perforatum* L. *J Ethnopharmacol*. 2010; 127: 468-477.

165) Süntar I, Küpeli-Akkol E, Keleş H, Oktem A, Başer KHC, Yeşilada E. A novel wound healing ointment: a formulation of *Hypericum perforatum* oil and sage and oregano essential oils based on traditional Turkish knowledge. *J Ethnopharmacol*. 2011; 134: 89-96.

166) Prisacaru AI, Andritoiu CV, Andriescu C, Havarneanu EC, Popa M, Motoc AGM, Sava A. Evaluation of the wound-healing effect of a novel *Hypericum perforatum* ointment in skin injury. *Rom J Morphol Embryol*. 2013; 54(4): 1053-1059.

167) Erdogan-Orhan I, Kartal M, Gülpinar AR, Yetkin G, Orlikova B, Diederich M, Tasdemir D. Inhibitory effect of St. John's wort oil macerates on TNFα-induced NF-κB activation and their fatty acid composition. *J Ethnopharmacol*. 2014; 155: 1086-1092.

168) Tanideh N, Namazi F, Andisheh Tadbir A, Ebrahimi H, Koohi-Hosseinabadi O. Comparative assessment of the therapeutic effects of the topical and systemic forms of *Hypericum perforatum* extract on induced oral mucositis in golden hamsters. *Int J Oral Maxillofac Surg*. 2014; 43: 1286-1292.

169) Peksen ME, Kurtı A, Soylu S, Tuzcu M, Bostancı ME, Mollaoğlu MC, Abakay U, Şen M. Comparison of the healing effects of silver sulfadiazine cream (1%) and the extract of *Hypericum perforatum* scald burn wound in a rat model. *Basic Clin Sci*. 2014; 3: 1-8.

170) Kıyan S, Uyanıkgil Y, Altuncı YA, Cavuşoğlu T, Çetin-Uyanıkgil EÖ, Karabey F. Investigation of acute effects of *Hypericum perforatum* (St. John's wort-Kantaron) treatment in experimental thermal burns and comparison with silver sulfadiazine treatment. *Ulus Travma Acil Cerrahi Derg*. 2015; 21(5): 323-336.

171) Yaşar M, Kaya A, Karaman H, Kavugudurmaz M, Polat H, Sağıt M, Özcan İ. Potential curative role of *Hypericum perforatum* in an experimental rat model of tympanic membrane perforation. *J Int Adv Otol*. 2016; 12(3): 252-256.

172) Yücel A, Kan Y, Yesilada E, Akın O. Effect of St. John's wort (*Hypericum perforatum*) oily extract for the care and treatment of pressure sores; a case report. *J Ethnopharmacol*. 2017; 196: 236-241.

173) Taher MM, Lammering GM, Hershey CM, Valerie KC. Mood-enhancing antidepressant St. John's wort inhibits the activation of human immunodeficiency virus gene expression by Ultaviolet Light. *IUBMB Life*. 2002; 54: 357-364.

174) Mitsopoulou KP, Vidali VP, Koliiopoulos G, Couladouros EA, Michaelakis A. Hyperforin and deoxycohumulone as a larvicidal agent against *Culex pipiens* (Diptera: Culicidae). *Chemosphere*. 2014; 100: 124-129.

175) Khan M, Subhan F, Khan A, Abbas M, Ali G, Rauf K, Gilani AH. Nature cures nature: *Hypericum perforatum* attenuates physical withdrawal signs in opium dependent rats. *Pharm Biol*. 2014; 52(5): 586-590.

176) Tian J, Tao R, Zhang X, Liu Q, He Y, Su Y, Ji T, Ye F. Effect of *Hypericum perforatum* L. extract on insulin resistance and lipid metabolic disorder in high-fat-diet induced obese mice. *Phytother Res*. 2015; 29: 86-92.

177) Yeşilada E, Honda G, Sezik E, Tabata M, Fujita T, Tanaka T, Takeda Y, Takaishi Y. Traditional medicine in Turkey. V. Folk medicine in the inner Taurus Mountains. *J Ethnopharmacol*. 1995; 46: 133-152.

178) Tuzlacı E, Tolon E. Turkish folk medicinal plants, part III: Şile (Istanbul). *Fitoterapia*. 2000; 71: 673-685.

179) Sezik E, Yeşilada E, Honda G, Takaishi Y, Takeda Y, Tanaka T. Traditional medicine in Turkey X. Folk medicine in central Anatolia. *J Ethnopharmacol*. 2001; 75: 95-115.

180) Tuzlacı E, Eryaşar-Aymaz P. Turkish folk medicinal plants, part IV: Gönen (Balıkesir). *Fitoterapia*. 2001; 72: 323-343.

181) Kültür Ş. Medicinal plants used in Kırklareli (Turkey). *J Ethnopharmacol*. 2007; 111: 341-364.

182) Cakilcioglu U, Turkoglu I. An ethnobotanical survey of medicinal plants in Sivrice (Elazığ-Turkey). *J Ethnopharmacol*. 2010; 132: 165-175.

183) Altundag E, Ozturk M. Ethnomedicinal studies on the plant resources of east Anatolia, Turkey. *Procedia Soc Behav Sci*. 2011; 19: 756-777.

184) Cakilcioglu U, Khatun S, Turkoglu I, Hayta S. Ethnopharmacological survey of medicinal plants in Maden (Elazig-Turkey). *J Ethnopharmacol*. 2011; 137: 469-486.

185) Polat R, Satıl F. An ethnobotanical survey of medicinal plants in Edremit Gulf (Balıkesir - Turkey). *J Ethnopharmacol*. 2012; 139: 626-641.
186) Bulut G, Tuzlaci E. An ethnobotanical study of medicinal plants in Turgutlu (Manisa - Turkey). *J Ethnopharmacol*. 2013; 149: 633-647.
187) Polat R, Cakilcioglu U, Satıl F. Traditional uses of medicinal plants in Solhan (Bingöl-Turkey). *J Ethnopharmacol*. 2013; 148: 951-963.
188) Güzel Y, Güzelşemme M, Miski M. Ethnobotany of medicinal plants used in Antakya: a multicultural district in Hatay province of Turkey. *J Ethnopharmacol*. 2015; 174: 118-152.
189) Özdemir E, Alpınar K. An ethnobotanical survey of medicinal plants in western part of central Taurus Mountains: Aladaglar (Nigde - Turkey). *J Ethnopharmacol*. 2015; 166: 53-65.
190) Polat R, Cakilcioglu U, Kaltalioğlu K, Ulusan MD, Türkmen Z. An ethnobotanical study on medicinal plants in Espiye and its surrounding (Giresun-Turkey). *J Ethnopharmacol*. 2015; 163: 1-11.
191) Sargin SA, Selvi S, Büyükcengiz M. Ethnomedicinal plants of Aydıncık district of Mersin, Turkey. *J Ethnopharmacol*. 2015; 174: 200-216.
192) Sargin SA, Selvi S, Lopez V. Ethnomedicinal plants of Sarigöl district (Manisa), Turkey. *J Ethnopharmacol*. 2015; 171: 64-84.
193) Bulut G. Medicinal and wild food plants of Marmara Island (Balikesir-Turkey). *Acta Soc Bot Pol*. 2016; 85(2): 3501-3516.
194) Sezik E, Yesilada E, Shadidoyatov H, Kulivey Z, Nigmatullaev AM, Aripov HN, Takaishi Y, Takeda Y, Honda G. Folk medicine in Uzbekistan I. Toshkent, Djizzax, and Samarqand provinces. *J Ethnopharmacol*. 2004; 92: 197-207.
195) Lardos A. The botanical *materia medica* of the *Iatrosophikon* - a collection of prescriptions from a monastery in Cyprus. *J Ethnopharmacol*. 2006; 104: 387-406.
196) Benitez G, Gonzalez-Tejero MR, Molero-Mesa J. Pharmaceutical ethnobotany in the western part of Granada province (southern Spain): ethnopharmacological synthesis. *J Ethnopharmacol*. 2010; 129: 87-105.
197) Jaric S, Popovic Z, Macukanovic-Jocic M, Djurdjevic L, Mijatovic M, Karadzic B, Mitrovic M, Pavlovic P. An ethnobotanical study on the usage of wild medicinal herbs from Kopaonik Mountain (Central Serbia). *J Ethnopharmacol*. 2007; 111: 160-175.

198) Gonzalez JA, Garcia-Barriuso M, Amich F. Ethnobotanical study of medicinal plants traditionally used in the Arribes del Duero, western Spain. *J Ethnopharmacol*. 2010; 131: 343-355.

199) Saric-Kundalic B, Dobes C, Klatte-Asselmeyer V, Saukel J. Ethnobotanical study on medicinal use of wild and cultivated plants in middle, south and west Bosnia and Herzegovina. *J Ethnopharmacol*. 2010; 131: 33-55.

200) Menkovic N, Savikin K, Tasic S, Zdunic G, Stesevic D, Milosavljevic S, Vincek D. Ethnobotanical study on traditional uses of wild medicinal plants in Prokletije Mountains (Montenegro). *J Ethnopharmacol*. 2011; 133: 97-107.

201) Savikin K, Zdunic G, Menkovic N, Zivkovic J, Cujic N, Terescenko M, Bigovic D. Ethnobotanical study on traditional use of medicinal plants in south-western Serbia, Zlatibor district. *J Ethnopharmacol*. 2013; 146: 803-810.

202) Cavero RY, Calco MI. Medicinal plants used for respiratory affections in Navarra and their pharmacological validation. *J Ethnopharmacol*. 2014; 158: 216-220.

203) Tuttolomondo T, Licata M, Leto C, Savo V, Bonsangue G, Gargano ML, Venturella G, La Bella S. Ethnobotanical investigation on wild medicinal plants in the Monti Sicani Regional Park (Sicily, Italy). *J Ethnopharmacol*. 2014; 153: 568-586.

204) Cavero RY, Calco MI. Medicinal plants used for musculoskeletal disorders in Navarra and their pharmacological validation. *J Ethnopharmacol*. 2015; 168: 255-259.

205) Jaric S, Macukanovic-Jocic M, Djurdjevic L, Mitrovic M, Kostic O, Karadzic B, Pavlovic P. An ethnobotanical survey of tradtionally used plants on Suva planina mountain (south-eastern Serbia). *J Ethnopharmacol*. 2015; 175: 93-108.

206) Mustafa B, Hajdari A, Pieroni A, Pulaj B, Koro X, Quave CL. A cross-cultural comparison of folk plant uses among Albanians, Bosniaks, Gorani, and Turks living in south Kosovo. *J Ethnobiol Ethnomed*. 2015; 11(39): 1-26.

207) Rigat M, Valles J, D'Ambrosio U, Gras A, Iglesias J, Garnatje T. Plants with topical uses in Ripolles district (Pyrenees, Catalonia, Iberian Peninsula): ethnobotanical survey and pharmacological validation in the literature. *J Ethnopharmacol*. 2015; 164: 162-179.

208) Jaiswal PK. *High-Performance Thin-Layer Chromatography in Food Analysis*. 1st ed. New Delhi: CBS Publishers & Distributors Pvt Ltd; 2010.

209) Striege MF, Hill J. *Thin-Layer Chromatography for Binding Media Analysis*. Los Angeles: The Getty Conservation Institute; 1996.

210) Shivatare RS, Nagore DH, Nipanikar SU. HPTLC an important tool in standardization of herbal medical product: a review. *J Sci Innov Res*. 2013; 2(6): 1086-1096.

211) Variyar PS, Chatterjee S, Sharma A. Fundamentals and Theory of HPTLC-Based Separation. In: Srivastava M, ed. *High-Performance Thin-Layer Chromatography (HPTLC)*. Berlin: Springer; 2011: 27-39.

212) Sherma J. Basic TLC Techniques, Materials, and Apparatus. In: Sherma J, Fried B, ed. *Handbook of Thin-Layer Chromatography*. 3rd ed. New York: Marcel Dekker; 2003: 1-61.

213) Harvey D. *Modern Analytical Chemistry*. USA: McGraw-Hill; 2000.

214) Hahn-Deinstrop E. *Applied Thin-Layer Chromatography*. 2nd ed. Germany: Wiley-VCH; 2007.

215) Rabel FM. Sorbents and Precoated Laters in Thin-Layer Chromatography. In: Sherma J, Fried B, ed. *Handbook of Thin-Layer Chromatography*. New York: Marcel Dekker; 2003: 129-175.

216) Andola HC, Purohit VK. High Performance Thin Layer Chromatography (HPTLC): a modern analytical tool for biological analysis. *Nat Sci*. 2010; 8(10): 58-61.

217) Patel RB, Patel MR, Batel BG. Experimental Aspects and Implementation of HPTLC. In: Srivastava M, ed. *High-Performance Thin-Layer Chromatography (HPTLC)*. Berlin: Springer; 2011: 41-54.

218) Srivastava MM. An Overview of HPTLC: A Modern Analytical Technique with Excellent Potential for Automation, Optimization, Hyphenation, and Multidimensional Applications. In: Srivastava M, ed. *High-Performance Thin-Layer Chromatography (HPTLC)*. Berlin: Springer; 2011: 3-24.

219) Kowalska T, Kaezmarski K, Prus W. Theory and Mechanism of Thin-Layer Chromatography. In: Sherma J, Fried B, ed. *Handbook of Thin-Layer Chromatography*. 3rd ed. New York: Marcel Dekker; 2003: 62-105.

220) Dzido TH, Tuzimski T. Chambers, Sample Application, and Chromatogram Development. In: Waksmundzka-Hajnos M, Sherma J, Kowalska T, ed. *Thin Layer Chromatography in Phytochemistry*. USA: CRC Press; 2008: 119-174.

221) Sonia K, Shree BB, Lakshmi KS. HPTLC method development and validation: an overview. *J Pharm Sci Res*. 2017; 9(5): 652-657.

222) Spangenberg B. Derivatization, Detection (Quantification), and Identification of Compounds Online. In: Waksmundzka-Hajnos M, Sherma J, Kowalska T, ed. *Thin Layer Chromatography in Phytochemistry*. USA: CRC Press; 2008: 175-191.

223) Quality Guidelines. ICH Harmonised Tripartite Guideline. Validation of Analytical Procedures: Text and Methodology Q2 (R1). Available from: www.ich.org/products/guidelines/quality/article/quality-guidelines.html. [cited 2017 January, 23].

224) Reich E, Schibli A, Debatt A. Validation of High-Performance Thin-Layer Chromatographic methods for the identification of botanicals in a cGMP environment. *J AOAC Int*. 2008; 91(1): 13-20.

225) Renger B, Vegh Z, Ferenczi-Fodor K. Validation of thin layer and high performance thin layer chromatographic methods. *J Chromatogr A*. 2011; 1218: 2712-2721.

226) Eserian JK, Lombardo M. Method validation in pharmaceutical analysis: from theory to practical optimization. *Inov Pharm*. 2015; 6(1): 1-3.

227) Adamovics JA. Regulatory Considerations for the Chromatographer. In: Adamovics JA, ed. *Chromatographic Analysis of Pharmaceuticals*. 2nd ed. USA: Marcel Dekker; 1997: 1-21.

228) Ravichandran V, Shalini S, Sundram KM, Harish R. Validation of analytical methods - strategies & importance. *Int J Pharm Pharm Sci*. 2010; 2(3): 18-22.

229) Rashmin P, Mrunali P, Nitin D, Nidhi D, Bharat P. HPTLC method development and validation: strategy to minimize methodological failures. *J Food Drug Anal*. 2012; 20(4): 794-804.

230) Dubey N, Dubey N, Mehta R. Validation of HPTLC and HPLC methods for the quantative determination of allyl disulfide in some polyherbal oils. *J AOAC Int*. 2012; 95(6): 1574-1578.

231) Starek M, Krzek J, Michnik S. TLC-densitometric analysis of β-sitosterol in pumpkin seed oil. *J Planar Chromatogr*. 2007; 20(5): 327-330.

232) Sheikh S, Asghar S, Ahmad S. Development of HPTLC qualitative fingerprinting profile of almond oil in marketed herbal cream. *Int J Res Pharm Sci*. 2013; 3(1): 85-92.

233) Arsic I, Zugic A, Antic DR, Zdunic G, Dekanski D, Markovic G, Tadic V. *Hypericum perforatum* L. Hypericaceae/Guttiferae sunflower, olive and palm oil extracts attenuate cold restraint stress - induced gastric lesions. *Molecules*. 2010; 15: 6688-6698.

234) *European Pharmacopoeia*. 8th ed. Strasbourg, France: Council of Europe; 2014.

235) Eroğlu E. *Hypericum pamphylicum Robson et Davis türünün hiperisin miktarı ve biyolojik aktivite yönünden incelenmesi* [dissertation]. İstanbul: İstanbul Üniversitesi; 2007.

236) Özkan E. *Drosanthe ve Taeniocarpium seksiyonlarına ait bazı endemik Hypericum türlerinin kimyasal bileşimi ve biyolojik aktivite yönünden incelenmesi* [dissertation]. İstanbul: İstanbul Üniversitesi; 2011.

237) Mohammad A, Bhawani SA, Sharma S. Analysis of herbal products by thin-layer chromatography: a review. *Int J Pharma Bio Sci*. 2010; 1(2): 1-50.

238) Bubueanu C, Grigore A, Pirvu L. HPTLC fingerprint use, an important step in plant-derived products quality control. *Sci Pap Ser B Horticulture*. 2015; 59: 437-442.

239) Bubueanu C, Pavaloiu R. HPTLC chromatographic polyphenolic fingerprints of plant species from Eastern Europe. *Malays J Med Biol Res*. 2016; 3(1): 41-44.

240) Males Z, Saric D, Bojic M. Quantitative determination of flavonoids and chlorogenic acid in the leaves of *Arbutus unedo* L. using thin layer chromatography. *J Anal Methods Chem*. 2013.

241) Cretu G, Gertrud M, Miron AR, Nechifor AC. A high-performance thin-layer chromatographic method for chlorogenic acid and hyperoside determination from berry extracts. *Rom Biotechnol Lett*. 2013; 18(5): 8657-8665.

242) Kisseleff N, Schmid T, Ryffel J, Lapke C. Flavonoids in glycerol macerates of *Ribes nigrum* L. 2014; Available from: phytotherapie2014.smgp.ch/QK09_ small.pdf. [cited 2017 Jan, 21].

243) Calina D, Olah NK, Patru E, Docea A, Popescu H, Bubulica M-V. Chromatographic analysis of the flavonoids from Robinia Pseudoacacia species. *Curr Health Sci J*. 2013; 39(4): 232-236.

244) Southwell IA, Bourke CA. Seasonal variation in hypericin content of *Hypericum perforatum* L. (St. John's Wort). *Phytochemistry*. 2001; 56: 437-441.

245) Gray DE, Pallardy SG, Garrett HE, Rottinghaus GE. Effect of acute drought stress and time of harvest on phytochemistry and dry weight of St. John's wort leaves and flowers. *Planta Med*. 2003; 69(11): 1024-1030.

246) Gruszczyk M, Kieltyka A. Yields and raw material quality of *Hypericum perforatum* L. and *Solidago virga aurea* L. from one-year and two-year plantations. *Herba Pol*. 2005; 51(1/2): 5-9.

247) Bruni R, Sacchetti G. Factors affecting polyphenol biosynthesis in wild and field grown St. John's wort (*Hypericum perforatum* L. Hypericaceae/Guttiferae). *Molecules*. 2009; 14: 682-725.

248) Verma N, Shukla S. Impact of various factors responsible for fluctuation in plant secondary metabolites. *J App Res Med Aromat Plants*. 2015; 2: 105-113.

249) *WHO Monographs on Selected Medicinal Plants*. Malta: World Health Organization; 2002.