ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

EFFECT OF PROCESSING STEPS ON THE PHENOLIC COMPOUNDS AND PROTEIN DIGESTIBILITY OF COWPEA

M.Sc. THESIS

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JUNE 2017

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ISTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

PROSES BASAMAKLARININ BÖRÜLCEDE BULUNAN FENOLİK BİLEŞİKLER VE PROTEİN SİNDİRİLEBİLİRLİĞİ ÜZERİNE ETKİSİ

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Damla Ezgi Uzun, a **M.Sc**. student of **ITU Graduate School of Science Engineering and Technology** student ID **506131532**, successfully defended the thesis/dissertation entitled "**EFFECT OF PROCESSING STEPS ON THE PHENOLIC COMPOUNDS AND PROTEIN DIGESTIBILITY OF COWPEA**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my family,

FOREWORD

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ABBREVIATIONS

ABTS : 2,2- azinobis 3-ethylbenzothiazoline-6-sulfonic acid
ANOVA : Analysis of variance
AOAC : Association Official of Analytical Chemists
CEA : Catechin Equivalent
Da : dalton
DPPH : 1,1-Diphenyl-2- picrylhydrazyl
DW : Dry weight
FAO : Food and Agriculture Organization of the United Nations
FRAP : Ferric Reducing Antioxidant Capacity
FW : Fresh weight
GAE : Gallic Acid Equivalent
GI : Gastrointestinal
HPLC : High Performance Liquid Chromatography
IN : After digestion dialyzed fraction of sample
IVPD : in vitro Protein Digestibility
OUT : After digestion non-dialyzed fraction of sample
SGF : Simulated Gastric Fluid electrolyte stock solution
SIF : Simulated Intestinal Fluid electrolyte stock solution
SPSS : Statistical Package for the Social Sciences
SSF : Simulated Salivary Fluid electrolyte stock solution
TAC : Total antioxidant capacity
TEAC : Trolox Equivalent Antioxidant Capacity
TFC : Total flavonoid content
TIA : Trypsin Inhibitor Activity
TIU : Trypsin Inhibitor Unit
TPC : Total phenolic content

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EFFECT OF PROCESSING STEPS ON THE PHENOLIC COMPOUNDS AND PROTEIN DIGECTIBILITY OF COWPEA

SUMMARY

Cowpeas, *Vigna unguiculata* (L.) Walpers, belong to the family Leguminosae, originated from sub-Saharan Africa. It is known as a source of protein as well as phenolic compounds and typically have been consumed as dry seeds, which is common especially in undeveloped countries. However, the consumption is limited by presence of some antinutritional compounds such as trypsin which adversely affect the digestibility and bioavailability of nutrients. ıt has been suggested through many studies that traditional processing steps contribute not only to the reduction of the adverse effects of antinutritional compounds, but also increase the nutritional value of cowpea.

In this study, in order to investigate the effect of both landrace and improved types of different varieties on the health-related constituents, the total phenolic, flavonoid, antioxidant capacity, major phenolic compounds were determined. Moreover, the effect of processing steps on total phenolic, flavonoid, antioxidant capacity, major phenolic compounds were determined for a selected cowpea variety along with simulation of in vitro gastrointestinal (GI) digestion as well as its protein content, protein digestibility and trypsin inhibitor activity were examined.

The variety results showed that the free total phenolic content ranged from 0.36 to 1.13 mg GAE/g dw while the bound total phenolic content ranged from 6.05 to 9.13 mg GAE/g dw. Bound total phenolic content was higher than total phenolic content of the free forms, and this for each of the varieties. Also, there was no significant difference in free total phenolic content between landrace and improved types of each varieties ($p>0.05$).

The total flavonoid content ranged from 0.41 to 1.48 mg CAE/g dw for the free fraction and 2.90 to 12.6 mg CAE/g dw for bound fraction in cowpea varieties. When compared free total flavonoid content between landrace and improved types of each varieties, no significant difference was observed ($p>0.05$).

The results of the total antioxidant capacity which was measured by three different methods (ABTS, DPPH and FRAP), show different trends. When compared the types of those varieties, landraces had highest free total antioxidant capacity. On the other hand, no significant difference was observed between landrace and improved types of each varieties for antioxidant capacity ($p>0.05$). In briefly, Asrat had highest TPC, TFC, DPPH and FRAP values but not the highest ABTS values. Moreover, the improvement application seems to be ineffective in terms of increasing amount of phenolic compounds and antioxidative capacity.

Results of processed cowpeas showed that raw cowpea had the highest protein content (39.06 g/100g dw), the protein contents were decreased with processing steps.

The highest total phenolic content was found after the fermentation process (1.97 mg GAE/g dw) compared to the raw samples (0.80 mg GAE/g dw). For total flavonoid content, the highest value was found in cooked cowpea (1.10 mg CAE/g dw). The total antioxidant capacity was found to be the highest in fermented cowpea, ranging from 15.96 mg TE/g dw for DPPH, 32.57 µmol TE/g dw for ABTS to 60.61 µmol Fe II/g dw for FRAP. Furthermore, the increases in TAC were found after cooking and germination by 76.29% and 41.13% for ABTS, and after cooking by 4.93% for FRAP compared raw cowpea.

The analyses of individual phenolic compounds in analyzed processed cowpea, belonging to phenolic acids and flavonoids. Luteolin was found in high quantities after soaking, dehulling, germination and fermentation process. Other phenolic compounds measured were vanillic acid, caffeic acid, sinapic acid, gallic acid, naringenin and p-coumaric acid, quercetin, catechin. The impact of processing on these phenolic compounds was different.

Also, other phenolic compounds measured were vanillic acid, caffeic acid, sinapic acid, gallic acid, naringenin and p-coumaric acid, quercetin, catechin. The impact of processing on these phenolic compounds was different.

According to the results of after *in vitro* gastrointestinal digestion, 38% of total phenolic content were present in the dialyzed fractions for raw cowpea while after dehulling (33%), soaking (25%), fermentation (23%), cooking (23%) and germination (21%) processes the recovery ratios were decreased. After the *in vitro* simulation of GI digestion, the dialyzed free flavonoid fraction represented 71% of the initial value for raw cowpea which had highest recovery. To determine the effect of *in vitro* gastrointestinal digestion on total antioxidant capacity, DPPH, ABTS and FRAP were used. Comparing the DPPH results, the lowest TAC of the dialyzed fractions was found in soaked (2.11 mg TE/g dw) and dehulled (1.74 mg TE/g dw) cowpea while raw sample had highest TAC of the dialyzed fraction (5.87 mg TE/g dw). After *in vitro* GI digestion, the highest recovery in the dialyzed fraction was measured after cooking process (107%). On the other hand, overall view of the results of ABTS, the amount of the dialyzed fraction was remained same after processing steps, except fermentation made an increase in amount of the dialyzed fraction (142.94 µmol/g dw) ($p<0.05$). Contrary to ABTS results, FRAP The amount of the dialyzed fraction decreased with processing steps, especially the lowest TAC was observed after dehulling.

Protein digestibility was significantly increased by the process steps and the highest increase was observed in cooked cowpea (5%). On the other hand, there was a significant reduction measured in the trypsin inhibitor activity after soaking (8%), germination (30%), cooking (89%) compared to raw cowpeas ($p<0.05$). After fermentation, no trypsin inhibitor activity was detected. However, an increased activity of the trypsin inhibitor was observed in dehulled cowpea (11%) compared to the raw samples.

According to SDS Page analysis, different process steps contained almost similar bands except cooked cowpea in the range of 21.5 kDa to 191 kDa. Moreover, when compared to bands belonging to trypsin inhibitor, the clearest band was seen in dehulled cowpea and followed by raw, soaked and germinated cowpea while there was no band equal to trypsin inhibitor in cooked cowpea and fermented cowpea.

PROSES BASAMAKLARININ BÖRÜLCEDE BULUNAN FENOLİK BİLEŞİKLER VE PROTEİN SİNDİRİLEBİLİRLİĞİ ÜZERİNE ETKİSİ

ÖZET

Börülce, *Vigna unguiculata* (L.) Walpers, Leguminosae familyasına aittir ve kökeni Afrika Sahra çölünün güney bölgesine aittir. Özellikle gelişmemiş ülkelerde yaygın olarak bulunan börülce tohumları özellikle kuru olarak tüketilmektedir ve protein ve fenolik bileşikler bakımından iyi bir kaynak olduğu bilinmektedir. Fakat, besinlerin sindirimine ve biyoyararlılığına olumsuz etki oluşturan tripsin gibi anti-besinsel bileşikleri barındırması sebebiyle tüketimi sınırlıdır. Geleneksel proses basamaklarının sadece anti-besinsel bileşiklerin olumsuz etkilerini azaltmada değil, ayrıca börülcenin besinsel değerlerini arttırmada da katkı sağladığı yapılmış çalışmalarda tespit edilmiştir.

Bu çalışmada, doğal yöntemlerle ve laboratuar ortamında iyileştirme çalışmalarıyla üretilmiş börülce farklı türlerinin fenolik, flavonoid, antioksidan kapasitesi ve başlıca fenolik bileşikleri ölçülmüştür. Ayrıca, farklı proses basamaklarının; protein içeriği, toplam fenolik miktarı, toplam flavonoid miktarı, antioksidan kapasite, başlıca fenolik bileşikler ve tripsin inhibitör etkisi gibi özellikler seçilmiş börülce türü üzerindeki araştırılmıştır. Daha sonra *in vitro* olarak mide ve bağırsak simulasyonu oluşturularak protein ve fenolik bileşiklerin sindirilebilirliği üzerine proses basamaklarının etkisi değerlendirilmiştir.

Farklı börülce türlere ait sonuçlar incelendiğinde, toplam serbest fenolik bileşik miktarı 0,36 ile 1,13 mg GAE/g kuru madde arasında, bağlı toplam fenolik miktarı ise 6,05 ile 9,13 mg GAE/g kuru madde arasında değiştiği görülmüştür. Bağlı toplam fenolik miktarı her bir tür için serbest toplam fenolik bileşik miktarından yüksek çıkmıştır. Ayrıca her bir tür için, doğal yöntemlerle üretilmiş ve laboratuar ortamında iyileştirilmiş börülce örnekleri arasında toplam fenolik bileşik miktarı açısından önemli bir fark olmadığı tespit edilmiştir ($p>0,05$). Bu sonuç ile iyileştirme çalışmalarının değerlendirelen özellikler bakımından etkili olmadığı görülmüştür.

Farklı börülce örneklerine ait toplam serbest flavonoid miktaları 0,41 ile 1,48 mg CAE/g kuru madde olduğu, bağlı flavonoid miktarları ise 2,90 ile 12,6 mg CAE/g arasında ölçülmüştür. Ayrıca her bir tür için, doğal yöntemlerle üretilmiş ve laboratuar ortamında iyileştirilmiş börülce örnekleri arasında toplam flavonoid bileşik miktarı açısından önemli bir fark olmadığı tespit edilmiştir ($p>0,05$).

Her bir börülce çeşiti için toplam antioksidan kapasiteleri 3 farklı yöntem ile (ABTS, DPPH ve FRAP) ölçülmüş ve her bir yöntemin farklı sonuçlar verdiği gözlenmiştir. Türlere ait üretim yöntemleri karşılaştırıldığında doğal yöntemlerle üretilmiş börülcelerin daha yüksek serbest antioksidan kapasitesine sahip olduğu ölçülmüştür. Fakat istatistiksel olarak doğal yöntemle üretilmiş ile iyileştirme yöntemleriyle

üretilmiş börülcelere ait antioksidan kapasiteleri arasında önemli bir fark olmadığı görülmüştür ($p>0,05$).

Özetle, *Asrat* türü en yüksek toplam fenolik miktarı, toplam flavonoid miktarı, DPPH ve FRAP değerlerine sahiptir. Fakat ABTS'de en yüksek değer serbest form için *TVU* (25,64 µmol TE/g kuru madde) türünde, bağlı form için ise *Black Eyed Peas* (485,15 µmol TE/g kuru madde) türünde görülmüştür. Ayrıca, iyileştirme çalışmalarının fenolik bileşik miktarları ve antioksidan kapasite değerleri açısından yetersiz kaldığı tespit edilmiştir.

İşlenmiş börülce örnek sonuçları incelendiğinde en yüksek protein içeriği (39,06 g/100g kuru madde) ham börülcede olduğu ve protein miktarının proses basamakları ile azaldığı ölçülmüştür.

Toplam fenolik madde miktarları, işlenmiş börülce örnekleri arasında karşılaştırıldığında en yüksek değer fermente edilmiş börülcede (1,97 mg GAE/g kuru madde), en yüksek flavonoid madde içeriği ise pişirilmiş börülce örneğinde (1,10 mg CAE/g kuru madde) tespit edilmiştir. Bu değerler ham börülce örneği için toplam fenolik madde miktarı 0,80 mg GAE/g kuru madde iken, toplam flavonoid miktarı ise 0,69 mg CAE/g kuru maddedir.

Toplam antioksidan kapasitesi ise DPPH (15,96 mg TE/g kuru madde), ABTS (32,57 µmol TE/g kuru madde), FRAP (60,61 µmol Fe II/g kuru madde) yöntemleri için en yüksek değerler fermente edilmiş börülce örneğinde görülmüştür. Bunun yanında, toplam antioksidan kapasitesinin pişirme işleminden sonra ABTS değerinde %76,29, FRAP değerinde %4,93; filizlendirme işleminden sonra ise ABTS değerinde %41,13 artış olduğu tespit edilmiştir.

Fenolik ve flavonoidlere ait bileşiklerden yaygın olarak bulunan bileşikler HPLC yardımı ile tespit edildi. Luteolin ıslatma, kabuk soyma, filizlendirme ve fermentasyon aşamalarından sonra en yüksek miktarda bulundu. Vanilik asit, kafeik asit, sinapik asit, gallik asit, naringenin, p-kumarik asit, kuersetin ve kateşin miktarlarının; proses basamakları ve sindirime bağlı olarak değişim gösterdiği tespit edildi.

in vitro mide ve bağırsak sindirim simülasyonu sonuçlarına göre, ham börülcedeki toplam fenolik madde miktarının %38'inin diyaliz fraksiyonuna geçtiği; kabuk ayıklama (%33), sulandırma (%25), fermentasyon (%23), pişirme (%23) ve filizlendirme (%21) basamaklarında sonra geri kazanım oranının azaldığı tespit edildi.

in vitro mide ve bağırsak sindirim simülasyonunun ardından, başlangıçtaki flavonoid miktarının diyaliz fraksiyonundaki maksimum geri kazanım değeri, ham börülce örneğinde (%71) belirlendi.

in vitro mide ve bağırsak sindiriminin, toplam antioksidan kapasitesi üzerindeki etkisini belirlemek amacıyla DPPH, ABTS ve FRAP yöntemleri kullanıldı. Diyaliz fraksiyonlara ait DPPH sonuçları karşılaştırıldığında en düşük toplam antioksidan kapasitesi sulandırılmış (2,11 mg TE/g kuru madde) ve kabukları ayrılmış (1,74 mg TE/g kuru madde) börülcede bulunurken, en yüksek toplam antioksidan kapasitesi ham börülce örneğinde (5,87 mg TE/g kuru madde) ölçülmüştür. Diyaliz fraksiyondaki en yüksek geri kazanım oranları karşılaştırıldığında ise en yüksek değer pişirilmiş börülce örneğinde (%107) hesaplanmıştır. Diğer yandan ABTS sonuçları karşılaştırıldığında, fermentasyon prosesi diyaliz fraksyiondaki antioksidan kapasitesinde artış sağlarken (142,94 µmol/g kuru madde), diğer proses

basamaklarından sonra elde edilen örnekler ile ham börülce örneği arasında istatistiksel olarak bir fark görülmemiştir ($p<0,05$). ABTS sonuçlarının aksine FRAP değerlerine göre, diyaliz fraksiyonun miktarında proses basamaklarıyla birlikte azalış görülmüştür, en düşük değer ise kabukları ayrılmış börülce örneğinde gözlenmiştir.

Protein sindirilebilirliği her bir proses basamağı ile artış göstermiştir ve bu artış en yüksek pişirilmiş börülce örneğinde (%5) hesaplanmıştır. Bu oranı %4 ile fermente edilmiş ve kabuğu ayrılmış börülce, %3 ile filizlendirilmil börülce ve %1 ile sulandırılmış börülce örneği takip etmektedir.

Tripsin inhibitör aktivitesi, ham börülce örneği ile karşılaştırıldığında, proses basamakları ile önemli düzeyde azalış göstermiştir ($p<0,05$). Tripsin inhibitör aktivitesi, ham börülce ile karşılaştırıldığında; sulandırma prosesinden sonra %8, filizlendirme prosesinden sonra %30 ve pişirme işleminden sonra %89 oranında azaldığı belirlenmiştir. Ayrıca, fermentasyon işleminden sonra börülce örneklerinde herhangi bir tripsin inhibitör aktivitesi tespit edilmemiştir. Fakat diğer proseslerin aksine, kabuk ayırma işleminden sonra, ham örneklere oranla tripsin inhibitör aktivitesinde %11'lik bir artış belirlenmiştir.

SDS Page jel elektroforezine göre, farklı proses basamaklarından elde edilen börülce örneklerinin 21,5 kDa ile 19kDa arasında benzer bantlara sahip olduğu fakat pişirilmiş börülce örneğinde tek ana bandın (31,5 kDa) olduğu görülmüştür. Bunun yanında tripsin inhibitöre ait bantlar karşılaştırıldığında, en belirgin bant kabukları ayrılmış börülce örneğinde görülürken; bunu ham, sulandırılmış ve filizlendirilmiş börülce örnekleri takip etmiştir. Fakat fermentasyon ve pişirme işlemlerinden sonra elde edilen örneklerde tripsin inhibitör bandına denk bir bant görülmemiştir.

1. INTRODUCTION

The family Leguminosae, better known as legumes, involves 13,000 different species. The family is the third largest family in size among flowering plants and the second in economic importance following the grasses (Sathe, 1996). Despite the thousands of species, only soybeans, dry beans, peas, chickpeas, broad beans, lentil, and cowpeas are widely grown for commercial purposes. Besides, chickpeas, beans, lentils are better known species and consumed in the developing and developed countries while cowpea is nearly only consumed in the undeveloped countries.

Cowpea (*Vigna unguiculata* (L.) Walp.) belongs to the family Leguminosae, subfamily Papilionoideae and formerly carried the name *Vigna sinensis* L. (Phillips, 2013; Bouker *et al*., 2015; Sathe, 1996). While the colors of cowpea vary from white to red to mottled, the seed coat can be thick and loose or thin and adhering (Phillips, 2013). Cowpea is originated in sub-Saharan Africa, as well as it is leading in Asia and certain parts of America (Phillips, 2013). As for the biggest cowpea producers, Nigeria, Niger, Burkino Faso, Tanzania, and Myanmar can be listed (FAO, 2013). Cowpea has a high protein content (25-36%) which can vary depending on the varieties; moreover, they are containing also high amounts of calcium abd potassium.

Phenolic compounds are produced against stress conditions such as external factors, including trauma, wounding, drought, and pathogen attack by plants as secondary metabolic products. Those bioactive compounds can prevent the development of various diseases, like atherosclerosis, cancer, diabetes, aging and cardiovascular diseases in food such as fruits, vegetables, grains, and legumes by virtue of the role in the protection against oxidative damage (Xu et al., 2007; Dueñas *et al*., 2005; Doblado *et al*., 2007; Naczk & Shadihi, 2014). It has been proven by various studies that there is a high amount of phenolic compounds in cowpea depending on numerous factors such as color, varieties as well as in other species of legumes (Oboh, 2006; Gutierrez-Uribe *et al*., 2011; Sreerama *et al*., 2012; Siddhuraju and Becker, 2007; Ojwang *et al*., 2012; Sparvoli *et al.*, 2015).

Although legumes contain high protein content and phenolic compounds, some antinutritional compounds involving protease inhibitors, trypsin inhibitors, tannins, phytic acid, α-amylase, can also be found (Ercan *et al*., 2016; Urbano *et al*., 2000; Gupta, 1987; Khattab, 2009). However, the concentration of these compounds may vary depending on many factors such as varieties, growth condition, location of collection (Bhat and Karim 2009). Proteins become ready for digestion thereby, trypsin breaks down proteins into smaller peptides by catalyzing the hydrolysis of peptide bonds (Rawlings *et al*., 1994; Polgár, 2005). The presence of protease inhibitor like trypsin inhibitor in the food matrix, decreases the nutritional quality of proteins in the diet disturbing the ability of body digestive enzymes to degrade dietary protein, and therefore limiting the intake of amino acids needed to construct new proteins. Yet, the content of inhibitors in varieties of cowpea and on the dose, frequency of consumption and some process steps can reduce the negative effects of antinutritional compounds (Sreerama *et al.,* 2012).

Bioactive compounds that are extracted following a gastrointestinal digestion (GI), must be biologically accessible in order to have potential health benefits for body. In vivo studies in human and animals are limited by ethical reasons, high costs (Holst & Williamson, 2008). For those reasons, *in vitro* simulation models which have good correlation with bioaccessibility results obtained from human and animal studies, have been developed and widely used for evaluation of food bioavailability.

A wide range of processing techniques have been developed and used to produce food for consumption, to provide product diversity, and to increase nutritional value for centuries (Azarpazhooh & Boye, 2013). The traditional process such as soaking, dehulling, cooking, germination and fermentation can contribute to increase the bioavailability of nutrients by means of inactivating antinutritional factors, improve flavor and deliciousness (Lopez, 2016; Carbonora, 2011). However, the effects of processing steps can be altered with many factors including process conditions, time, type of food and characteristics of compounds. The effects of process steps on nutritional values must be examined since cowpea is not consumed in the raw form. It is known that there are over than 20 different foods involving at least one processing steps, to consumption of cowpea.

However, there are many studies investigating the effect of traditional process steps on legumes, the number of studies with cowpea, which compares the effects of

different process steps, is rather limited. In addition, no studies have been reported on simulation of *in vitro* gastrointestinal (GI) digestion of cowpea.

Within this context, the aim of this research was:

I. To determine free and bound phenolic content and antioxidant capacities of different cowpea varieties;

II. To examine the influence of traditional process steps on total phenolic, flavonoid content, major phenolic compounds, and antioxidant capacity as well as trypsin inhibitor activity of cowpea;

III. To evaluate the influence of traditional process steps on total phenolic, flavonoid content, major phenolic compounds, antioxidant capacity of digested cowpea and protein digestibility, using simulated *in vitro* models.

2. LITERATURE STUDY

2.1 Legumes

The term legume subsumes more than 13,000 different species, belong to the family Leguminosae. Among flowering plants, the Leguminosae is the third largest family (after Compositae and Orchidaceae) in size, and in economic importance second following the family of the grasses (Gramineae) (Sathe, 1996). Legumes are a substantial food group in the diets of humans around the world. Of the thousands of species, however, only a few are widely grown for commercial purposes: soybeans, dry beans, peas, cowpeas, broad beans, chickpeas, and lentils. The botanical and common names for the different legumes are summarized in Figure 1. Soybean is by far the most widely produced. The soybean is undoubtedly the world's most valuable crop, used as feed by billions of livestock, as a source of dietary protein and especially its oil is used by millions of people and in the industrial manufacture of thousands of products. Because of this popularity, soybean is usually kept separate from the others (Sathe, 1996; Sparvoli *et al*., 2015). In the following, soybeans will not be used further to compare the composition of cowpeas with other legumes, as soybeans are very extensively studied and thus well-known.

Legumes are planted and harvested mainly for their mature or immature seeds, are known as a source of dietary protein and carbohydrates. Although it is accepted in India where religion or local customs prevent consumption of meat or dairy products, consumption of legumes is commonly associated with poverty in many parts of the world, especially in the tropics. Although most legume seeds contain an optimum amount of sulphur-containing amino acids, meat and fish are more commonly consumed as a protein sources, despite the very many health-promoting properties of properly cooked legumes (Sathe, 1996).

Scientists have started to focus on different food sources because of several reasons: inverse proportion between the number of people and the amount of food; inadequacy and expensive animal protein; the limited land areas which can be used

for production of food crops while farming systems are changing towards specialized cereal and oilseed production; deliberate reduction in red meat consumption for health reasons (Sparvoli *et al*., 2015).

Despite the high nutritional value of legumes, lentil, bean, and chickpea species are more known and consumed, while cowpea is not prevalent and common especially in developing and developed countries.

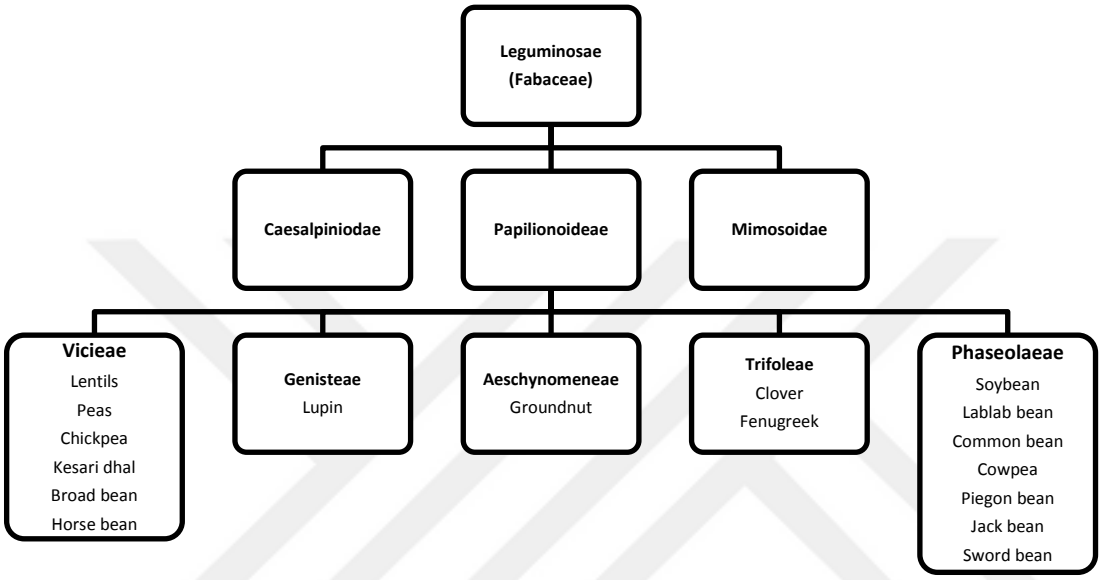


Figure 2. 1 : Botanical classification of food legumes (Sathe, 1996).

2.2 Cowpea

Cowpea, *Vigna unguiculata* (L.) Walpers, belongs to the family Leguminosae, subfamily Papilionoideae and formerly carried the name *Vigna sinensis* L. (Bouker et al. 2015; Phillips, 2013; Sathe, 1996) Seeds of cowpea vary in appearance from white to red to black to mottled, and the seed coat from thick and loose to thick and tightly adhering to thin, wrinkled and tightly adhering (Phillips, 2013).

Cowpea is one of the most important pulse legumes in sub-Saharan Africa, from where it originated, but it is also of importance in Asia and certain parts of the Americas (Phillips, 2013). Cowpea is grown in the tropical lowlands, especially in dry areas, but also in warm temperate regions. For 2013, the total area under cultivation and total world production was 11.32 million hectares and 5.72 million metric tons, respectively (FAO 2013). According to FAO (2013), the top 5 producers

of cowpea are: Nigeria (2.5 million tons), Niger (1.3 million tons), Burkino Faso (0.58 million tons), Tanzania (0.18 million tons), and Myanmar (0.17 million tons).

Cowpea seed is composed of (g/100g fresh weight): moisture (11.95); protein (16-36); fat (1-1.3); carbohydrate (56-68) and ash (2-3.5) (Bouker *et al.* 2015; USDA, 2016). Evans and Boulter (1974) studied different cowpea varieties and reported a protein content ranged between 21 to 34 per 100g fresh weight, while Nielsen et al. (1993) reported a similar protein content ranging from 22.9 to 32.9 per 100g fresh weight.

In general, legumes contain high amounts of protein (Table 2.1). The highest protein contents are observed in lentil and cowpea varieties (Table 2.1). But when compared to the two types of high-protein content legumes, lentils are more popular both in terms of not only production but also consumption across the globe. Therefore, the number of studies on lentils is quite high while the studies on cowpea and the awareness of people about it are insufficient.

Table 2. 1 : Proximate composition of legumes (Sparvoli et al., 2015; Bouker *et al.* 2015; USDA, 2016).

Species	Protein	Fat	Ash	Carbohydrates
Cowpea	25-36	1-1.3	2-3.5	56-68
Lentil	20.6-32	1-2.1	2.5-3.4	54-58
Pea	21.9-31	1.3-3	2-3.3	52-62
Chickpea	16-28	3.1-7	2-3.5	54-66
Common bean	20.9-30.1	1.3-2.5	1.8-3.2	54-64
Faba bean	24.3-32.3	1.1-4	2.2-2.8	57-60
Pigeon bean	15.9-24.1	1.2-1.6	4-4.5	57-58
Mung bean	23.3-27.7	0.7-2.4	3.2-4	61-62

When the mineral contents of cowpeas are compared with those of other legumes (Table 2.2), cowpea have the highest calcium and potassium content (110 and 1112 mg/100g fw, respectively) after beans. Besides, iron, magnesium, phosphorus, and sodium content of cowpea (8.27, 184, 424 and 16 mg/100g fw, respectively) are quite higher than for the others (Table 2.2). However, it can be mentioned that legumes are not main source in terms of vitamin content (Table 2.3).

Table 2. 2 : Mineral content of different legumes (mg per 100g fresh weight) (USDA, 2016).

Minerals	Species					
	Cowpea	*Lentil*	*Pea*	*Chickpea*	*Black Bean*	*Navy Bean*
Calcium, Ca	110	35	25	57	123	147
Iron, Fe	8.27	6.51	1.47	4.31	5.2	5.49
Magnesium, Mg	184	47	33	79	171	175
Phosphorus, P	424	281	108	252	352	407
Potassium, K	1112	677	244	718	1483	1185
Sodium, Na	16	6	5	24	5	5
Zinc, Zn	3.37	3.27	1.24	2.76	3.65	3.65

Table 2. 3 : Vitamins content of different legumes (per 100g fresh weight) (USDA, 2016).

Vitamins	Unit	Species					
		Cowpea	*Lentil*	*Pea*	*Chickpea*	*Black Bean*	*Navy Bean*
Vitamin C, Total ascorbic acid	mg	1.5	4.5	40	4.0	ND	ND
Thiamin	mg	0.85	0.87	0.27	0.48	0.90	0.78
Riboflavin	mg	0.23	0.21	0.13	0.21	0.19	0.16
Niacin	mg	2.08	2.23	2.09	1.98	1.96	2.19
Vitamin B-6	mg	0.36	0.54	0.17	0.54	0.29	0.43
Folate, DFE	µg	633	479	65	557	444	364
Vitamin A, RAE	µg	3	2	38	3	ND	ND
Vitamin A, IU	IU	50	39	765	67	17	ND
Vitamin E (alpha-tocopherol)	mg	0.39	0.49	0.13	0.82	0.21	0.02
Vitamin K (phylloquinone)	µg	5.0	5.0	24.08	9.0	5.6	2.5

2.1.1 Phenolic compounds of cowpea

Phenolic compounds are produced by plants as secondary metabolic products to protect plants against unfavorable environmental conditions such as stress, injury. Phenolic compounds play an important role in the protection against oxidative damage caused by free radicals, radical scavengers, reducing agents, potential complexes of pro-oxidant metals and quenchers of singlet oxygen formation. They have also antimutagenic activity (Dueñas *et al*., 2005; Doblado *et al*., 2007; Xu et al., 2007). Those bioactive compounds can prevent the development of various

diseases, like atherosclerosis, cancer, diabetes, aging and cardiovascular diseases (Dueñas *et al*., 2005; Naczk & Shadihi, 2014). Phenolic compounds are mostly volatile, as found for phenolic acids (or phenolcarboxylic acids), flavonoids and simple phenols in plants (Naczk & Shadihi, 2014).

A phenolic compound consists of a hydroxyl group (-OH) bonded directly to an aromatic hydrocarbon group. Figure 2 shows the simplest structure of the phenolic compounds, carbolic acid (C_6H_5OH). Although the basic skeleton remains the same, their variation depends on the numbers and positions of the hydroxyl groups on the aromatic ring (Rappoport, 2003). Based on these substitutions several classes of phenolic compounds are defined, of which phenolic acids and flavonoids representing only two classes of the phenolic compounds.

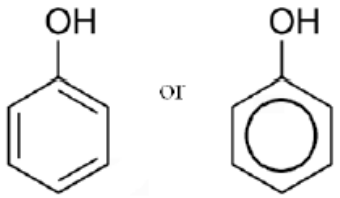


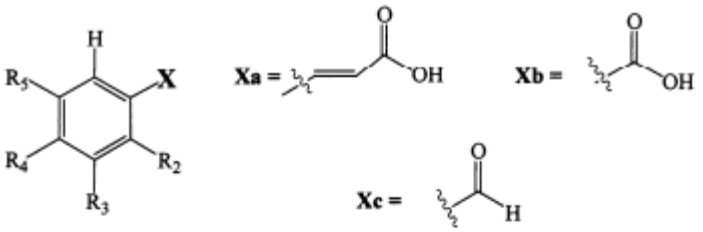
Figure 2. 2 : The simplest phenol structure (Robbins 2003).

2.1.1.1 Phenolic acids

Phenolic acids can mainly be divided into two classes: derivatives of benzoic acid and derivatives of cinnamic acid (Robbins, 2003). The structures of the major naturally occurring phenolic acids are shown in the Table 4. Most common type of hydroxybenzoic acids are gallic, *p*-hydroxybenzoic, protocatechuic, vanillic and syringic acids having C6–C1 structure; while, hydroxycinnamic acids are aromatic compounds with a three-carbon side chain (C6–C3), with caffeic, ferulic, *p*-coumaric and sinapic acids as the most important representative compounds (Balasundram *et al*., 2006).

Legumes, especially dark varieties like red common bean, black gram, black eyed peas, have a high amount of phenolic acids. The total phenolic content (TPC) of legumes ranged from 0.57 to 7.53 mg GAE/g fw; the highest quantity of total phenolic content was reported in seeds of lentil and black bean (Table 2.5) (Amarowicz & Pegg, 2008; Han *et al*., 2008; Sparvoli *et al*., 2015).

Table 2. 4 : Structures of the prominent naturally occurring phenolic acids (Robbins, 2003).



R_2	R_3	R_4	R_5	X	code	common name
H	H	H	H	a	1	cinnamic acid
–OH	H	H	H	a	2	*o*–coumaric acid
H	H	–OH	H	a	3	*p*–coumaric acid
H	–OH	H	H	a	4	*m*–coumaric acid
H	$–OCH_3$	–OH	H	a	5	ferulic acid
H	$–OCH_3$	–OH	$–OCH_3$	a	6	sinapic acid
H	–OH	–OH	H	a	7	caffeic acid
H	H	H	H	b	8	benzoic acid
–OH	H	H	H	b	9	salicylic acid
H	H	–OH	H	b	10	*p*–hydroxybenzoic acid
H	$–OCH_3$	–OH	H	b	11	vanillic acid
H	$–OCH_3$	–OH	$–OCH_3$	b	12	syringic acid
H	–OH	–OH	H	b	13	protocatechuic acid
–OH	H	H	–OH	b	14	gentisic acid
–OH	–OH	–OH	–OH	b	15	gallic acid
H	$–OCH_3$	$–OCH_3$	H	b	16	veratric acid
H	$–OCH_3$	–OH	$–OCH_3$	c	17	syringealdehyde
H	$–OCH_3$	–OH	H	c	18	vanillin

Oboh (2006) reported that the total phenolic content of cowpea ranges from 0.3 mg GAE/g fw in a white cultivar to 1.0 mg GAE/g fw. in a brown variety, and likewise Gutierrez-Uribe *et al*., (2011) found a total phenolic content of 0.75 mg GAE/g dw. When compared with Kalogeropoulos *et al*. (2010), they found a lower total phenolic content for cowpea (0.15 mg GAE/ g fw) while Sreerama *et al*., (2012) reported higher amounts (12.16 mg GAE/g dw).

However, Siddhuraju and Becker (2007) found different TPC values for light brown variety content (16.4, 13.3 g tannic acid equivalents per 100 g dry weight basis) and for dark brown variety (13.3 g tannic acid equivalents per 100 g dry weight basis) for cowpea.

Legumes contain phenolic acids including gallic, protocatechuic, p-hydroxybenzoic, vanilic, coumaric, ferulic, sinapic, gentisic and syringic asids (Amarowicz & Pegg, 2008; Han *et al*., 2008; Sparvoli *et al*., 2015); although the amount of these individual phenolic compounds may change depending on the variety of the legumes. p-Hydroxybenzoic acid is most commonly available in legumes followed by coumaric, procatechuic and ferulic acid (Magalhaes *et al*, 2017; Amarowicz & Pegg,

2008). Gallic acid is the major phenolic acid in pea, and chickpea (148, 137 mg/100g fw, respectively) while coumaric acid is the major one in lentils (322-342 mg/100g dw) and trans-ferulic acid is dominant in bean (342-366 mg/100g dw). Those are followed by trans-p-coumaric acid (37.7-41.5mg/100g dw), protocatechuic acid (32 mg/100 g fw) in pea; p-hydroxybenzoic acid (93.6-100 mg/100g dw) and protocatechuic acid (49.9-52.3 mg/100g dw) in lentil; gallic acid (153 mg/100g fw), protocatechuic and p-hydroxybenzoic acid (32.8-41.4, 32.3-36.1 mg/100g dw, respectively) in bean; trans-ferulic acid (131 mg/100g fw) in chickpea (Table 2.6).

Protocatechuic acid is a major phenolic acid in cowpea, present in esterified forms. Depending on the variety of cowpea the amount of protocatechuic acid ranges between 9,3 – 92,7 mg/100g of flour. The other important phenolic acids are p-hydroxybenzoic acid, caffeic acid, p-coumaric acid, ferulic acid, 2,4-dimethoxybenzoic acid, and cinnamic acid. These phenolic acids are mostly distributed in their free acid forms at levels lower than 7 mg/100 g of flour. Total phenolic contents were different among the varieties ranging from 34.6 to 376.6 mg/100 g of flour (Cai *et al*., 2003).

2.1.1.2 Flavonoids

Flavonoids, as one of the predominant classes among the phenolic compounds, possess significant antioxidant activity (Xu *et al*., 2007). Flavonoids represent the largest group of plant phenolic compounds, including more than 50% of the eight thousand naturally occurring phenolic compounds. Flavonoids are low molecular weight compounds, consisting of fifteen carbon atoms, arranged in a C6–C3–C6 configuration. Basically, the structure is made up of two aromatic rings A and B, joined by a 3-carbon bridge, usually in the form of a heterocyclic ring, C (Figure 2.3). Depending on replacement patterns to ring C, variation results in the major flavonoid classes i.e. flavonols, flavones, flavanones, flavanols, isoflavones, flavanonols, and anthocyanidins (Figure 2.4) of which flavones and flavonols are the most widely occurring and structurally diverse (Heim *et al*, 2002; Balasundram *et al*., 2006).

The content of total flavonoids in seeds from legumes, cowpea, lentil, navy bean, black bean, and chickpea ranged from 0.09 to 4.54 mg CAE/g fw; the highest quantity of total flavonoids was determined in seeds of lentil and black bean (Table 2.5).

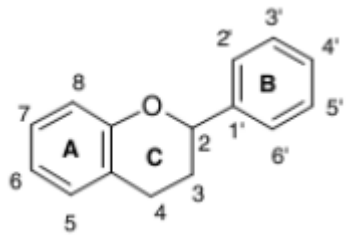


Figure 2. 3 : Structure of a flavonoid molecule (Balasundram *et al*., 2006).

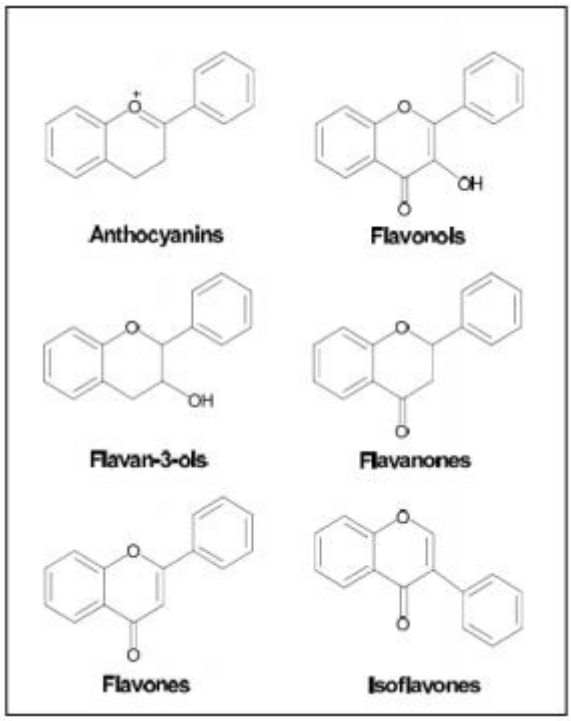


Figure 2. 4 : Major classes of flavonoids (Shahidi & Nazck, 2004).

Ojwang *et al*. (2012) found that red varieties of cowpea had the highest flavanol content (0.88- 1.06 mg CAE/g fw), whereas green and white varieties of it had the lowest (0.27-0.35 mg CAE/g fw). Sparvoli *et al.* (2015) measured a total flavonoid content (TFC) of cowpea 0.27-2.09 mg CAE/g fw while Gutierrez-Uribe *et al*. (2011) reported that total flavonoid content of cowpea is 0.98 mg quercetin equivalents per g dry weight basis.

Flavonoids in foods exist primarily as 3-*O*-glycosides and polymers. Flavonoids generally present in legumes belong to flavanols, flavan-3-ols, flavones and anthocyanidin (Carbonaro, 2011); quercetin, catechin, kaempferol, myricetin and luteolin and their derivate are commonly found. Quercetin 3-O-galactoside,

kaempferol and catechin are forms, most dominantly present in pea; luteolin glucoside, quercetin glucoside and catechin in lentil; catechin, myricetin-3-O-rhamnoside and quercetin 3-O- in bean; myricetin-3-O-rhamnoside, quercetin 3-O-galactoside, quercetin-3-O-rhamnoside in chickpea (Table 2.7) (Amarowicz *et al*., 2008; Amarowicz & Pegg, 2008; Cai *et al.*, 2003; Lopez-Amaroz *et al.*, 2006; Lopez et al., 2016; Magalhaes *et al*., 2017; Parmar *et al*., 2016).

The major flavonoids in cowpea are myricetin 3-O-glucoside, quercetin 3-O-galactoside, quercetin diglycoside, quercetin 3-O-glucoside (Dueñas *et al*., 2005; Amarowicz & Pegg, 2008). On the other hand, Ojwang *et al*. (2012) reported that mono-, di-, and tri(acyl)glycosides of quercetin were predominant in most varieties of cowpea; myricetin and kaempferol glycosides were present only in specific varieties and only black varieties had anthocyanins, predominantly delphinidin and cyanidin 3-O-glucosides (Table 2.7).

Table 2. 5 : Phenolic contents in seeds of different legumes (Amarowicz & Pegg, 2008; Han *et al*., 2008; Kalogeropoulos *et al*., 2010; Sparvoli *et al*., 2015).

Material	TPC (mg GAE/g fw)	TFC (mg CAE/g fw)
Cowpea	0.15-1.0	0.27-2.09
Pea	0.86-1.14	0.09-0.17
Chickpea	0.98	0.72
Lentil	8.86-9.6	3.04-4.54
Navy bean	0.57	0.92
Black bean	3.37-6.99	2.51-3.3

GAE, gallic acid equivalents; CAE, catechin equivalents.

Table 2. 6 : Content of phenolic acids in legume seeds.

Compounds	Species									
	Cowpea		Pea		Lentil		Bean		Chickpea	
Gallic	0.016 [5]	mg/100g fw	148[1]	mg/100g fw	ND[5]		153[1]	mg/100g fw	137[1]	mg/100g
Protocatechuic	9.3–92.7[6]	mg/100g fw	32[1]	mg/100g fw	49.9–52.3[5]	mg/100 g dw	32.8–41.4[5]	mg/100 g dw	61[1]	mg/100g
p-Hydroxybenzoic	4.49[6]	mg/100g fw	0.05[3]	mg/100g dw	93.6–100[5]	mg/100 g dw	32.3–36.1[5]	mg/100 g dw	3.51[7]	mg/100g dw
Vanillic	2.51[6]	mg/100g fw	0.021-0.067[3]	mg/100g dw	13.3–15.3[5]	mg/100 g dw	90.9[3]	µg/100g dw	64[1]	mg/100g fw
trans-p-Coumaric	0.86[6]	mg/100g fw	37.7-41.5[3]	mg/100g dw	322–342[5]	mg/100 g dw	19.00[1]	mg/100g fw	5.00[1]	mg/100g fw
trans-Ferulic	1.6[6]	mg/100g fw	1.82b / 9.1[3]	mg/100g dw	20.9–25.7[5]	mg/100 g dw	342–366[5]	mg/100 g dw	131[1]	mg/100g fw
cis-Ferulic	1.24[6]	mg/100g fw					74.1-79.1[3]	µg/100g dw		
trans-Feruloylaldaric	4.01[6]	mg/100g fw								
trans-p-Coumaroyaldaric	3.66[6]	mg/100g fw								
caffeic acid	<7 [6]	mg/100g fw								
2,4-dimethoxybenzoic acid	<7 [6]	mg/100g fw								
p-hydroxybenzoic acid	<7 [6]	mg/100g fw								
cinnamic acid	<7 [6]	mg/100g fw								
Sinapic			8.00[1]	mg/100g fw			9.00[1]	mg/100g fw	2.00[1]	mg/100g fw
Gentisic acid									2.6[7]	mg/100g dw
Syringic acid									4.59[7]	mg/100g dw

1: (Parmar *et al.*, 2016); 2: (Amarowicz *et al*., 2008); 3: (Lopez-Amaroz *et al*., 2006); 4: (Lopez *et al.*, 2016); 5: (Amarowicz & Pegg, 2008); 6: (Cai *et al*., 2003); 7: (Magalhaes *et al*., 2017).

Table 2. 7 : Content of flavonoids in legume seeds.

Compounds	**Species**									
	Cowpea		**Peas**		**Lentil**		**Bean**		**Chickpea**	
Quercetin 3-O-galactoside	364[5]	mg/100g	14[5]	mg/100g extract			643.18[8]	mg/100g	0.72[7]	mg/100g dw
Quercetin 3-O-glucoside	114.5[5]	mg/100g			0.14[4]	mg/100g	ND[9]			
Quercetin rutinoside							ND[9]			
Quercetin arabinoglucoside							ND[9]			
Quercetin diglycoside	180[5]	mg/100g								
Quercetin-3-O-rhamnoside									0.5[7]	mg/100g dw
Myricetin 3-O-glucoside	964[5]	mg/100g					115.72[8]	mg/100g		
Myricetin-3-O-rhamnoside							212[2]	µg/g extract	0.74[7]	mg/100g dw
Kaempferol			51[5]	mg/100g extract	0.05[4]	mg/100g	1.06[9]	µg/g dw		
(+)-Catechin			39[1]	mg/100g	0.1–0.3[5]	mg/100g dw	123[1]	mg/100g fw		
Luteolin-8-C-glucosid					2.26[4]	mg/100g			0.12[7]	mg/100g dw

1: (Parmar *et al*., 2016); 2: (Amarowicz *et al*., 2008); 3: (Lopez-Amaroz *et al*., 2006); 4: Lopez A. et al., 2016; 5: Amarowicz & Pegg (2008); 6: (Cai *et al.*, 2003); 7: (Magalhaes *et al*., 2017) 8: (Sibul *et al.*, 2015); 9: (Duenas *et al*., 2015).

2.1.2 Antioxidant capacity

Besides the positive effects that developing technology has on our lives, there are also some negative effects. People are exposed to the health risk associated with oxidative damage caused by reactive oxygen species. These reactive oxygen species are resulting from automobile exhaust fumes, industrial plants, waste incinerations, and cigarette smoking, to indirect environment consequences, such as UV-light exposure through a depleted ozone layer. Oxidation of several biological substances can lead to many diseases such as AIDS, Alzheimer's disease, atherosclerosis, cancer, cataracts, diabetes, inflammation, liver disease, and Parkinson's disease, as well as aging (Karadag *et al*., 2009; Rebollo *et al.*, 2014; Shibamoto, 2013; Sparvoli *et al*., 2015).

Antioxidants are naturally found in plants as vitamins and phenolic compounds (Shibamoto, 2013). The high phenolic contents correlate with the highest antioxidant activities, as assessed with different evaluation methods. It is known that phenolic compounds in plants protect them against active oxygen-induced and free radical mediated oxidation of biological molecules. Therefore, a great interest is present in the quantification of antioxidants and the determination of antioxidant capacities of several specific food compounds that might help to prevent the diseases (Shibamoto, 2013; Sparvoli *et al*., 2015).

Lots of different kind of methods have been developed and used among scientists to measure antioxidant capacity, nevertheless, using only one of these methods cannot be accurately showing the mechanism of action of radical sources or antioxidants in a complicated system. The antioxidant capacity methods have been divided into two classes; hydrogen atom transfer reaction (HAT) and electron transfer reaction (ET) methods. Spectrophotometrically methods measuring a product formed by oxidation, associated with electron or radical scavenging, are most commonly used due to their simplicity and fast reaction times. Prevalent assays of them are DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay, ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6 sulfonic acid)) radical cation scavenging activity and FRAP (ferric reducing/antioxidant power) (Karadag *et al*., 2009; Shibamoto, 2009).

In the determination of the antioxidant activity of food components, DPPH assay is the most commonly and widely used method. DPPH readily forms a stable radical

(DPPH•), which accepts hydrogen from an antioxidant. The disappearance of DPPH•, which is proportional to the antioxidant effect, is monitored by a spectrophotometer at 517 nm to determine antioxidant activity (Shibamoto 2009). ABTS, the other significant assay, is based on the theory that decolorization of the ABTS radical cation is caused by the action of antioxidants. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) usually is used as antioxidant standard, and the results of both assays are expressed in terms of Trolox equivalent antioxidant capacity (TEAC). While for another assay i.e. FRAP (the ferric reducing/antioxidant power) concerning oxidation of ferrous to ferric ions in an organometallic complex, iron (II) sulfate is used as standard. The theory of the assay is that color changes of a solution occur when ferrous ions are oxidized (Shibamoto, 2009).

In literature, DPHH, ABTS and FRAP are frequently used for the determination of the antioxidant capacity of legumes. Marathe *et al.* (2011) studied different legumes varieties and has found varying antioxidant capacities values depending on seeds color (Table 2.8). Furthermore, DPPH results are sorted in a way that cowpea (brown) have the highest value, followed by common bean (red), common bean (black), common bean (brown) and cowpea (red). Additionally, Han *et al.* (2008) compared antioxidant capacities of different legumes. They found using the ABTS assay that the results were highest in lentils at 14 μmol TEAC/g fw and lowest in green peas at 1.9 μmol TEAC/g fw. Sparvoli *et al.* (2015) summarized antioxidant capacities of different legumes. Lentil and black bean have had highest DPPH and FRAP values while yellow and green pea have lowest.

Zia-Ul-Haq, (2013) measured antioxidant capacity of cowpea varieties (DPPH value 25.1-27.9 μmol TEAC/g fw, FRAP value 13.2-15.5 mmol Fe (II)/g fw) while Marathe *et al*., (2011) found different FRAP (14.26-68.03 μmol Fe(II)/g fw) and ABTS (9.34-46.26 μmol TEAC/g fw) values of cowpea depending on their color.

2.1.3 Bioaccessibility

In addition to the phenolic content, one of the fundamental issues concerning the beneficial effects of polyphenols is their bioavailability. Bioavailability is defined as the absorption of nutrients and bioactive compounds in the food after ingestion of the food, reaching the cells and used or stored for normal metabolic and physiological functions

(El, 2016). The Food and Drug Administration also defines bioavailability as 'the rate and the extent to which the therapeutic moiety is absorbed and becomes available to the site of drug action' (Holst & Williamson, 2008). Another term used is bioaccessibility, which is defined as the amount of food compounds which can come out of the food matrix and ready for absorption in the small intestine, after the food is digested (El, 2016).

Table 2. 8 : Phenolic contents in seeds of different legumes.

Material	DPPH (µmol TEAC/g fw)	FRAP (µmol Fe (II)/g fw)	ORAC (µmol TEAC/g fw)	ABTS (µmol TEAC/g fw)
Cowpea	25.1-27.9 [3]	14.26 -68.03[4]	83.8-86.7 [3]	11.26-68.03 [4]
Pea	0.57-2.65 [1]	5.81-9.0 [1,4]	3.26-12.8 [1]	1.8 [2]-6.18 [4]
Chickpea	1.26 [1]	4.12-8 [1,4]	9.26 [1]	2.2 [2]-6.56 [4]
Lentil	19.07-19.87 [1]	9.6 [4] -87.5 [1]	59.55-95.19 [1]	6.47 [4]-14.8 [2,4]
Navy bean	1.48 [1]	12.7 [1]	13.3 [1]	6.03 [4]
Black bean	14.49-18.95 [1]	30.9[1]-60.5[4]	48.91-92.73 [1]	8.32 [4]

DPPH, 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay; FRAP, ferric reducing antioxidant power; ORAC, oxygen radical absorbance capacity; ABTS; radical cation scavenging activity

1: (Sparvoli *et al.*, 2015) 2: (Han *et al.*, 2008) 3: (Zia-Ul-Haq, 2013) 4: (Marathe *et al*., 2011)

Bioactive compounds obtained from the diet, must be biologically accessible to show the potential health benefits. The compounds are extracted following a gastrointestinal digestion (GI). Bioaccessibility specifically refers to the amount of antioxidants which are potentially presented to the intestine for absorption. In vivo studies in human and animals are not chosen in a first step because of the ethical limitations, complicated trials, and costliness reasons. Despite that, the in vitro digestion models are well correlated with bioaccessibility results which are obtained from human and animal studies, also they are more rapid and easy methods to evaluate phytochemical stability. The in vitro digestion model simulates the stomach and mimickes the intestinal digestion using pepsin, pancreatin and bile salts (El, 2016; Holst & Williamson, 2008).

To our knowledge, no study exists determining in vitro bioaccessibility phenolic compounds and content of cowpea variety using the in vitro gastrointestinal digestion (GI).

2.1.4 Antinutritional compounds

Although legume seeds contain high amount of proteins, calories, certain minerals and vitamins, their use in food is still limited by the presence of several antinutritional factors (ANFs). In the active form, these compounds are toxic or

deleterious for the growth of most animal species (Carbonaro, 2011). These include antivitamins, allergens, cyanogenic glycosides, flatulence factors, goitrogens, hydrogen cyanide, lectins, lysinoalanine, oligosaccharides, phytate, protease inhibitors, saponins, trypsin inhibitors, tannins, phytic acid, α-amylase and flatulence-causing oligosaccharides and several high-molecular- weight phenolic compounds (Ercan *et al*., 2016; Urbano *et al*., 2000; Gupta, 1987; Khattab, 2009). However, the concentration and level of these antinutrients might vary between legumes and among varieties of the same legume source depending on the location of collection, stage of development, and availability (Bhat and Karim 2009).

Nevertheless, it should be considered that some of the commonly considered antinutrient compounds like phenols and tannins are known as potential antioxidants with health promoting effects (Bhat and Karim 2009).

Elimination or reduction of antinutritional factors can be achieved by classical breeding, molecular biology techniques and by several technological treatments, often used in combination. Most commonly used post-harvesting treatments are; dry and moist thermal treatments, extrusion cooking, steaming, soaking, germination, fermentation, dehulling, enzymatic treatment (Carbonaro, 2011).

One of the most important antinutritional factor in legumes is trypsin inhibitors. Some more attentions to this antinutritional compound are given below.

2.1.4.1 Trypsin Inhibitor Activity

Trypsin is a serine protease from PA clan superfamily, found in the digestive system of many vertebrates, where it hydrolyses proteins. Trypsin is formed in the small intestine when its proenzyme form, the trypsinogen produced by the pancreas, is activated. The enzyme cleaves peptide chains mainly at the carboxyl side of the amino acids. It has many biotechnological processes, generally referred to as trypsin proteolysis or trypsinisation. In the duodenum, trypsin catalyzes the hydrolysis of peptide bonds, breaking down proteins into smaller peptides. Tryptic digestion is very important for the digestion and absorption of proteins (Rawlings *et al*., 1994; Polgár, 2005).

Protease inhibitors form stable complexes with digestive enzymes and inhibit their activity. The presence of protease inhibitors in food decreases the known nutritional quality of proteins in the diet disturbing the ability of body digestive enzymes to

degrade dietary protein, and therefore limiting the intake of amino acids needed to construct new proteins. The best examples are trypsin inhibitors found in legumes (Sreerama *et al.*, 2012).

Trypsin inhibitor adversely affects the enzymes having a role in protein digestion; inhibit trypsin activity, induce hypersecretion of pancreatic enzymes, stimulate pancreatic hypertrophy and in the end, reduce digestion and absorption of dietary protein. These negative modifications in digestive functions result in lower retention of nitrogen and sulphur (Sreerama et al., 2012; Pisulewska et al., 2000).

However, in certain conditions the effects of inhibitors on protein digestion might be advantageous, for instance by improving the intact absorption of some therapeutic proteins, such as orally delivered insulin. Moreover, the control of protease activity is considered to play a critical role in a wide range of biological processes and malfunctioning related to cancer progression.

On the other hand, the positive or negative effect of the enzyme inhibitor depends on its content in the different varieties of cowpea and on the dose and frequency of consumption (Sreerama et al., 2012).

2.3 Processing Steps

For centuries, people have developed a wide range of processing techniques including cooking, germination, soaking, dehulling and fermentation, to produce food for consumption, to provide product diversity, and to increase nutritional value (Azarpazhooh & Boye, 2013). The food processing is needed before ingestion to increase the bioavailability of nutrients by means of inactivating antinutritional factors, in addition to improve flavor and deliciousness (Lopez, 2016; Carbonora, 2011). As with most food types, legumes are generally ready to be consumed after they have been passed through various processing steps. Effects of pretreatments on nutrient composition of legumes have been considerably studied in recent years, resulting in different outcomes.

It is described that there are over more than 20 different foods, made from whole seeds, flours, pastes etc., using cowpea across the regions. The recipes apply series of processes, many of them use more than one. Among the most popular cowpea-based foods in West Africa, is *akara* (fried cowpea paste). In India, cowpea is integrated

with traditional foods such as prapad, an extruded, deep-fat fried dough, idhli, and dhosal. In Brazil, a fried fritter, like *akara* and called *acaraje*, is produced in the northeastern part of the country. In the United States, cowpea is consumed as dry, canned, or frozen seed and utilization of cowpea is generally as cooked whole seeds. Cowpeas are commonly consumed as part of meals with a variety of vegetables including sweet corn, tomatoes, okra, and often with baked cornbread or fried corn cakes (Phillips, 2013).

Below, as well as in Table 2.9, an overview is given of the effect of different processing steps on the impact on protein content and protein digestibility, phenolic compounds and antinutritional factors. It must be mentioned that a comparison over different studies is very difficult. Most of the time, units are quite different to express the antioxidant capacity or the activity of the antinutritional enzymes among studies. Besides results for the different parameters are expressed in per 100g, not always defining if this is per 100g dry matter, or fresh weight. Recalculation to per 100g dry matter is not always possible as in many studies the dry matter content was not given. This makes the overview of different studies not clear, and does not allow us to make general conclusions on the impact of the processing steps on different nutritional important parameters.

2.3.1 Soaking Process

Soaking of legumes is an important part of processing operations. For instances, this process also contributes to increase the nutritional profile of legumes. Process of soaking before cooking aids to reduce cooking time and increases the moisture content of seeds, allowing chemical reactions such as starch gelatinization and protein denaturation during cooking. Also, the water absorption causes seeds to become softer and more uniform in texture (Azarpozhooh and Boye, 2013).

Chemical Composition

The soaking process can cause changes in the chemical composition of legume seeds. Khalil (2001) reported a decrease after soaking process for beans as well as Elmaki et al. (1999) found a slight decrease in protein content expressed on dry weight basis for sorghum samples. Though, Rehman & Shah (2005) found that the protein content of legumes, expressed on dry weight basis, including lentils, chick peas, red kidney beans, white kidney beans and black grams was not affected by soaking. Ekpenyong

and Borchers (1980) investigated the effect on the chemical composition and digestibility of winged beans and found that there was an increase in protein content, total carbohydrates, and digestibility in the samples after soaking. Similarly, Edijala (1980) studied the effects of soaking on protein content of cowpea varieties and found small increases in protein contents for cowpea varieties.

Phenolic Compounds

The soaking process also affects the phenolic compounds in legumes. Boateng *et al.* (2007) found a significant reduction in the TPC of pinto beans after soaking but this difference was not observed for kidney beans. However, looking to the average TPC on a range of different legumes, it could be observed that there was a decrease in general. Luthria and Pastor-Corrales (2006) reported that only 2% of the total phenolic compounds are lost in the soaking water, while 83% remain in beans and 15% are probably lost during cooking. On the contrary, there were no significant changes measured in TPC and antioxidant capacity in soaked navy and pinto beans by Anton *et al.* (2008). However, Adb El-Hady and Habiba (2003) suggested that there was a decrease in TPC because of leaching of water soluble phenolic compounds into the soaking water.

Also, Ranilla *et al.* (2009) reported a relationship between the reduction of phenolic compounds and reduced antioxidant activity in soaked beans. Interestingly, Boateng *et al*. (2007) measured DPPH and FRAP values of soaked beans; FRAP values increased with the soaking process while DPPH values decreased.

Protein Digestibility

Moreover, in vitro protein digestibility increases with soaking process in legumes (Azarpozhooh and Boye, 2013). Alonso *et al.* (2000) measured in vitro protein digestibility of soaked beans (71.4%) which was clearly higher than of raw samples (68.1%). Also, Rehman and Shah (2001) reported similar results.

Antinutritional Compounds

Like cooking, soaking process may also reduce the content of some antinutrients. Sathe and Salunkhe (1981) suggested that trypsin inhibitor activity of winged beans was reduced by soaking. Onwuka (2006) reported that soaking black cowpeas for 18 hours reduced trypsin inhibitors compared to raw sample. Also, Ogun *et al.* (1989) found similar results with cowpea.

2.3.2 Dehulling process

Dehulling is the removal of the seed coat of legumes prior to their processing and consumption, to improve their cooking and nutritional properties. It also helps to remove antinutritional compounds which are located in the seed coat. Loosening the hulls is traditionally achieved either by wet or dry methods during dehulling (Sreerama *et al*., 2009).

Chemical Composition

Decortication can affect the values of proximate composition. Ghavidel and Prakash (2007) measured that dehulled bean, cowpea, lentil, and chickpea had higher protein and fat content than raw samples like Alonso *et al.* (2000). Also, Edijala (1980) and Aroba *et al*. (1996) found higher values in dehulled cowpea varieties.

Phenolic Compounds

Sosulski and Dabrowski (1984) suggested that the dehulling greatly reduced TPC of seeds for pigeon pea, faba bean, mung bean, and lentil, while it had only a slight effect on TPC of samples from field pea, navy bean, and chickpea. Additionally, Towo *et al*. (2003) measured lower TPC values of cowpea, mung bean and kidney bean after dehulling.

Protein Digestibility

Dehulled seeds generally have higher *in vitro* protein digestibility by 3–4 % (Deshpande et al. 1982; Ghavidel & Prakash, 2007). Alonso *et al.* (2000) measured *in vitro* protein digestibility of dehulled beans (71.6%) which was higher than in raw samples (68.1%).

Antinutritional Compounds

In contrast to the other processes, dehulling significantly increased the trypsin and α-amylase inhibitory activities in small white, dark red kidney and light red kidney beans (Deshpande et al. 1982). Attia *et al.* (1994) also reported that dehulling did not significantly reduce trypsin inhibitor activity in chickpea, percentage losses in trypsin inhibitor activity were in the range of 53.6–59.9%.

2.3.3 Cooking process

Cooking is one of the most common and ancient processes to prepare food for consumption, brings about several changes in physical characteristics and chemical compositions of legumes. The most known types of cooking are boiling, pressure boiling, steaming, and high pressure cooking technology which may provide high quality of products (Xu & Chang, 2008).

Chemical Composition

Attia *et al*. (1994) found a noteworthy increase in reducing sugars, crude protein, starch contents and in vitro digestibility of chickpea after cooking and this is attributed to the inactivation of proteinaceous antinutritional factors (Van der Poel, 1990). Also, Ekpenyong and Borchers (1980) reported an increase in protein content, total carbohydrates and digestibility in winged beans subjected to cooking. Also, according to Edijala (1980), who studied the effects of cooking of a pasta product on protein content of cowpea varieties, cooking showed small increases in protein contents of cowpea varieties. In contrast to the mentioned reports, Rehman and Salariya (2005) also, Clawson and Taylor (1993) reported that protein and starch contents of some legumes decreased after cooking.

Phenolic Compounds

According to reports of Pinelo *et al.* (2004), Turkmen *et al.* (2005) and Siddhuraju (2006) reported that the cooking process mainly results in a decrease in the phenolic compounds and so in their antioxidant ability. Adebooye and Singh (2007) worked with different cowpea varieties and the comparison showed that cooking resulted in 19-37% losses in total phenolic contents in the cowpea varieties. Barroga *et al*. (1985) found that cooking reduced the quantity of phenolic compounds in mung bean by 73% and the diffusion of phenolic compounds into the cotyledons was remarkably. The reduction in the content of quercetin and kaempferol in common bean seeds after cooking ranged from 12 to 65% and from 5 to 71% (Barroga *et al*., 1985).

The reasons of decreasing of phenolic compounds may be due to the entirely destruction or breakdown or conversion of phenolic compounds to other metabolites during cooking, or it can also be attributed to the fact that some phenolic compounds

might have formed complexes with proteins and carbohydrates and thus become unextractable (Siddhuraju & Becker, 2007) or can be oxidized (Nderitu *et al*., 2013).

However, Zhang & Hamauzu (2004) measured the TPC content and found an increase from 134 to 154% after cooking in lentils while the TFC of peas was significantly reduced. In the case of green beans, the TPC increased after cooking. This is presumably due to the higher extractability of phenolic compounds from the processed material.

In the other studies, Amarowicz and Pegg (2008) asserted that the total antioxidant capacity of green beans increased during the cooking procedure, while the total antioxidant capacity of peas remained the same as for fresh samples. Xu and Chang (2008) measured the effect of cooking on DPPH scavenging activity and ORAC of different legumes including green pea, yellow pea, chickpea, and lentil and they found that the process of cooking caused significant decreases in DPPH and ORAC values as compared to the fresh samples. Also for cowpea samples, light brown varieties had lowest whereas dark brown varieties had highest total antioxidant capacity values compared to raw samples (Siddhuraju and Becker, 2007)

Interestingly, some phenolic compounds which are not detected in raw samples, can be observed in cooked samples while some of them cannot be detected in cooked samples although they were detected in raw ones. For instance, epicatechin, ferulic acid and quercetin-3,7-diglucoside which were not detected in raw cowpea samples, were found in cooked cowpea by Nderitu *et al*. (2013).

Protein Digestibility

On the other hand, the cooking process provides valuable improvement in protein digestibility on various legumes. Rehman and Shah (2005) reported that protein digestibility of uncooked legumes ranged from 33.8 to 37.6% whereas for cooked legumes it was improved by 86.0-93.3%. However, in the same study a decrease was seen in protein digestibility when cooking time was increased.

Antinutritional Compounds

The process of cooking contributes to the reduction of antinutritional compounds such as trypsin and α-amylase inhibitor. Onwuka (2006) found that cooking significantly reduced the trypsin inhibitor by 58 to 70% when compared with raw pigeon pea seed and raw cowpea. Also, Khattab and Amtfield (2009) measured the

effect of cooking on trypsin inhibitor activities of different cowpea varieties. In raw samples, trypsin inhibitor activity ranged from 16-24 TUI/mg, while it could not be determined in cooked samples. Furthermore, Ranilla *et al*. (2010) reported that a significant decrease in the α-amylase inhibition was observed after thermal treatment such as that raw bean samples exhibited higher α-amylase inhibitor than cooked samples.

2.3.4 Germination process

Germination is a process in which seeds are sprouted by enzymes in the presence of suitable temperature, oxygen, and water content. Germination process has been extensively used for enhancing the functionality of seeds due to the increase in bioactive compounds as well as to improve the nutritional quality of legumes, decrease the effect of antinutritional factors present, owing to increasing usable carbohydrates, free amino acids, dietary fiber, and other components. (Fernandez-Orozco et al. 2006; Lopez-Amoros et al. 2006). In virtue of germination during enzymatic action, the synthesis of new cellular compounds occurs, which induces biochemical, nutritional and sensorial changes (Lopez-Amaros, 2016).

Chemical Composition

Germination process causes changes in chemical composition. Uwaegbute *et al.*, (2000) measured an increase in protein content of cowpea after germination. Also, Ghavidel (2007) reported that germinated chickpea and lentil had higher protein content when compared with raw chickpea and lentil.

Phenolic Compounds

The process of germination provides significant changes in the phenolic compounds of legumes, as a result of the action of endogenous enzymes and the complex biochemical metabolism during the process (Lopez, 2016).

Khang *et al.* (2016) measured TPC and DPPH values of germinated legumes. The results showed that germinated black bean, mung bean and white cowpea had higher TPC and DPPH values compared to raw seeds. Also, TPC values increased with prolongation of germination. For instance, 120 h germinated black bean, mung bean, white cowpea had highest TPC than 24 h germinated one.

The metabolism can change the content of phenolic acids such as *p*-hydroxybenzoic, vanillic, *p*-coumaric, and ferulic acid decreased after germination of legumes while vanillic and -*p*-hydroxybenzoic acids were found only in germinated seeds as well as the presence of flavonol glycosides such as quercetin-3- rutinoside, quercetin-3-rhamnoside, kaempferol-3-rutinoside, and kaempferol-3-glucoside was detected only in the germinated seeds. Besides, germinated beans have higher quercetin content compared to raw bean (Lopez *et al*. 2006).

Moreover, germination process affected the antiradical value against DPPH of legumes. Lopez *et al*. (2006) and Zielinski (2002) reported that bean and pea showed higher antiradical capacity whereas lentil had a weaker antiradical capacity than raw seeds after germination.

Protein Digestibility

On the other hand, there is important correlation between the process of germination and in vitro protein digestibility (Phillips, 2013). Nnanna and Phillips (1990) found that germination for 24 h significantly increased the digestibility of protein. However, Portori *et al*. (2005) reported that in vitro protein digestibility of chickpea values showed a small reduction after germination of 6 days, a fact that could be related to the variation in the composition of the protein fractions.

Antinutritional Compounds

Contents of some antinutritional compounds also decreased with the process of germination. The most known example, trypsin inhibitor activities of legumes reduce with germination (Phillips, 2013). Also, Uwaegbute et al. (2000) measured that trypsin inhibitor activity of germinated cowpea decreased with germination.

2.3.5 Fermentation process

Fermentation is one of the cheapest and the simplest processes to improve the quality and palatability of legumes. Fermentation process has been used since ancient times to produce more digestible and palatable vegetables, cereals, and legumes and to improve the nutrient density and bioavailability. The lactic acid fermentation method is desirable due to its low cost, high yield, and its formation of new flavors for human consumption. Proteolytic activity, which affects the amounts of amino acids,

modifies amino acid composition may occur after fermentation (Azarpazhooh & Boye, 2013).

It is reported that in virtue of fermentation in common beans, amounts of potassium, soluble fiber, and some amino acids were decreased whereas fatty acids, soluble sugar and vitamin content were increased (Barampama and Simard, 1995).

Chemical Composition

Xiao et al. (2015) measured the crude protein content of fermented chickpea (26.43g/100g dw) which was higher than for raw samples (22.3g/100g dw) as well as they found a higher fat content. But, Prinyawiwatkul et al. (1996) did not observe significant changes on the proximate composition of fermented cowpea compared with raw samples.

Phenolic Compounds

Duenas *et al*. (2005) observed an increase in TPC after natural and induced fermentation by *L. plantarum* of lentil and cowpea varieties. Also, they reported that complex polyphenols are hydrolyzed to other simpler and biologically more active compounds during fermentation. Torino *et al*. (2013) found that lentils performed with *B. subtilis* fermentation led to a significant increase in TPC, from 24 mg GAE/g fw to 34mg GAE/g fw after fermentation. In addition, the antioxidant capacity increased with fermentation, although longer fermentation time did not induce significant changes. Likewise, Limon et al. (2015) found an increase in TPC value of kidney beans after fermentation with natural flora and *L. plantarum*. Similar increase was found in naturally and inoculated fermented cowpea extract (Kapravelou *et al*., 2015). Xiao (2014) measured TPC of *C. militaris* fermented mung bean (2.97 mg GAE/g dw) which had higher values than raw samples (1.37 mg GAE/g dw).

Fermentation of legumes can modify their phenolic composition. In spontaneously fermented lentils, *p*-hydroxybenzoic, protocatechuic acids and catechin increased whereas hydroxycinnamic acids and procyanidin dimers decreased (Bartolome *et al*., 1997). Fermentation of cowpeas with natural microflora and with *L. plantarum* modified the content of phenolic compounds in a different way. Tyrosol and quercetin, which were not detected in the raw cowpea flour, was found after fermentation with *L. plantarum*. The levels of these two compounds were more

abundant after inoculation of the cowpeas than by spontaneous fermentation. Decreasing in quercetin 3-*O*-glucoside and quercetin 3-*O*-galactoside was associated with the increase in quercetin levels. The free phenolic acids increased after spontaneous fermentation. Nevertheless, after fermentation with *L. plantarum*, a decrease in *trans*-*p*-coumaric and *cis*-ferulic acids was observed. Xiao (2014) suggested that some phenolic acids (shikimic acid, p-coumaric acid) and flavonoids (rutin, daidzein, genistein and biochanin) were increased while others could decrease with fermentation process in chickpea extract.

The bacterial strains employed in the experiment were probably able to impart phenolic acid decarboxylase activity. The microorganisms participating in natural fermentation produce a consistent pH decrease, which could activate some enzymes that hydrolyse the quercetin glycosides, consequently yielding quercetin (Amorowicz & Pegg, 2008).

An increase was observed in the antioxidant activity of phenolic compounds present in cowpea seeds as assayed by the DPPH-method after spontaneous and inoculated fermentation (Duenas *et al*., 2005). Yet, after pigeon pea fermentation, a 4% reduction was observed in the total antioxidant capacity by Torres *et al.* (2006).

Protein Digestibility

On the other hand, fermentation provides improvement of protein efficiency with increasing in vitro protein digestibility. Xiao (2015) measured in vitro protein digestibility of *C. militaris* fermented chickpea (85.82%), which was higher compared with non-fermented one (82.22%). Also, Mbithi-Mwikya *et al.* (2002) reported improvements in protein digestibility and biological value of legumes after fermentation.

Antinutritional Compounds

Trypsin inhibitors showed a reduction with fermentation time (Phillips, 2013). But, Egounlety (2003) did not found any activity during fermentation in cowpea compared to raw samples. Khattab & Arntfield (2009) studied different legumes and measured their trypsin inhibitor activities, and fermented cowpea, kidney bean and pea varieties had lower trypsin inhibitor activities than raw varieties.

Table 2. 9 : Summaries of protein content, total phenolic content, in vitro protein digestibility and trypsin inhibitor activity of legumes.

Material	Protein	TPC	*In vitro* Protein Digestibility (%)	Trypsin Inhibitory
Cowpea				
Raw	*24-25.2 g/100g fw* [1]	3.5-13.5 µg GAE per g dw [4] 7.71 mg GAE/g dw[5]	71.286	12.4 TIU / mg fw[1] 27.6 TIU / mg fw[3] 1.5-2.4 TIU / g dw[8] 12.55 mg/g dw[9]
Soaking			736	25.7 TIU / mg fw[3]
Dehulling	*28.40 g/100g dw* [2]		73.8[6]	1.3-3.1 TIU/g dw[8] 26.6 TIU / mg fw[3]
Cooking	*24.70 g/100g fw* [3]	2.7-8.1 µg GAE per g dw4	786	1.81 mg/g dw[9] ND[8]
Germination (48h)	*26.1-27.1* g/100g fw [1,3]	14.06 mg GAE/g dw[5]	756	10.5 mg/g dw[9] ND[9]
Fermentation	*25.60 g/100g fw* [3]		85.826	0.9-2.2 TIU /g dw[8]

Material	Protein	*In vitro* Protein Digestibility (%)
Lentil		
Raw	26.5 g/100g dw 2	65.6[2]
Soaking		
Dehulling	29.6 g/100g dw 2	78.8[2]
Cooking		
Germination (48h)	28.4 g/100g dw 2	75.1[2]
Fermentation		

Table 2. 9 (continuing): Summaries of protein content, total phenolic content, in vitro protein digestibility and trypsin inhibitor activity of legumes.

Material	Protein	TPC	*in vitro* Protein Digestibility (%)	Trypsin Inhibitory
Bean				
Raw	16-22 g/100g fw [10]	0.42-1.92 mg GAE/g dw [12]	68.1 [10]	216 [14]
	27.5 g/100g dw [11]	6.12 mg GAE/g dw [13]		3.1-3.5 TIU/g dw [8]
Soaking	27 g/100g dw [11]	5.36 mg GAE/g dw [13]	71.4 [10]	1.1-2.5 TIU/g dw [8]
Dehulling	27.6 g/100g dw [11]	0.46-1.98 mg GAE/g dw [12]	70.3 [10]	0.26 TIU/g dw [14]
Cooking	26.8 g/100g dw [11]		84.4 [15]	ND [14]
Germination (48h)	30 g/100g dw [11]	12.32-15.59 mg GAE/ g dw [5]	73.4 [10]	
Fermentation				1.7-2.1 TIU/g dw [8]

Material	Protein	*In vitro* Protein Digestibility (%)
Chickpea		
Raw	22.10 g/100g dw [2]	64.2 [2]
Soaking		
Dehulling	27.20 g/100g dw [2]	77.6 [2]
Cooking		
Germination (48h)	*24.2 g/100g dw* [2]	73.4 [2]
Fermentation		

Table 2. 9 (continuing): Summaries of protein content, total phenolic content, in vitro protein digestibility and trypsin inhibitor activity of legumes.

Material	Protein	*In vitro* Protein Digestibility (%)
Lentil		
Raw	26.5 g/100g dw 2	65.6[2]
Soaking		
Dehulling	29.6 g/100g dw 2	78.8[2]
Cooking		
Germination (48h)	28.4 g/100g dw 2	75.1[2]
Fermentation		

1: (*Uwaegbute et al., 2000*); 2: *(Ghavidel, 2007)*; 3: (Phillips, 2013); 4: (Hachibamba, 2013); 5: (Khang *et al*., 2016); 6: (Xiao, 2015); 8: (Khattab & Arntfield, 2009); 9: (Egounlety & Aworh, 2003); 10: (Azarpazhooh, 2013); 11: (Mubarak, 2005); 12: (Anton *et al.*, 2008); 13: (Boateng et al, 2007); 14: (Deshpande, 1982); 15: (Park, 2010).

3. MATERIAL AND METHODS

3.2 Materials

3.2.2 Chemicals and Reagents

An overview of the different chemicals needed for the performed analysis is listed in Table 3.1.

Table 3. 1 : List of chemicals.

Fermentation	Extraction	TPC	TFC
L. plantarum	80% Methanol	Folin-Ciocalteu's reagent	Sodium nitrate ($NaNO_3$)
MRS Broth	Gallic acid	Sodium carbonate (Na_2CO_3)	Aluminum chloride ($AlCl_3$)
	Sodium hydroxide (NaOH)	Sodium hydroxide (NaOH)	
	Hydrogen chloride (HCl)	Catechin	
	Formic acid (CH_2O_2)		

in vitro Digestion	HPLC
Dialyses bag	Triflouroacetic acid (TFA)
Sodium chloride (NaCl)	HPLC grade water
α-Amylase from porcine pancreas	HPLC grade methanol
Pepsin from porcine gastric mucosa	Sodium bicarbonate ($NaHCO_3$)
Pancreatin	Gallic acid
Bile	Vanillic acid
Sodium bicarbonate ($NaHCO_{3)}$	p-Coumaric acid
Potassium chloride (KCl)	Naringenin
Monopotassium phosphate ($KH_2PO_{4)}$	Catechin
Magnesium chloride hexahydrate ($MgCl_2(H_2O)_6$)	Caffeic acid
	Ferrulic acid
Ammonium carbonate ($(NH_4)_2CO_{3)}$	Luteolin
Calcium chloride dihydrate ($CaCl_2(H_2O)_{2)}$	Protocatechnic acid
	o-coumaric acid
	Quercetin
	Syringic acid
	Sinnapic acid
	Salicylic acid

Table 3. 2 (continuing): List of chemicals.

DPPH	ABTS	FRAP	SDS Page
DPPH	ABTS	$C_2H_3NaO_2•3H_2O$ (sodium acetate)	Biosafe Coomassie blue G250
(1,1-diphenyl-2-picrylhydrazyl)	potassium persulfate (K2S2O8)	Acetic acid (C2H4O2)	Non-reducing sample buffer: XT
90% methanol	TPTZ (2,4,6-tripyridyl-s-triazine)	**Trypsin Inhibitor Activity**	Reducing sample buffer:
Trolox	Hydrogen chloride (HCl)	BAPNA (Benzyl – DL – arginine –	990 µl XT (4x, Biorad) + 10 µl XT red (20x)
(6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid)	Iron(III) Chloride Hexahydrate ($FeCl_3•6H_2O$)	*p* – nitroanilide hydrochloride)	Markers
***In vitro* Protein Digestibility**	Iron(II) sulfate heptahydrate	dimethyl sulphoxide ((CH3)2SO)	
a-Chymotrypsin from bovine pancreas	($FeSO_4.7H_2O$)	Tris(hydroxymethyl)	
Trypsin from porcine pancreas	Trolox	aminomethane	
Protease from Streptomyces griseus		Calcium chloride ($CaCl_2$)	
		acetic acid (CH3COOH)	
		Trypsin from porcine pancreas	

3.2.3 Cowpea seed samples

Seed samples of seven improved and landrace cowpea varieties (*Vigna unguiculata* L. Walp) (*Asebot, Asrat, Bekur, Black eyed-pea, Bole, TVU and White wonder*), grown under similar agronomic practices and management conditions were obtained from Melkasa Agricultural Research Center in Ethiopia. One variety (*Bekur),* that was proven to have relatively better nutritional composition as compared to the other varieties was chosen to evaluate the effect of domestic (traditional) processing on nutritional composition, antioxidant compounds and *in vitro* antioxidative properties. Among the traditional processing techniques, soaking, dehulling, cooking and germination were done in the laboratory of the Center for Food Science and Nutrition, Addis Ababa

University, Ethiopia while controlled fermentation using *Lactobacillus plantarum* starter culture was done in the laboratory of Food Microbiology and Biotechnology at Ghent University, Belgium.

Figure 3. 1 : Cowpea (*Vigna unguiculata* L. Walp*)* varieties.

3.3 Methods

3.3.1 Traditional processing techniques

3.3.1.1 Soaking (hydration)

Whole cowpea seeds were soaked in distilled water 12 h (overnight) at room temperature (22ºC ± 2) with a seed to water ratio of 1:5 (w/v). The water was drained off and then, soaked seeds were rinsed twice with distilled water, dried at 55ºC for 12 hours in hot air oven to a constant weight and milled. Samples were stored at -18ºC for further analysis.

3.3.1.2 Dehulling (decoating)

After soaking the seeds overnight (12 h), the seed coats were removed manually. Then decoated cowpea seeds were dried at 55ºCfor 12 hours in hot air oven to a constant weight and milled. Samples were stored at -18ºC for further analysis.

3.3.1.3 Traditional cooking

Whole cowpea seeds were cooked in distilled water (1:10 w/v) in a beaker with a condenser at boiling temperature (100ºC). The seeds were cooked until soft as felt by pressing between the forefinger and thumb. Cooked cowpea seeds were rinsed with distilled water and dried in hot air oven at 55ºC for 12 hours and samples were stored at -18ºC for further analysis.

3.3.1.4 Germination

The cowpeas seeds were soaked in distilled water (1:5; w/v) for 12 hours. Then, seeds were rinsed with distilled water and sterilized with 2 % ethanol to control mold development during germination. The soaked seeds were placed in a moistened thin cloth and kept in dark and allowed to germinate for 48 hours at room temperature (22ºC ± 2). The seeds were moistened with distilled water at regular intervals of 12 h. Finally, the sprouts were rinsed with distilled water and dried in hot air oven at 55ºC to constant weight and milled. In all processing, the flour samples were kept in air tight plastic containers and samples were stored at -18ºC for further analysis.

3.3.1.5 Fermentation

Lactobacillus plantarum was used as a starter culture for the fermentation. An inoculum (10^6 CFU/ml) was prepared from a stock culture in MRS broth. Milled cowpea flours were suspended in sterile distilled water (1:10 w/v). Then the inoculum of *L. plantarum* was added so an initial load of 10^6 CFU/mlwas obtained. This suspension was incubated for 24 h at 37ºC. After fermentation, fermented samples were stored at -18ºC for future analysis.

3.3.2 Proximate analysis

Proximate composition of all flours was performed according to AOAC Official Methods 925.10 (moisture), 923.03 (ash), 920.85 (lipid) and 920.87 (crude protein using %N×6,25) (AOAC, 2003). Carbohydrate content was determined based on percent differential from 100%.

3.3.3 Extraction of soluble phenolic compounds

Methanol extracts of cowpea flours were prepared according to Dewanto et al. (2002) by adding 1 g flour to 15 ml of methanol (80%) and were mixed using an

Ultra-Turrax homogenizer (IKA® T 18, Staufen, Germany) at 8000 rpm for 30s and then were putted on ice bath for 15 min. The extracts were centrifuged at 13,000g for 15 min at 10ºC before the supernatant was collected. The residues were again re-extracted with 10ml of methanol (80%) and were mixed using an Ultra-Turrax homogenizer at 8000 rpm for 20s as well. After pooling the supernatants, the volume was adjusted to 25 ml using methanol (80%) and stored in storage bottles covered with aluminum foil at -18 ^{0}C in a freezer (GSN 58 AW 30, Belgium) until used in the analysis of further analyses. The remaining residue was dried over night at room temperature and used in the extraction of bound phenolic compounds.

3.3.4 Extraction of bound phenolic compounds

Bound phenolic compounds in cowpea seed flour were extracted using alkaline hydrolysis, after drying the residue of soluble phenolic compounds at room temperature overnight (Gonzales *et al*., 2014). Briefly, dried pellet (ca. 0.1 g) obtained after extraction of soluble phenolic compounds was placed in the screw-capped falcon tubes. Then, the pellet was hydrolyzed with 2 mL of 2M NaOH and vortexed (Model G56 0E, VORTEZ GGENIE, Scientific Industries, Inc, U.S.A), sonicated (UP 400S, Ultraschallprozessor, dr. Hielscher, GhmB, Teltow, Germany) in warm water with sonication amplitude (SA) of 50 %, pulse cycle (PC) of 24 kHz at 60 ^{0}C for 30 min. After sonication, the pH of the mixture was adjusted to neutral (pH 7) using digital pH Meter (HI-2002 Edge®, HANNA Instruments, Romania) with HCl (2M) and extracted twice with 0.1 % formic acid in methanol for 2 minutes under vigorous vortex mix. Then, the tubes with their contents were centrifuged (Z 300 K, Hermel Labortechnik, GmbH, Germany) for 10 min at 10,000Xg and 10 ^{0}C. Supernatants of first and second round extraction were combined and the volume was adjusted to 20 mL with methanol (80 %) and stored in storage bottles covered with aluminum foil at -18 ^{0}C in a freezer (GSN 58 AW 30, Belgium) until used in the analysis of TPC, TFC, DPPH, FRAP, and ABTS.

3.3.5 Determination of total phenolic content (TPC)

Total phenolic contents (TPC) were determined for cowpea flour extracts using Folin-Ciocalteu's reagent based on the method of Dewanto et al. (2002). Briefly, 1 ml of extracts were mixed with 0.5 ml of Folin-Ciocalteu's reagent (1:10 v/v with distilled water). After incubation for 6 min, 1.5 ml sodium carbonate (20% w/v) and

1ml deionized distilled water were added to neutralize the reaction mixture, then the solution was thoroughly mixed and allowed to stand for 2 h in the dark. The absorption of the solutions was measured at 760 nm using a spectrophotometer. Gallic acid was used as standard, and the total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per gram dry weight sample (mg GAE/g dw).

3.3.6 Determination of total flavonoid content (TFC)

Total flavonoid contents (TFC) were analyzed for cowpea flour extracts according to aluminum chloride method of Dewanto et al. (2002). In brief, 1 ml of extracts were mixed with 75 µl sodium nitrate (5%). After incubation for 6 min, 150 µl aluminum chloride (10%) added. After an incubation for 5min, 1M NaOH was added and left for 15min. The absorption of the solutions was measured at 510 nm using a spectrophotometer. Catechin was used as standard, and the total flavonoid content was expressed as milligrams of Catechin equivalents per gram dry weight sample (mg catechin/g dw).

3.3.7 Measurements of antioxidant activities

3.3.7.1 Determination of DPPH radical scavenging activity

The DPPH (1,1-diphenyl-2- picrylhydrazyl) assay was performed as described by Kumaran et al. (2006). Aliquot (2 mL) of cowpea seed extracts was mixed with 2 ml of 0.1 mM DPPH solution. After shaking for 5 s, the mixtures were incubated at room temperature for 30 min in the dark and the absorbance was measured at 517 nm. The Trolox calibration curve was plotted as a function of percentage of DPPH radical scavenging activity. The results were expressed as milligram of Trolox (TE) per gram dry weight sample (mg TEAC/g dw).

3.3.7.2 Evaluation of ABTS radical cation scavenging activity

The ABTS radical cation ($ABTS^{\cdot+}$) scavenging activity of the extracts was analyzed as described by Re *et al.* (1999). $ABTS^{\cdot+}$ was generated by the reaction of 7 mM aqueous solution of ABTS with 2.45 mM aqueous solution of potassium persulfate which was allowed to stand in dark at room temperature for 16 h before use. The $ABTS^{\cdot+}$ solution was diluted with methanol (90%) to an absorbance of 0.70±0.02 at 734 nm and equilibrated at 30ºC. Then it was diluted in proportion of 1:52.5 (v/v)

with methanol (90%). Then, 2 ml of $ABTS^{\cdot+}$ was mixed with 20 µl of extracts, methanol was used for blank. The absorbance was recorded at 734 nm after 6 min at 25ºC. The results were expressed as micromol of Trolox (TE) per gram dry weight sample (µmol TEAC/g dw).

3.3.7.3 Estimation of ferric reducing antioxidant power (FRAP)

Ferric reducing antioxidant power of extracts were carried out according to the method as described by Benzie *et al.* (1996). FRAP working solution was prepared by mixing 25 ml acetate buffer (pH 3.6, 300mmol/l) with 2.5ml TPTZ solution (40 mmol /l HCl) and 2.5ml ferric chloride (20mmol/l). The FRAP working solution was prepared daily and warmed at 37ºC for 10min before use. Briefly, 100 µl of extracts was mixed with 300 µl of HPLC water and 5 ml of working FRAP solution and shaked for 5 seconds. The mixtures were incubated in the dark for 20min and absorbance readings were recorded at 593 nm. The measurements were compared with calibration curve of ferrous sulfate solution, and the results were expressed as µmol Fe (II) equivalents per gram of dry weight sample (µmol Fe (II)/g dw).

3.3.8 HPLC Analysis of major individual phenolic compounds

Major individual phenolic compounds were determined following the method of Wen *et al*. (2005). Extracts were filtered through a 0.45 µm membrane filter and analyzed by the HPLC system W600 Waters HPLC system coupled to a Waters 996 PDA detector and compounds were separated using a Supelcosil LC-18 column (250 × 4.6 mm, 5 µ). Mobile phase A was water containing 0.02% trifluoroacetic acid (TFA), and phase B was methanol containing 0.02% TFA. The water and methanol used were HPLC grade. A linear gradient was used as follows: at 0-5 min, 25% B; at 5-10 min, 25-30% B; at 10-16 min, 30-45% B; at 16-18 min, 45% B; at 18-25 min, 45-80% B; at 25-30 min, 80% B; at 30-40 min, 80-25% B; and at 40 min returns to its initial conditions. The flow rate was 0.5 ml/min. Detection was done at 254, 275, 305, and 320 nm. Peak identification was done by comparing absorbance spectra and retention times of eluting peaks with available standards. All analyses were performed in duplicate and the obtained data were expressed as µg dry weight.

3.3.9 Simulated in vitro gastrointestinal (GI) digestion

In order to simulate the *in vitro* GI digestion conditions, and to determine the amount of free soluble phenolic compounds potentially available for further uptake, the procedure adapted from Minekus *et al*. (2014) was followed. To follow the release of phenolic compounds from sample matrices, free dialyzable content and non-dialyzable content were analyzed, respectively.

The stock solutions of simulated digestion fluids (Simulated Salivary Fluid (SSF), Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) electrolyte stock solutions) were prepared according to Table 3.2 and 3.3.

Dialyses bags were cut into a length of 15.5 cm and were filled up 5.5 ml of 0.9% NaCl and 5.5mL of 0.5M $NaHCO_3$ and clipped from both sides to prevent leak of solution.

3.3.9.1 Oral phase

In a 250 ml glass beaker, 1.5 g dw of samples were mixed with 3.5 ml SSF electrolyte stock solution and 0.5 ml of salivary α-amylase solution, 1 ml of 0.3M $CaCl_2$ were added. After that, the pH was adjusted to 7 by adding 6M HCl. The mixture was incubated for 2 min at 37ºC in a shaking water bath (Nürnberg, Germany).

3.3.9.2 Gastric phase

After 2 mins, 7.5 ml of SGF electrolyte stock solution, 1.6 ml of porcine pepsin stock solution and 0.7 ml of 0.3M $CaCl_2$ were added and pH adjusted to 3. After incubation at 37◦C for 1.5h in a shaking water bath, the dialysis bags were inserted into beakers, followed by 30 min incubation at 37◦C under shaking conditions.

3.3.9.3 Intestinal phase

After incubation, 11 ml of SSF electrolyte stock solution, 5 ml of pancreatin solution, 2.5 ml of fresh bile and 40 µl of 0.3 M $CaCl_2$ were added into the beaker. After that, pH was adjusted to 7 and 1.31 ml water was added. After incubation for 2 h at 37◦C, the dialysis bags were removed, cleaned and properly dried before removing the contents. The solution in the dialysis tubing was taken as the IN sample representing the material that entered the serum and the solution outside the dialysis bags were

taken as the OUT sample representing material that remained in the gastrointestinal tract. IN and OUT samples were stored at -18ºC until further analysis.

Table 3. 3 : Preparation of stock solutions of simulated digestion fluids.

Constituent	Stock concentration		SSF pH 7		SGF pH 3		SIF pH 7	
			Vol. of stock	Conc. in SSF	Vol. of stock	Conc. in SGF	Vol. of stock	Conc. in SIF
	g/L	mol/L	ml	mmol/L	ml	mmol/L	ml	mmol/L
KCl	37.3	0.5	15.1	15.1	6.9	6.9	6.8	6.8
KH_2PO_4	68	0.5	3.7	3.7	0.9	0.9	0.8	0.8
$NaHCO_3$	84	1	6.8	13.6	12.5	25	42.5	85
NaCl	117	2	-	-	11.8	47.2	9.6	38.4
$MgCl_2(H_2O)_6$	30.5	0.15	0.5	0.15	0.4	0.1	1.1	0.33
$(NH_4)_2CO_3$	48	0.5	0.06	0.06	0.5	0.5	-	-
$CaCl_2(H_2O)_2$*	44.1	0.3		1.5		0.15		0.6

* $CaCl_2(H_2O)_2$ was added to the final mixture of simulated digestion fluids.
The fluids were stored -18◦C and brought to temperature of 37 ^oC at the point of mixing with food matrix before using.

Table 3. 4 : Preparation of the enzymes, bile, and dialysis fluids.

Compounds	Concentration
Enzymes	
α-amylase	0.0468 g/ml SSF
Pepsine	0.0078 g/ml SGF
Pancreatin (100 U/mL trypsin activity)	0.0019 g/ml SIF
Bile	11 mg/ml SIF
NaCl for dialysis	0.9%
$NaHCO_3$ for dialysis	0.5M

The fluids were stored -18◦C and brought to temperature of 37 ^oC at the point of mixing with food matrix before using.

3.3.10 Trypsin inhibitor activity (TIA)

Trypsin inhibitory activity was measured with the method was modified from Dwivedi *et al.* (2015) and Senanatake *et al.* (2013), using BAPNA as substrate. Briefly, BAPNA solution was prepared by dissolving 40mg of BAPNA (Benzyl – DL – arginine – *p* – nitroanilide hydrochloride) in 1ml of dimethyl sulphoxide and

diluted to 100 ml with prewarmed (37ºC) 0.05M tris buffer containing 0.02 M $CaCl_2$ at pH 8.2. Trypsin inhibitor was extracted from 1 g of flour, using 50 ml of 0.01 N NaOH for 3 h, and a dilution series with distilled water (0, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2 ml extract/ml) was prepared. In the tubes containing 2 ml of diluted extract, 2 ml porcine pancreas trypsin solution (0,12mg/0.001N HCl) was added and preincubated at 37◦C for 5 min. Then, 5 ml of BAPNA solution was added and vortexed to start the reaction. After incubating for 10 min at 37◦C, 1 ml acetic acid (30%) was added and vortexed to terminate the reaction. The reaction mixture was centrifuged at 4000g for 10min. The absorbance of the mixture was measured at 410 nm, which was possible due to the formation of *p*-nitroaniline. One unit of trypsin inhibitor was measured as the decrease of 0.01 in absorbance at 410 nm under assay conditions compared with the control (buffer instead of extract).

3.3.11 *In vitro* protein digestibility

The *in vitro* protein digestibility of cowpeas was determined using a three-enzyme method (trypsin, chymotrypsin and protease) of Hsu et al. (1977). The enzyme mixture (1.6mg/l porcine pancreatic trypsin, 3.1 mg/l bovine pancreatic chymotrypsin and 1.3 mg/l porcine intestinal peptidase with double distilled water) was maintained in an ice bath and adjusted to pH 8.0 and prewarmed to 37ºC before use. The samples were weighed as 1g of nitrogen must be in 1 ml of double distilled water according to the amount of nitrogen previously measured (1mg N/ml) and was mixed with 50ml double distilled water and vortexed. After incubation for 1 h at 37ºC in the shaking water bath, pH was adjusted to 8 and when 5ml of the enzyme mixture was added, pH was measured immediately. Then, incubation was continued at 37ºC in the shaking water bath for the 10 min and recorded pH exactly after 10 min. The percentage of protein digestibility was calculated by the equation:

$$\text{In vitro protein digestibility \%} = 210.46 - 18.10*(pH_{\text{initial}} - \Delta pH_{10\ \text{min}}) \quad (3.1)$$

3.3.12 SDS-Page

SDS-PAGE analysis was performed based on BioRad (2016). Protein samples are prepared using heat and SDS to denature the proteins. SDS minimizes charge variability among proteins, giving them the same charge to mass ratio and forcing them into rod-like shapes. This effectively eliminates the effects of protein

conformation and native charge density on the electrophoretic migration distance. Under reducing conditions the denaturing sample buffer eliminate protein secondary structure by reducing disulfide bonds.

3.3.12.1 Sample preparation

Cowpea protein concentrates were prepared by the isoelectric precipitation method (Mune *et al*., 2015). An aliquot (10 g) of sample was mixed with 100 mL distilled water, the pH was adjusted to 10 using a 1.0 M NaOH solution and the mixture was stirred for 2 h at room temperature. The resultant slurry was then centrifuged at 4,000 g for 30 min and at 4◦C. The pH of the resultant supernatant was adjusted to 4.5 using a 1.0 M HCl solution with consistent stirring; the precipitated proteins were recovered by centrifugation at 4,000 g for 30 min (4◦C). The proteins were resuspended in distilled water and the pH was adjusted to 7.0 with constant stirring.

3.3.12.2 Gel Loading, electrophoresis, and staining

The precast gel cassette was removed from the BioRad package, rinsed with deionized water, the tape in the bottom of the cassette was peeled off and the comb was pulled out of the cassette with care. Then the gels were placed into the slots of the Criterion Cell.

The upper and lower buffer chamber were filled with 1X running buffer. 20 µl sample was loaded into the wells and electrophoresis was performed for 1 hour in the following conditions: 160V, 300A, 300W. After 1 hour duration, the gels were gently removed from the cassettes.

The gels were first washed with demineralized water and they were put in fresh demineralized water twice for 10 minutes by gently shaking. Then the gels were stained during maximum 60 minutes in staining solution (Biosafe Coomassie blue G250) with gentle mixing. Afterwards, the staining solution was removed and demineralized water was added. The demineralized water refreshed after 10-30-15 minutes washing and finally the gels were left for de-staining overnight on a shaker. Stained gels were rapidly scanned to prevent drying of the gels after covering with a cellophane membrane.

3.3.13 Statistical Analysis

Data were collected from two independent extractions for each fraction and reported as mean ± SD. For multiple comparisons and correlations, data were subjected to statistical analysis using SPSS software (version 16.0 for Windows, SPSS Inc.) for the analysis of variance (ANOVA). Processing steps were compared using one-way analysis of variance (ANOVA) followed by Tukey post hoc test, and $p < 0.05$ was considered significant. 2-way interaction terms were used for varieties x types of varieties and type of process x digestion fractions.

4. RESULTS AND DISCUSSION

4.1 Total Phenolic Content, Total Flavonoid Content, and Antioxidant Capacity of Cowpea Varieties

4.1.2 Total phenolic content (TPC) of cowpea varieties

The total phenolic contents of cowpea varieties are shown in Table 4.1. All data are given in terms of mg GAE/g on dry matter basis. The free fraction TPC values ranged from 0.36 to 1.13 mg GAE/g dw while the bound fraction TPC values ranged from 6.05 to 9.13 mg GAE/g dw. No significant effect of the 2-way interaction terms (varieties x types) was observed ($p>0.05$). The results showed that Asrat, White Wonder and Bekur had higher free fraction TPC values than Bole, Asebot, TVU and Black eyed peas varieties. On the other hand, there is no significant difference in free TPC values between landrace and improved types of each varieties ($p>0.05$). The highest free TPC was found in improved type of Asrat (1.13 GAE mg/g dw). Bound TPC values were higher than the TPC values of the free forms, and this for each of the varieties. When compared bound TPC results, types of Asrat had the highest TPC values while types of Bole had lowest values. In addition, the highest bound TPC was measured in improved type of White Wonder (9.13 mg GAE/g dw).

The phenolic compounds contents may vary depending on numerous factors such as varieties, types, growth conditions, metabolic states, initial compound level and intensity of stress (Shibamoto, 2013; Sparvoli *et al*., 2015; Rebollo *et al.*, 2014). Thus, different TPC values ranging from 0.36 to 1.13 mg GAE/g dw for free form, were found in this study. It is difficult to compare the results with those reported in the literature because they express the results in different units, and not always all information is available to recalcute the reported values in mg GAE/g dw. Oboh (2006) reported that the free TPC of cowpea ranges from 0.3 mg GAE/g fw in a white cultivar to 1.0 mg GAE/g fw and likewise Gutierrez-Uribe *et al*., (2011) found a total phenolic content of 0.75 mg GAE/g dw. Also, Kalogeropoulos *et al*. (2010) found a lower free total phenolic content for cowpea (0.15 mg GAE/ g fw) while Sreerama *et al*., (2012) reported higher amounts (12.16 mg GAE/g dw). Also, the

way of extracting the phenolic compounds from the plant matrix will affect the reported values.

Table 4. 1 : Total phenolic contents (TPC) of cowpea varieties.

Varieties	Type	TPC (mg GAE/g dw)			
		free		bound	
Bole	Landrace	0.45±0.01	a	7.02±0.55	a
	Improved	0.47±0.05		4.62±0.32	
Asebot	Landrace	0.53±0.15	a	7.69±0.01	abc
	Improved	0.69±0.10		6.48±0.91	
Asrat	Landrace	0.74±0.03	b	8.32±0.65	c
	Improved	1.13±0.04		8.73±0.22	
White Wonder	Landrace	0.62±0.03	b	7.73±0.14	bc
	Improved	0.97±0.16		9.13±0.37	
Bekur	Landrace	0.75±0.02	b	8.37±0.41	bc
	Improved	1.09±0.05		7.87±1.53	
TVU	Landrace	0.77±0.04	a	8.73±0.62	bc
	Improved	0.36±0.02		6.83±0.31	
Black Eyed Peas	Landrace	0.58±0.02	a	7.75±0.83	abc
	Improved	0.54.0.07		6.05±0.39	

Data represent average quantities standard deviation of 2 independent samples. Different letters in columns within each variety represent statistically significant differences ($p<0.05$).

4.1.3 Total flavonoid content (TFC) of cowpea varieties

The total flavonoid content ranged from 0.41 to 1.48 mg CAE/g dw for the free fraction and 2.90 to 12.6 mg CAE/g dw for bound fraction in cowpea varieties (Table 4.2). No significant effect of the 2-way interaction terms (varieties x types) was observed ($p>0.05$). Yet, among seven varieties in free fraction, types of Asrat showed the highest TFC values. When compared free TFC values between landrace and improved types of each varieties, no significant difference was observed ($p>0.05$). On the other hand, types of Bole variety which landrace type (12.61 mg CAE/g dw) richer than improved type (10.64 mg CAE/g dw), had the highest TFC values in bound fraction while Black eyed peas had lowest TFC values. However, there was no significant difference between improved and landrace types of each varieties for bound form ($p>0.05$).

It is very difficult to compare the TFC results with literature data, as often results are expressed in fw, or other standards were used to express the results. Ojwang *et al*. (2012) found that flavonoid content ranged from 0.27-0.35 mg CAE/g fw to 0.88-

1.06 mg CAE/g fw depending on the color of seeds. Sparvoli *et al.* (2015) measured TFC of cowpea in the range 0.27-2.09 mg CAE/g fw while Gutierrez-Uribe *et al.* (2011) reported that TFC of cowpea is 0.98 mg quercetin equivalents per g dry weight basis. In addition, Yeo and Shahid (2017) found higher TPC and TFC values in bound fraction than free fraction in lentil.

Table 4. 2 : Total flavonoid content (TFC) of cowpea varieties.

Varieties	**Type**	**TFC (mg CAE/g dw)**	
		free	bound
Bole	Landrace	0.41±0.02 ^{a}	12.61±0.94 ^{c}
	Improved	0.72±0.03	10.64±1.60
Asebot	Landrace	0.95±0.18 ^{b}	9.39±2.64 ^{abc}
	Improved	0.82±0.03	4.98±0.67
Asrat	Landrace	1.48±0.02 ^{c}	11.05±6.01 ^{bc}
	Improved	1.22±0.03	7.51±0.43
White Wonder	Landrace	0.91±0.06 ^{b}	10.87±0.09 ^{bc}
	Improved	0.96±0.02	8.57±0.04
Bekur	Landrace	1.13±0.01 ^{b}	8.24±2.54 ^{abc}
	Improved	0.69±0.02	6.17±1.75
TVU	Landrace	0.71±0.03 ^{a}	8.25±1.19 ^{ab}
	Improved	0.59±0.02	3.05±0.63
Black Eyed Peas	Landrace	0.48±0.04 ^{a}	4.64±2.28 ^{a}
	Improved	0.57±0.01	2.90±0.10

Data represent average quantities standard deviation of 2 independent samples. Different letters in columns within each variety represent statistically significant differences ($p<0.05$).

4.1.4 Total antioxidant capacity (TAC) of cowpea varieties

The results of the total antioxidant capacity which was measured by three different methods (ABTS, DPPH and FRAP), show different trends (Table 4.3). No significant effect of the 2-way interaction terms (varieties x types) was observed, as well as no influence of varieties and types ($p>0.05$).

In agreement with the above results, the highest TAC was found in Bekur as measured by DPPH, in Asebot for the ABTS and in Asrat by FRAP method, and this for the free fraction of varieties. When compared the types of those varieties, landraces had highest free TAC values. However, for the bound fraction, Asrat showed the highest value for DPPH, while White Wonder had highest TAC values as

measured by ABTS and FRAP. When compared the types of those varieties, landraces had highest bound TAC values. On the other hand, no significant difference was observed between landrace and improved types of each varieties ($p>0.05$).

According to the results, the maximum antioxidant capacity in each method was not represented by one variety. Asrat had highest TPC, TFC, DPPH and FRAP values but not the highest ABTS values. In literature, DPPH values between 25.1-27.9 μmol TEA/g fw (Zia-Ul-Haq, 2013), FRAP values in a range of 14.26-68.03 μmol Fe (II)/g fw (Marathe *et al*., 2011), and ABTS values between 11.26-68.03 μmol TEA/g fw (Marathe *et al*., 2011) were reported for the free fraction. When comparing the results with literature, the results of DPPH were higher values while results of ABTS and FRAP were among the same range as literature values.

Moreover, the improvement application that targeting some quality parameters related to yield and yield components, nutritional value, and many other factors, seems to be ineffective in terms of increasing amount of phenolic compounds and antioxidative capacity.

In this study, in terms of reflecting the changes as a result of different varieties, the best correlated methods were free fraction of DPPH and free fraction of ABTS, and free fraction of DPPH and bound fraction of ABTS assays (Table 4.3). The measurement of antioxidant activities, cannot be evaluated sufficiently by a single method. Therefore, it is advisable to use more than one method because differences may be observed even among methods with the same principle and it is suggested to apply several test procedures to evaluate antioxidant activities.

Table 4. 3 : Total antioxidant capacity of cowpea varieties.

Varieties	Type	DPPH (mg TEAC/g dw)			ABTS (µmol TEAC/g dw)			FRAP (µmol Fe II/g dw)	
			free	bound		free	bound		free	bound
Bole	Landrace	a	1.12±0.28	4.07±0.04	a	10.73±0.40	112.58±1.87	ab	56.48±0.07	529.91±41.09
	Improved		0.84±0.01	3.11±0.82		6.10±0.36	90.44±6.42		54.53±1.22	486.05±81.58
Asebot	Landrace	b	1.87±0.42	6.31±1.33	c	19.19±0.01	170.11±1.87	ab	55.45±0.81	524.41±1.60
	Improved		1.27±0.13	5.38±1.00		10.13±1.05	128.29±27.27		55.04±0.02	451.27±62.45
Asrat	Landrace	c	3.82±0.16	11.08±7.43	d	22.67±1.25	186.80±26.87	b	59.22±1.87	503.64±63.52
	Improved		2.34±0.30	8.34±5.63		14.42±0.40	145.48±25.38		56.94±1.73	553.43±6.14
White Wonder	Landrace	c	2.83±0.05	6.84±2.92	d	18.07±2.70	194.58±9.08	ab	59.31±1.64	593.80±2.63
	Improved		2.37±0.37	9.57±1.02		16.55±0.78	164.02±13.59		56.39±2.09	530.72±37.17
Bekur	Landrace	c	4.01±0.23	9.48±5.48	d	22.24±0.15	174.03±38.85	b	55.35±0.96a	518.21±39.17
	Improved		1.88±0.19	5.89±0.93		12.37±0.36	140.78±8.08		54.89±0.86	463.37±96.91
TVU	Landrace	ab	2.04±0.18	3.63±2.23	d	25.64±0.09	158.00±11.63	ab	58.24±0.88	572.74±0.40
	Improved		0.94±0.33	4.11±0.13		10.91±0.60	114.55±3.75		54.74±0.06	537.76±13.39
Black Eyed Peas	Landrace	ab	1.22±0.14	3.83±057	b	11.90±0.16[b]	485.15±56.25	a	54.80±2.65	519.03±61.94
	Improved		0.98±0.18	3.96±0.29		9.51±0.24	121.51±9.97		54.51±0.45	510.60±6.05

Data represent average quantities standard deviation of 2 independent samples. Different letters in columns within each variety represent statistically significant differences ($p<0.05$)

4.1.5 The Correlation Coefficients for Spectrophotometric Assays

Pearson correlation coefficients for spectrophotometric assays ranged from 0.012 to 0.822 (Table 4.4). Parameters that showed a linear relation with a high correlation coefficient were: TFC free and DPPH free; TFC free and DPPH bound; DPPH free and DPPH bound; DPPH free and ABTS free; DPPH free and ABTS and bound. Among all those assays, the highest correlation was seen between TFC and DPPH for free fraction (0.822) ($p<0.01$). These results indicate that not only free fraction of phenolic compounds but also bound fractions are associated with the antioxidant capacity of the studied cowpea varieties.

Table 4. 4 : The correlation coefficients for spectrophotometric assays.

	TPC (free)	TPC (bound)	TFC (free)	DPPH (free)	DPPH (bound)	ABTS (free)	ABTS (bound)	FRAP (free)	FRAP (bound)
TPC (free)	1	0.576**	0.496**	0.461*	0.446*	0.301	0.371	0.234	-0.07
TPC (bound)	0.576**	1	0.447*	0.615**	0.408*	0.711**	0.642**	0.470*	0.513**
TFC (free)	0.496**	0.447*	1	0.822**	0.666**	0.584**	0.648**	0.463*	0.043
TFC (bound)	-0.027	0.217	0.287	0.345	0.012	0.315	0.194	0.409*	0.389*
DPPH (free)	0.461*	0.615**	0.822**	1	0.643**	0.784**	0.797**	0.530**	0.179
DPPH (bound)	0.446*	0.408*	0.666**	0.643**	1	0.412*	0.723**	0.510**	-0.123
ABTS (free)	0.301	0.711**	0.584**	0.784**	0.412*	1	0.767**	0.591**	0.355
ABTS (bound)	0.371	0.642**	0.648**	0.797**	0.723**	0.767**	1	0.681**	0.287
FRAP (free)	0.234	0.470*	0.463*	0.530**	0.510**	0.591**	0.681**	1	0.471*
FRAP (bound)	-0.07	0.513**	0.043	0.179	-0.123	0.355	0.287	0.471*	1

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

4.2 Effect of Processing Steps

4.2.1 Proximate composition

Proximate composition of the samples after different processing techniques are presented in Table 4.5. The processing steps caused a significant difference in protein content between raw and processed samples ($p<0.05$), though, no significant difference was observed in other parameters. Raw cowpea had the highest protein

content (39.06 g/100g dw), the protein contents were decreased with processing steps, followed by germinated (33.32 g/100g dw), soaked (32.69 g/100g dw), dehulled (30.96 g/100g dw), cooked (29.67 g/100g dw) and fermented cowpea (28.50 g/100g dw).

Table 4. 5 : Proximate composition of processed cowpea.

Material	Moisture (g/100g fw)	Protein (g/100g dw)	Lipid (g/100g dw)	Ash (g/100g dw)	Carbohydrate* (g/100g dw)
Raw Cowpea	7.6±0.28	39.40±0.38^{e}	1.34±0.38	3.65±0.30	50.86±4.30
Soaked Cowpea	5.5±0.42	32.69±0.01^{d}	1.84±0.52	3.18±0.31	59.27±3.97
Dehulled Cowpea	5.3±0.42	30.96±0.27^{c}	2.17±0.68	2.74±0.29	61.10±4.32
Cooked Cowpea	5.6±0.28	29.67±0.17^{b}	1.85±0.37	2.12±0.01	63.25±4.51
Germinated Cowpea	6.5±0.42	33.32±0.15^{d}	3.21±0.01	1.71±0.01	58.07±4.40
Fermented Cowpea	6.4±0.20	28.50±0.12^{a}	2.48±0.15	2.51±0.02	62.11±3.50

Values expressed are mean ± standard deviation. (n=2)
* Calculated by percent differential from 100%.
Different letters in columns represent statistically significant differences ($p<0.05$).

Some possible explanations for the observed changes in protein content after processing is given below. The total protein content in the samples was measured by using Kjeldahl methods which the principle of this is to measure total N content of sample. Some enzymes may become active with increasing water content, cutting the proteins in smaller molecules which are become better water soluble. These protein degradation products are probably migrated in the soaking water, resulting in a lower N amount in the sample. Khalil (2001) and Barampama (1995) reported a decrease after the soaking process for beans as well as Elmaki et al. (1999) found a slight decrease in protein content for sorghum samples. Rehman & Shah (2005) mentioned that soaking process did not affect protein contents of legumes. Nevertheless, Ekpenyong and Borchers (1980) and Edijala (1980) found small increases in protein content of legumes.

The proteins found in the hulls are removed from the seed together with the removal of the hulls in dehulling process. Chibber et al (1978) reported a decreased protein content after dehulling in sorghum while Khalil (2001) and Ghavidel and Prakash (2007) found an increase with dehulling for legume samples.

The decrease in the amount of protein may have been observed at the end of the cooking process due to denaturation of proteins at high temperature. Attia *et al*.

(1994), Ekpenyong and Borchers (1980) and Edijala (1980) found an increase in crude protein contents after cooking chickpea whereas Rehman and Salariya (2005), and Clawson and Taylor (1993) reported that the protein content of some legumes decreased.

On the other hand, enzymes participated in germination break down proteins to allow them to be utilized for the growth and development of the embryo (Elmaki *et al*., 1999) as well as bacteria involved in the fermentation use nitrogen as sources and thus, protein structures may change resulting in a decrease in total protein content.

Uwaegbute *et al.*, (2000) measured an increase in protein content of cowpea and Ghavidel (2007) reported similar results for lentil after germination. Also, Xiao et al. (2015) measured a higher protein content in fermented chickpea while Prinyawiwatkul et al. (1996) did not observe significant changes on the proximate composition of fermented cowpea. The differences between the results here and reported literature might be due to several reasons such as different process times, temperatures, condition of processes, varieties of legumes.

4.2.2 Total phenolic content (TPC) and total flavonoid content (TFC)

TPC and TFC of all samples are expressed on dry weight (dw) basis (Table 4.6). The results presented here are the phenolic compounds and flavonoids measured in the methanol extract, i.e. thus the soluble fractions. Results showed that TPC was increased and had the highest value after the fermentation process (1.97 mg GAE/g dw) compared to the raw samples (0.80 mg GAE/g dw). Also, after cooking, germination and soaking TPC were increased (1.48, 1.41, 1.02 mg GAE/g dw, respectively). However, a decrease was observed after dehulling process (0.72 mg GAE/g dw).

For TFC the highest value was found in cooked cowpea (1.10 mg CAE/g dw), which was significantly different compared to the TFC value of the raw and other processed samples ($p<0.05$). A small decrease in TFC compared to the raw cowpea as a result of soaking was found (0.66 mg CAE/g dw) ($p>0.05$), whereas, non-significant increases were observed in dehulled, germinated and fermented cowpea (0.72, 0.72, 0.80 mg CAE/g dw, respectively) compared to the raw samples ($p>0.05$).

Table 4. 6 : Total phenolic content (TPC) and total flavonoid content (TFC) of processed cowpea.

Material	Total Phenolic Content (mg GAE/g dw)	Total Flavonoid Content (mg CAE/g dw)
Raw Cowpea	0.80 ± 0.14^{a}	0.69 ± 0.02^{a}
Soaked Cowpea	1.02 ± 0.01^{ab}	0.66 ± 0.02^{a}
Dehulled Cowpea	0.72 ± 0.01^{a}	0.72 ± 0.08^{a}
Cooked Cowpea	1.48 ± 0.01^{bc}	1.10 ± 0.12^{b}
Germinated Cowpea	1.41 ± 0.06^{b}	0.72 ± 0.03^{a}
Fermented Cowpea	1.97 ± 0.26^{c}	0.80 ± 0.02^{a}

Values expressed are mean ± standard deviation. (n=2).
Different letters in columns represent statistically significant differences ($p<0.05$).

The fermentation process can assist the hydrolyzation of complex polyphenols and the release of bound phenolic compounds. This can result in the formation of more soluble and smaller compounds. Also, cooking probably causes the formation of more extractable phenolic compounds. Pinelo *et al.* (2004), Turkmen *et al.* (2005), Barroga *et al*. (1985) and Siddhuraju (2006) reported that the cooking process results in a decrease in the phenolic compounds. Adebooye and Singh (2007) found that cooking resulted in 19-37% losses in total phenolic contents in cowpea varieties. Yet, Zhang & Hamauzu (2004) measured the TPC content and found an increase from 134 to 154% after cooking in lentils while the TFC of peas was significantly reduced. Moreover, Duenas *et al*. (2005) and Kapravelou *et al*. (2015) observed an increase in TPC after fermentation of lentil and cowpea varieties as well as Torino *et al*. (2013) found that fermentation of lentils led to a significant increase in TPC. During the germination, a great amount of energy, new compounds such as phenolics can be generated as a result of the complex biochemical metabolisms due to the enzymes. Similar to the results here, Khang et al. (2016), Lopez *et al*. (2006) and Zielinski (2002) measured a higher TPC value of germinated legumes compared to raw seeds.

The removal of the hulls did not cause any significant difference in this experiment. However, Anton *et al.* (2008) and Towo *et al*. (2003) as well as Sosulski and Dabrowski (1984) suggested that the dehulling greatly reduced TPC of legume seeds. Besides, there were no significant changes measured in TPC after soaking process. Also, similar result was reported by Xu and Chang (2008).

4.2.3 Total antioxidant capacity (TAC)

Total antioxidant capacity was measured using three different methods (DPPH, ABTS and FRAP). Results of the different methods show different tendencies (Table 4.7). In agreement with the above results, and independent of the methods, TAC was found to be the highest in fermented cowpea, ranging from 15.96 mg TEAC/g dw for DPPH, 32.57 µmol TEAC/g dw for ABTS to 60.61 µmol Fe II/g dw for FRAP. Furthermore, the increases in TAC were found after cooking and germination by 76.29% and 41.13% for ABTS, and after cooking by 4.93% for FRAP compared to raw cowpea.

Table 4. 7 : Total antioxidant capacity of processed cowpea.

Material	DPPH (mg TEAC/g dw)	ABTS (µmol TEAC/g dw)	FRAP (µmol Fe II/g dw)
Raw Cowpea	6.07±0.06^{b}	12.40±0.40^{a}	55.35±0.96^{ab}
Soaked Cowpea	5.96±0.08^{b}	13.97±1.36^{a}	55.20±0.01^{ab}
Dehulled Cowpea	5.82±0.13^{ab}	13.94±1.24^{a}	54.72±0.05^{a}
Cooked Cowpea	5.12±0.25^{a}	21.86±0.18^{ab}	58.08±0.14^{c}
Germinated Cowpea	5.93±0.09^{ab}	17.50±0.20^{a}	56.54±0.04^{bc}
Fermented Cowpea	15.96±0.35^{c}	32.57±6.68^{b}	60.61±0.12^{d}

Values expressed are mean ± standard deviation. (n=2)
Different letters in columns within each cultivar represent statistically significant differences ($p<0.05$).

Antioxidant properties of phenolic compounds depends on their activity to chelate pro-oxidant metal ions, scavenge radicals, and inhibit some enzymes (Shibamoto, 2013). Antioxidant capacity of plant (extracts) varies depending on species, cultivar, developmental stage, metabolic state, initial compound level, duration, and intensity of the stress (Shibamoto, 2013; Sparvoli *et al*., 2015; Rebollo *et al.*, 2014).

The results of this study showed that the TAC increased with fermentation process in cowpea. There is a quiet important correlation between the amount of phenolic compounds and the antioxidant capacity. It appears that some complex phenolic compounds undergo a change to more active or extractable compounds by virtue of the microorganisms involved in fermentation or the endogenous enzymes that becomes more active due to the specific fermentation conditions. The increase in TAC after fermentation has also been reported for other legumes. Fermentation process provided a significant increase (53.6% to 93.9%) of antioxidant activity in

date varieties (Sanjukta *et al.,* 2015; Limon *et al*., 2015; Amorowicz & Pegg, 2008). Limon *et al*. (2015) explained that this increase was due to one of the bacterial proteolytic activity leads to the hydrolysis of phenolic compounds to more simple forms.

Moreover, the increase was observed after cooking for ABTS and FRAP methods. In contrast to the results, Amarowicz *et al.* (2008) and Adebooye *et al.* (2007) reported a decrease in TAC after cooking for some legumes while Zhang and Hamauzu (2004) measured an increase in TAC after cooking of lentils. The explanation for the increasing TAC values is the formation of maillard reaction products during cooking process, as well as the higher extractability of phenolic compounds with cooking process.

On the other hand, after soaking and dehulling process there was no significant loss of antioxidant activity of cowpea. But, in some studies, small decreases of TAC were reported for other legumes (Boateng *et al.,* 2007; Adb El-Hady and Habiba, 2003; Ranilla *et al.,* 2009).

In addition, germination process did not significantly affected TAC of cowpea in this study. According to Khang et al. (2016), Lopez *et al*. (2006) and Zielinski (2002), germination process contributed to an increase of TAC of legumes as a result of endogenous enzymes action and the complex biochemical metabolism during the process. Yet, germination time is one of the most important parameters for the change of phenolic compounds and TAC was increased with extension time of germination. In this study, germination time may not have been long enough to cause a change.

In this study, in terms of reflecting the changes as a result of process steps, there was a good correlation between the different methods (Table 2.4). The measurement of antioxidant activities, cannot be evaluated sufficiently by a single method. Therefore, it is advisable to use more than one method because differences are observed even among methods with the same principle such as DPPH and FRAP.

4.3.4 Correlation Between Spectrophotometric Assays

Pearson correlation coefficients for spectrophotometric assays ranged from 0.417 to 0.951 (Table 4.8). TPC, ABTS and FRAP showed a linear relationship with a high

correlation coefficient. Among all three assays, the highest correlation was demonstrated between ABTS and FRAP (0.951), followed by TPC and FRAP (0.945), TPC and ABTS (0.913) and ABTS and DPPH (0.857) ($p<0.01$). These results suggest that phenolic compounds contributed to the antioxidant capacity of the investigated processed cowpea.

Table 4. 8 : The correlation coefficients for spectrophotometric assays.

	TPC	TFC	DPPH	ABTS	FRAP
TPC	1	0.417	0.708**	0.913**	0.945**
TFC	0.417	1	-0.005	0.344	0.491
DPPH	0.708**	-0.005	1	0.857**	0.796**
ABTS	0.913**	0.344	0.857**	1	0.951**
FRAP	0.945**	0.491	0.796**	0.951**	1

**. Pearson Correlation is significant at the 0.01 level (2-tailed).

4.3.5 Major Individual Phenolic Compounds

In order to have a general perspective on the change of the content of phenolic compounds in the process steps, selected standards were used for HPLC analysis. Major phenolic compounds of processed cowpeas and their digestion fractions are reported in Table 4.9, belonging to phenolic acids and flavonoids. It was observed that processing steps affected phenolic compounds found in cowpea. As a consequence of process steps, new phenolic compounds that were not found in the raw sample showed up, while some compounds found in the raw sample were not found or their amount decreased due to processsing.

One of the important results is that, luteolin was found in high quantities after soaking, dehulling, germination and fermentation process as well as vanillic acid was also seen in different fractions of dehulled, germinated and fermented samples, although they were not detected in raw sample. Also, in small amounts caffeic acid occurred in initial fractions of soaked, dehulled, germinated samples. In addition to that, there was an increase in the amount of some phenolic compounds after processing such as sinapic acid after germination, gallic acid, naringenin and p-coumaric acid after fermentation. Interestingly, quercetin that was not found in raw sample, was measured only after dehulling and germination process.

Despite the positive effect of processing steps, not only a decrease in the amount of some compounds was observed but also, losses were observed after process steps.

One of the most remarkable losses was that catechin was not found after dehulling, cooking, germination, and fermentation. Rosmarinic acid was found in small amount compared raw cowpea and different fractions of soaked, cooked, germinated and fermented sample. Besides, the amount of p-coumaric acid was increased after fermentation whereas, a decrease was observed in the others processes.

Nderitu *et al.* (2013) reported a decrease in TPC value after cooking process in cowpea. However, some phenolic compounds increased with cooking process while some compounds disappeared like quercetin derivatives. They mentioned some reasons for this alteration i.e. some phenolic compounds might have formed complexes with proteins and carbohydrates after thermal treatment and thus became unextractable. Some phenolic compounds could also have been oxidized during cooking (Siddhuraju & Becker, 2007).

Duenas *et al.* (2005) reported that phenolic composition of cowpea was slightly modified with fermentation process, some phenolic compounds increased (procatechuin acid, hydroxybenzoic acid and vanillic acid) while others decreased (quercetin glycosides, quercetin 3-*O*-glucoside and quercetin 3-*O*-galactoside) and some remained the same. Fermentation also gave rise to some phenolic compounds (tyrosol and some quercetin derivatives) not detected in raw samples. Contrary to these results, a general decrease in gallic acid was observed after fermentation with *L. plantarum.* Besides, Bishnoi and Khetarpaul (1998) found a decrease and losses in concentration of phenolic compounds after soaking and dehulling in cowpea.

Table 4. 9 : Major individual phenolic compounds in processed cowpea before and after *in vitro* digestion (The results were expressed as µg/g dw).

	RCP			**SCP**			**DCP**		
	INITIAL	**IN**	**OUT**	**INITIAL**	**IN**	**OUT**	**INITIAL**	**IN**	**OUT**
Vanilic Acid								3.71	
Luteolin				2944	145		412	260	
Gallic acid		16	73		8.18	54		12	
Cathechin	12	22	18	2.14	33				
Syringic acid		43	56		79		5.03	0.54	
Naringenin	86		549	199	81		427	125	202
Quercetin							40	11	
Cafeic acid				1.56			15	ND	
p-coumaric acid	729			58			138		
Sinapic acid	1.30	7.05					16		
Rosmarinic acid	216			133					

Table 4. 9 (continuing) : Major individual phenolic compounds in processed cowpea before and after *in vitro* digestion (The results were expressed as µg/g dw).

	CCP			GCP			FCP		
	INITIAL	**IN**	**OUT**	**INITIAL**	**IN**	**OUT**	**INITIAL**	**IN**	**OUT**
					ıg/g dw				
Vanilic Acid			22			20	44		
Luteolin					278			1096	
Gallic acid	25	15	133		11		0.30	66	198
Cathechin									
Syringic acid				7.21	17	87			
Naringenin	209	171		447	91	404	1342	674	3562
Quercetin				36	11	36			
Cafeic acid				25					
p-coumaric	515			311			1645		
Sinapic acid	17			17					
Rosmarinic acid	0.22			16.15	3.01				21

4.3.6 *In vitro* gastrointestinal (GI) digestion

The impact of processing steps on *in vitro* GI digestion of TPC is shown in Figure 4.1. The compounds in the dialyzed free fractions (IN) and non-dialyzed free fractions (OUT) were evaluated after digestion. Processing steps significantly affected the amount of IN and OUT fractions of cowpea.

The amount of TPC in IN and OUT fraction deceased with soaking, dehulling and germination process while an increase was observed after fermentation ($p<0.05$).

When TPC values after digestion was analyzed compared with each process steps, 38% of the compounds were present in IN fractions for raw cowpea while after dehulling (33%), soaking (25%), fermentation (23%), cooking (23%) and germination (21%) processes; the recovery, which the relative amount of the fractions to the initial value, were decreased.

Similarly, when recovery ratios of OUT fractions were compared, the highest recovery was found in raw cowpea (139%) and followed by dehulled cowpea (117%), fermented cowpea (95%), soaked cowpea (89%), cooked cowpea (72%) and germinated cowpea (68%).

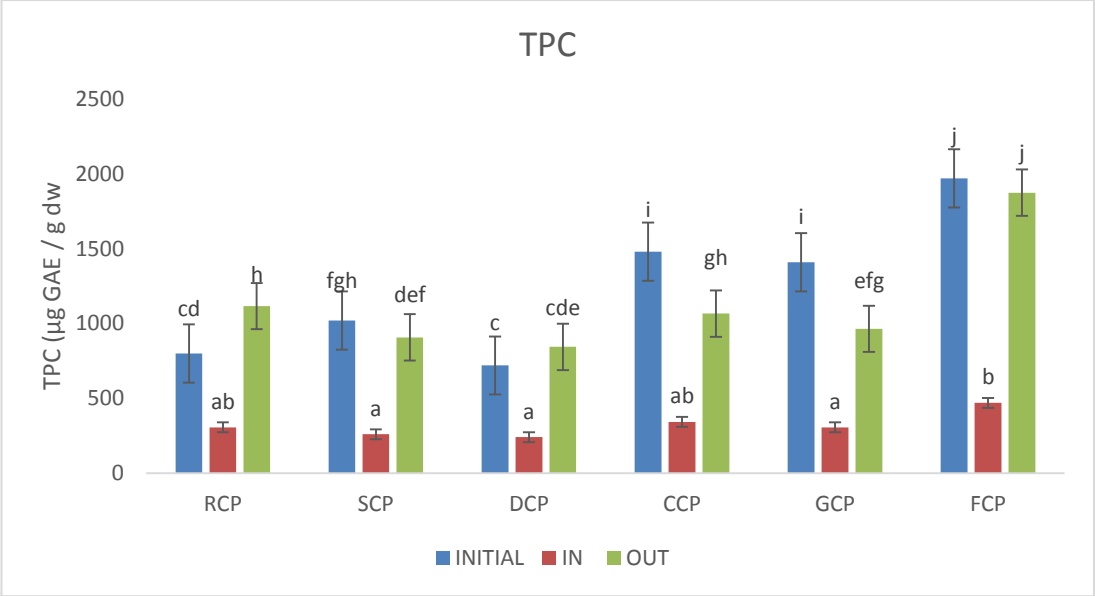


Figure 4. 1 : TPC of processed cowpea, expressed as GAE per g dw.

The terms represent; Initial, as initially determined from cowpea; IN, dialyzed free fraction after intestinal digestion; OUT, non-dialyzed free fraction after intestinal digestion. Data represents average values present error indicators as standard deviation of 2 independent samples. Different letters represent statistically significant differences ($p<0.05$).

Although there were much higher phenolic compounds in OUT fractions, IN fractions have quite low TPC values. This can be explained by the fact that the interaction of phenolic compounds with other compounds had high molecular weight such as fibers, may have an adverse effect on the absorption of the polyphenolic compounds.

The theory behind the Folin-Ciocalteau method, it strongly relies on the reduction of the Folin-Ciocalteau reagent. For this reason, any reducing component such as small peptides or reducing sugars formed by digestion or fermentation can interfere in the Folin-Ciocalteau assay (Ikawa *et al*., 2003). However, in this study there was no big differences in the observed TPC values between Initial and OUT fractions of all samples, but after fermentation process an increase in OUT fraction of cowpea was observed proportionally with initial TPC of fermented cowpea.

After the *in vitro* simulation of GI digestion, for cooking, germination, and fermentation process, same TFC values was observed in IN free fractions with raw cowpea while dehulled cowpea had the lowest free TFC values in IN fraction (Figure 4.2) ($p<0.05$). Also, the dialyzed flavonoid fraction (IN) represented 71% of the initial free TFC value for raw cowpea which had highest recovery. If compared with process steps, a decrease was observed in IN fractions for fermentation (67%),

soaked (42%), germination (36%) and the lowest recovery was found after dehulling (28%) process. The recovery ratio of fermented cowpea (491%) in free OUT fraction was higher whereas the other process steps were lower than free OUT fraction of raw sample (475%).

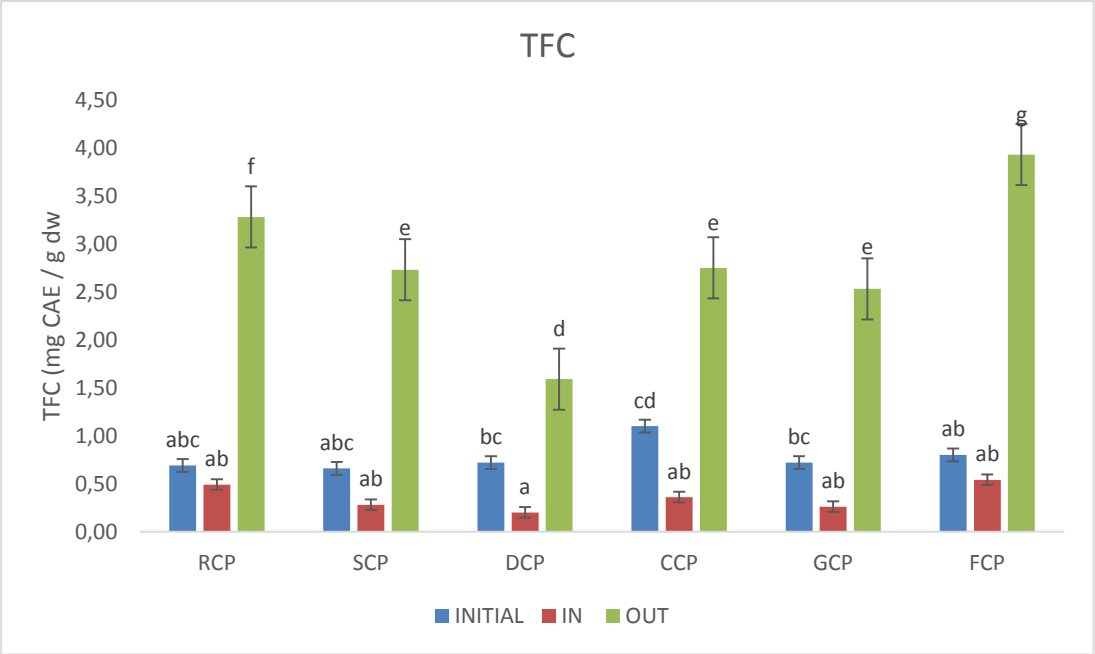


Figure 4. 2 : TFC of processed cowpea, expressed as CAE per g dw.

The terms represent; Initial, as initially determined from cowpea; IN, dialyzed free fraction after intestinal digestion; OUT, non-dialyzed free fraction after intestinal digestion. Data represents average values present error indicators as standard deviation of 2 independent samples. Different letters represent statistically significant differences ($p<0.05$).

According to the results of TFC, though the process steps did not affect IN fractions of cowpea after *in vitro* digestion, OUT fraction increased with fermentation process. Also, the increase in OUT fractions compared with the others fractions, it may be associated with the release of flavonoids bound to some structures like protein as a result of more effective protein digestion by both the pepsin and pancreatic protease (trypsin) with their varying specificities the simulated digestion (Apea-Bah *et al.*, 2016).

In order to determine the effect of *in vitro* GI digestion on TAC; DPPH, ABTS and FRAP assays which showed different trends according to the results, were performed (Figure 4.3a). Comparing the DPPH results, the lowest TAC of IN fractions was found in soaked (2.11 mg TEAC/g dw) and dehulled (1.74 mg TEAC/g dw) cowpea while raw sample had highest TAC of IN fraction (5.87 mg TEAC/g dw).

On the other hand, overall view of the results of ABTS, there was quite big increase in the amount of IN and OUT fractions of samples compared initial values (Figure 4.3b). The amount of IN fraction remained the same after processing steps, except fermentation made an increase in amount of IN fraction (142.94 µmol/g dw) as well as OUT fraction ($p<0.05$).

Contrary to ABTS results, after *in vitro* (GI) digestion, IN and OUT fractions in FRAP were lower than initial TAC values (Figure 4.3c). The amount of IN fraction decreased with processing steps, especially the lowest TAC was observed after dehulling and followed by fermentation.

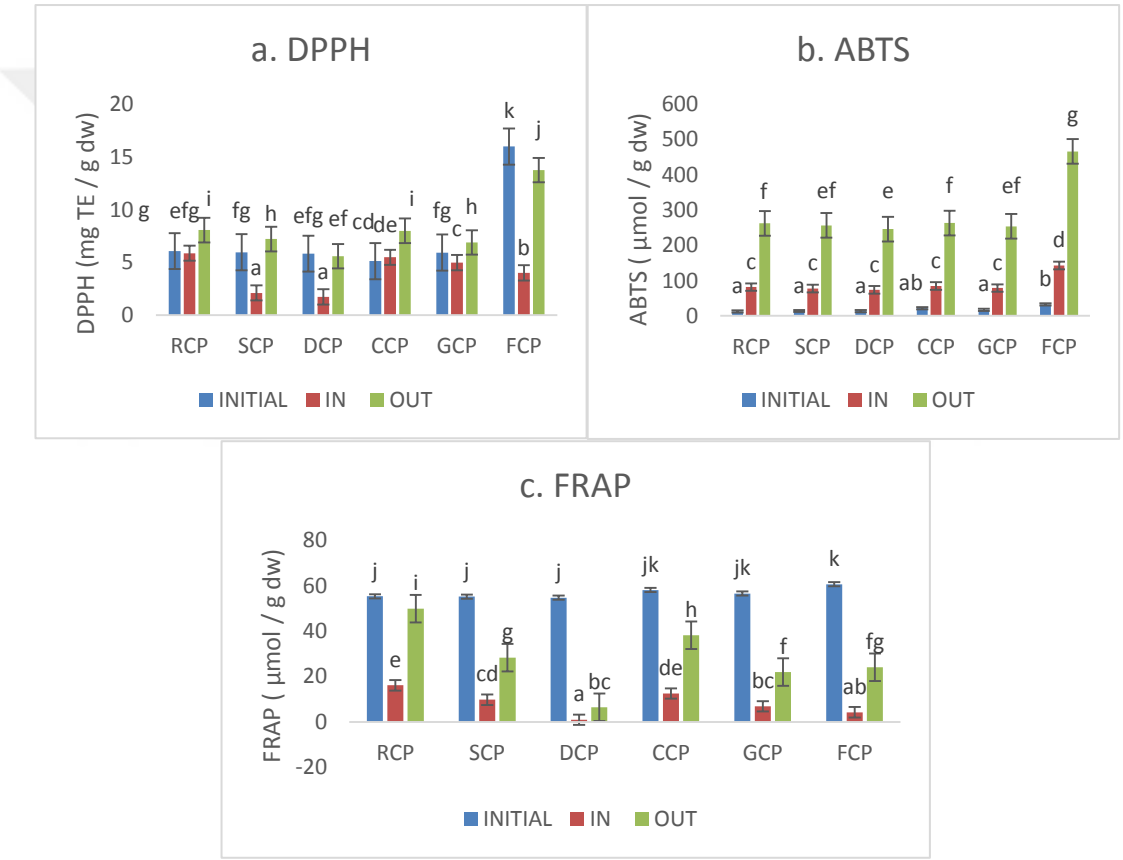


Figure 4. 3 : Antioxidant capacities of processed cowpea.

The terms represent; Initial, as initially determined from cowpea; IN, dialyzed fraction after intestinal digestion; OUT, non-dialyzed fraction after intestinal digestion. Data represents average values present error indicators as standard deviation of 2 independent samples. Different letters represent statistically significant differences ($p<0.05$).

The basic reason of the increase in OUT fractions may be that bound phenolic compounds may became soluble in dialyze fluids especially due to break down reactions during the fermentation. This also may be attributed to the release of extractable phenolic compounds bound to proteins during the simulated gastric and

duodenal phase. Although the released phenolic compounds may have interacted with peptides resulting from protein digestion, they exhibited higher radical scavenging properties than when bound to unhydrolyzed, denatured protein in the undigested cowpea (Apea-Bag *et al*., 2016). In literature, it was mentioned that FRAP and ABTS assays were both electron transfer based methods (Chen *et al*., 2015), nonetheless, initial values of FRAP was higher than other fractions. It may be because of the chemical compounds found in the digestive fluid which may prevented the oxidation of ferrous ions. Also, similar graphs were seen in both TPC and DPPH. This may be owing to the similar reaction mechanisms of the TPC and DPPH which are based on electron transfer (Wootton-Beard *et al.*, 2011).

On the other hand, comparing the individual phenolic compounds after digestion, the IN fractions of processed with raw cowpea, luteolin was formed in processed cowpea except cooked sample; the highest luteolin content measured was in fermented cowpea (1096 µg/g dw) among IN fractions. The amount of gallic acid in IN fraction increased with both cooking and fermentation while a decrease in IN fraction was observed after soaking, dehulling and germination. However, naringenin was not detected in raw sample, it emerged IN fractions of all process steps; especially fermented cowpea had highest naringenin content (3562 µg/g dw) among the others. Also, quercetin was detected just after dehulling and germination process.

Furthermore, viewing the results of the OUT fractions of processed and raw cowpea, luteolin was not observed in any of the OUT fraction of samples, while syringic acid was just found in raw and germinated samples. The amount of naringenin decreased with both dehulling and germination process, also it was not found after soaking, whereas an increase in the amount after fermentation compared with raw sample was observed. Interestingly, quercetin was only observed in germinated samples, while rosmarinic acid was just detected in fermented cowpea among OUT fractions.

4.3.7 *In vitro* protein digestibility (IVPD)

To identify the possible impact of processing on protein digestibility, the multi-enzyme (trypsin, chymotrypsin and peptidase) method was used as a measurement for the in vitro protein digestibility. IVPD of processed cowpeas is summarized in Table 4.10. Protein digestibility was significantly increased by the process steps and

the highest increase was observed in cooked cowpea (5%) and followed by fermented cowpea (4%), dehulled cowpea (4%), germinated cowpea (3%) and lowest increase was found in soaked cowpea (1%).

Table 4. 10 : *in vitro* protein digestibility of processed cowpea.

Material	*In Vitro* Protein Digestibility (%)
Raw Cowpea	76.97 ± 0.13^{a}
Soaked Cowpea	78.06 ± 1.79^{b}
Dehulled Cowpea	80.05 ± 0.26^{c}
Cooked Cowpea	81.05 ± 0.64^{d}
Germinated Cowpea	79.80 ± 0.64^{c}
Fermented Cowpea	80.51 ± 0.25^{cd}

Values expressed are mean ± standard deviation. (n=2)

Increased IVPD by cooking process can be attributed to the inactivation of antinutritional compounds such as trypsin inhibitors, lectins, and the reduction of antinutritional chemicals such as tannins, phytates present in cowpea as also mentioned by Park *et al.* (2010) for others legumes. Also, the breakdown of proteins occurs with increased temperature, which can contribute positively to the digestion of proteins.

Moreover, owing to the microorganisms involved in fermentation, proteins in complex structure are converted into compounds more suitable for digestion as well as some enzymes become active, degrading proteins to small structures during germination. Ghavidel and Prakash (2007) and Azarpazhooh and Boye (2013) also reported an increased protein digestibility after germination (2-4%). Additionally, the increase was reported with soaking by Alonso *et al.* (2000) (4.85%) and Rehman and Shah (2001).

Legumes provide an excellent plant protein resource for human diets, however, their proteins are less readily digestible than animal proteins. Hence, the process steps such cooking, fermentation, germination, dehulling and soaking for increasing the digestibility of proteins are highly effective.

4.3.8 Trypsin inhibitor activity (TIA)

Table 4.11 summarizes the effect of different process steps on trypsin inhibitor activity in cowpea. There was a significant reduction measured in the trypsin

inhibitor activity after soaking (8%), germination (30%), cooking (89%) compared to raw cowpeas ($p<0.05$). After fermentation, no trypsin inhibitor activity was detected. However, an increased activity of the trypsin inhibitor was observed in dehulled cowpea (11%) compared to the raw samples.

Table 4. 11 : Trypsin inhibitor activity of processed cowpea.

Material	Trypsin Inhibitor Activity (TIU/mg dw)
Raw Cowpea	36.74 ± 0.27^{e}
Soaked Cowpea	33.91 ± 0.08^{d}
Dehulled Cowpea	40.96 ± 0.06^{f}
Germinated Cowpea	25.71 ± 0.09^{c}
Cooked Cowpea	4.13 ± 0.06^{b}
Fermented Cowpea	ND^{a}

ND: not detected

The trypsin inhibitor can be altered with increasing temperature because of its protein structure. Thus, cooking usually inactivates heat sensitive factors such as trypsin inhibitors as a result of denaturation of these heat-liable proteins. Also, Boye *et al.* (2010) and Weder *et al.* (1983) reported that trypsin inhibitor activity was significantly reduced after soaking and boiling steps in cowpea.

Trypsin inhibitor activity was significantly affected during fermentation and its activity decreased. It may be attributed to the proteolytic activity that is associated with fermentation processes, affecting the protein structures (Azarpazhooh and Boye, 2013). Also, a similar change in trypsin inhibitor activity from raw to fermented legumes was reported by Phillips (2013) and Khattab and Arntfield (2009).

Germination has many catabolic reactions as the reserved substances present in the cotyledon like trypsin inhibitors are used for the development and growth of the embryo. Sangronis and Machado (2007) indicate that there is a possibility that the trypsin inhibitors could be utilized as an energy source during the early stages of germination. Akpapunam and Sefa-Dedeh (1997) mentioned that the germination of jack bean was more effective than cooking on the reduction of the trypsin inhibitor activity. On the contrary, Khaleque et al. (1985) reported that germination of jack bean did not significantly modify the trypsin inhibitor activity whereas Oloyo (2004) determined an increase in the trypsin inhibitor activity in pigeon bean.

On the other hand, the increase in the trypsin inhibitor activity with the removal of the hulls, is due to the fact that the trypsin inhibitors may be characteristically present in the cotyledon fractions of the seed. Also, removing the hulls may lead to an increase in the concentration of trypsin inhibitor activity on a unit weight basis. The increase in trypsin inhibitor activity after dehulling as opposed to the other processes, was also reported by Deshpande *et al*. (1982).

4.3.9 SDS-PAGE

SDS Page analysis was carried out to evaluate the effect of different process steps on the protein profile of cowpea. The gels are presented in Figure 4.4 and as protein standard, marker was used to compare the molecular weight ranges of the gel bands. The electrophoretogram showed that different process steps contained almost similar bands except cooked cowpea in the range of 21.5 kDa to 191 kDa. For raw and soaked cowpea 5 major bands at 26.40, 31.50, 52.3, 62 and 70.5 kDa as the molecular sizes of the main polypeptides were observed while germinated, dehulled and fermented cowpea had 6 major bands at 26.40, 31.50, 52.3, 62, 70.5, also 28.6 kDa. However, cooked cowpea had just one major band at 31.5 kDa. They had also same minor bands at 21.5, 36, 95.5, 117 and 191 kDa.

Moreover, when compared to bands found to be trypsin inhibitors by molecular weight, the clearest band was seen in dehulled cowpea and followed by raw, soaked and germinated cowpea while there was no band which molecular weight (21.5 kDa) equal to trypsin inhibitor in cooked cowpea and fermented cowpea.

Mune (2007) found that 3 major bands about 40, 65 and 72 kDa and minor bands of about 60,70 and 98 kDa in Bambara beans and 3 major bands and 6 minor bands in cowpea protein isolates while Lopez *et al.* (1991) reported that chickpea had bands range from 16.6 to 66.4 kDa. Similar bands were also reported for lentil protein isolates (Joshi *et al*., 2011). Furthermore, 3 major bands at 40, 60 66.2 kDa and 3 minor bands in the range of 10 to 20 kDa and 80 to 200 kDa was found in cowpea protein isolates by Horax *et al*. (2004).

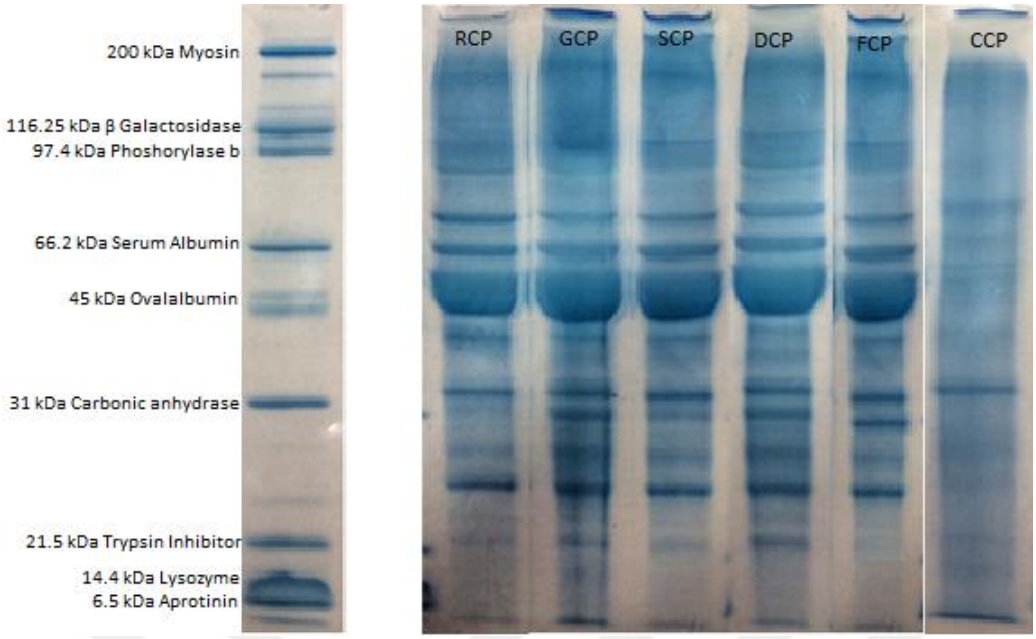


Figure 4. 4 : SDS Page analysis of processed cowpeas.

RCP: raw cowpea; GCP: germinated cowpea; SCP: soaked cowpea; DCP: dehulled cowpea; FCP: fermented cowpea; CCP: cooked cowpea

5. CONCLUSION

Traditional process steps have been used for many years not only to prepare food to be edible, provide product diversity and create richness in tastes, but also improve the nutritional values while inactive antinutritional factors. In this study, changes that occur in the health beneficial components of cowpea as result of processing steps involving soaking, dehulling, cooking, germination and fermentation were investigated.

It was observed that both free and bound fraction of the total phenolic content (TPC), total flavonoid content (TFC) and antioxidant capacity showed significant differences depending on the varieties of cowpea. However, the improvement application targeting some quality parameters related to yield and yield components, nutritional value, and many other factors, seems to be ineffective in terms of increasing the amount of phenolic compounds and antioxidative capacity.

One of the major findings of this study was an increase both in TPC and TFC of cowpeas after fermentation, cooking and germination process while there was no significant difference after soaking and dehulling step with raw cowpeas.

With regard to the result of HPLC analysis for individual phenolic compounds, some compounds (luteolin, vanillic acid and caffeic acid) appeared and the amount of some compounds (naringenin, sipanpic acid, gallic acid and p-coumaric acid) increased whereas some of them (catechin) were not detected after some processing steps.

The total antioxidant capacity (TAC) of cowpea, a quite increase after fermentation process and slight increase after cooking process was observed compared to raw cowpeas, while the other processing steps not significantly affected the TAC according to DPPH, ABTS and FRAP methods.

After simulated in vitro gastrointestinal (GI) digestion, the recovery that the ratio of the amount found in the dialyzed fraction of initial TPC to initial TPC decreased after processing steps. But, the amount in the dialyzed fraction of TPC increased in

fermented cowpea while the dialyzed fractions of other steps remained same compared the dialyzed fraction of raw cowpea.

It was observed that the amount found in the dialyzed fraction of initial total flavonoid content decreased with dehulling steps whereas after the other steps TFC found in the dialyzed fraction remained same with raw cowpea. Besides, the recovery of TFC decreased with process steps.

According to DPPH that one of the methods used to determine total antioxidant capacity (TAC) of digested cowpeas, there was the decrease in dialyzed fraction of TAC after soaking and dehulling, the other steps did not change the value compared raw cowpea. The results of ABTS, the amount of the dialyzed fraction was remained same after processing steps, except fermentation made an increase in amount of the dialyzed fraction. Contrary to ABTS results, FRAP The amount of the dialyzed fraction decreased with all processing steps.

Cowpea samples were evaluated on the *in vitro* protein digestibility (IVPD) and it was found that IVPD was significantly increased by each processing steps, and the highest value was found after cooking. However, protein content of cowpea was decrease with all processing steps.

In addition to the effect of the process steps on the nutritional values, the effect on antinutritional compounds such as trypsin inhibitor activity was also examined. It was found that the trypsin inhibitor activity significantly decreased with processing steps; especially there was no inhibitor activity after cooking and quite low inhibitor activity after fermentation, while an increase was observed after dehulling.

In conclusion, this study focused on the effect of processing phenolic compounds, antioxidant activity, bioavailability and antinutritional compounds of cowpea. Although the results obtained with the model of simulated *in vitro* GI digestion cannot directly predict the human *in vivo* conditions, still this model is helpful for investigating the bioavailability of phenolic compounds. In further studies, it would be interesting to focus on not only other nutritional compounds such as carotenoids, proanthocyanidin and anthocyanin, but also other antinutritional compounds such as lectins, phytate, protease inhibitors, saponins, tannins, phytic acid. Also, the effect of novel food technology process on nutritional and antinutritional compounds in cowpea also can be remarkable. Furthermore, the studies related to amylase enzyme,

which is thought to be related to obesity, which is in a notable position with increasing consumer consciousness.

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APPENDICES

APPENDIX A: Calibration Curves

APPENDIX B: HPLC Chromatograms

APPENDIX C: ANOVA Tables

APPENDIX A

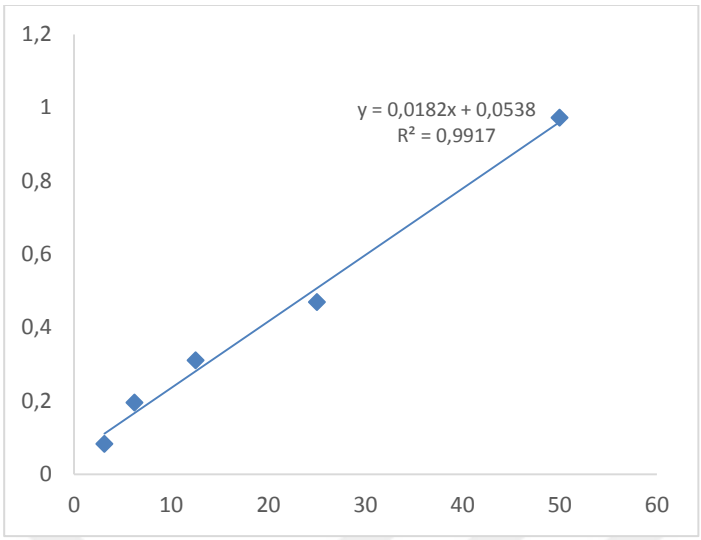

Figure A. 1 : Calibration curve for free fraction of TPC of cowpea varieties.

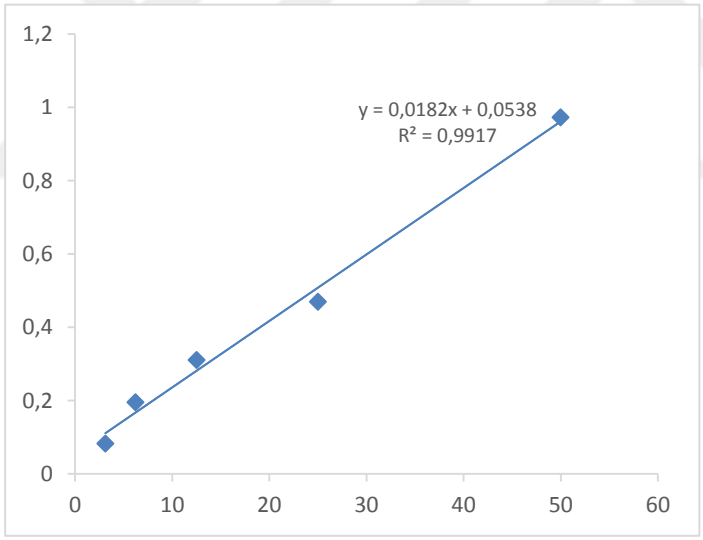

Figure A. 2 : Calibration curve for bound fraction of TPC of cowpea varieties.

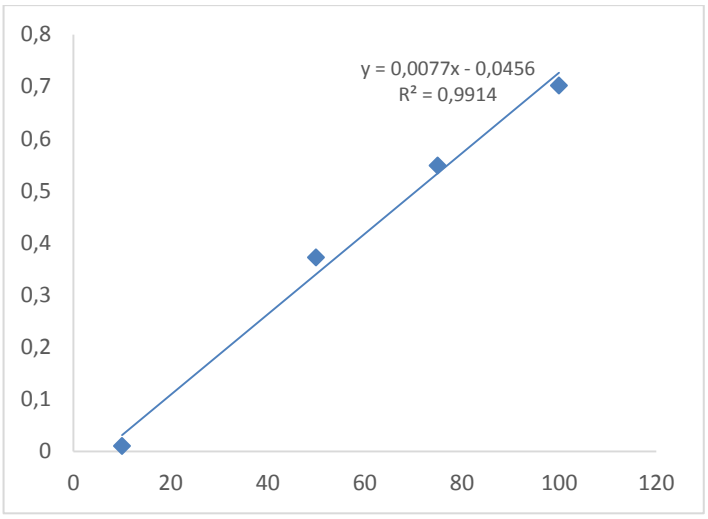

Figure A. 3 : Calibration curve for free fraction of TFC of cowpea varieties.

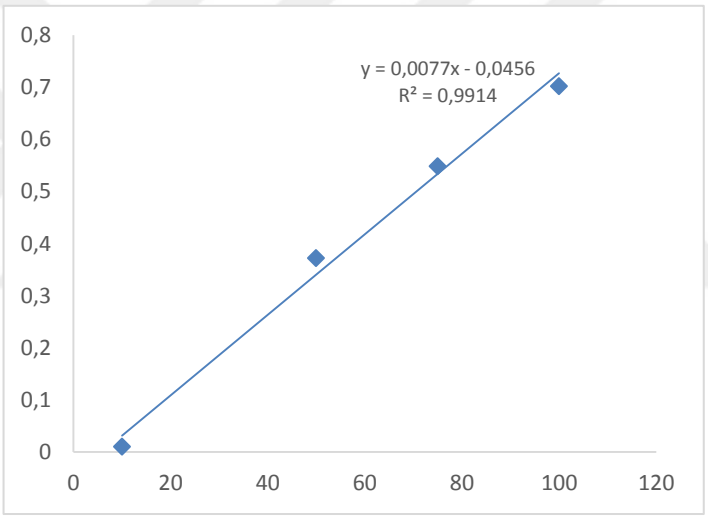

Figure A. 4 : Calibration curve for bound fraction of TFC of cowpea varieties.

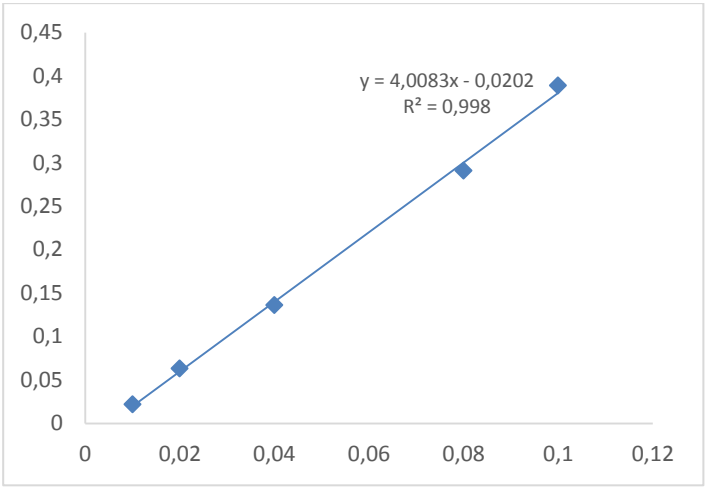

Figure A. 5 : Calibration curve for free fraction of DPPH of cowpea varieties.

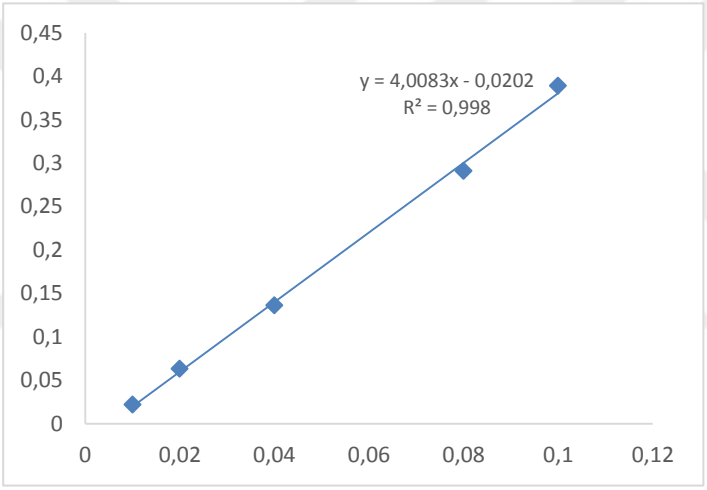

Figure A. 6 : Calibration curve for bound fraction of DPPH of cowpea varieties.

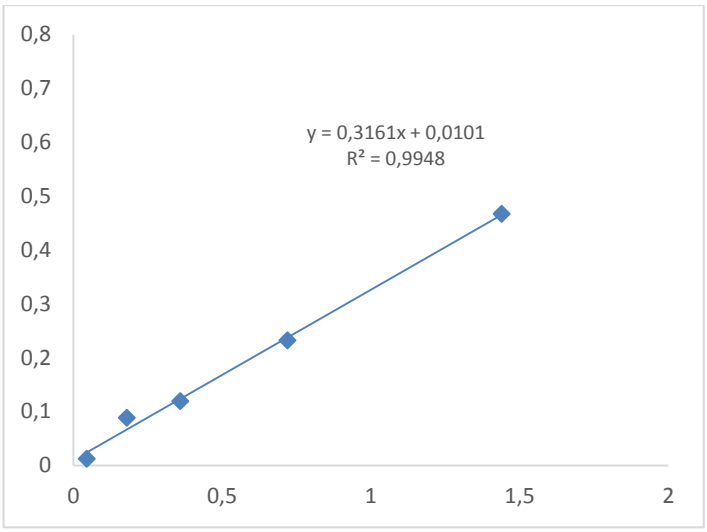

Figure A. 7 : Calibration curve for free fraction of ABTS of cowpea varieties.

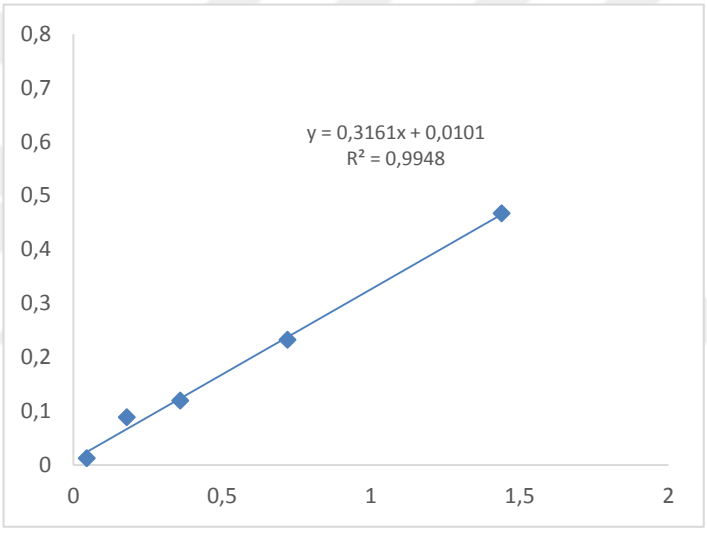

Figure A. 8 : Calibration curve for bound fraction of ABTS of cowpea varieties.

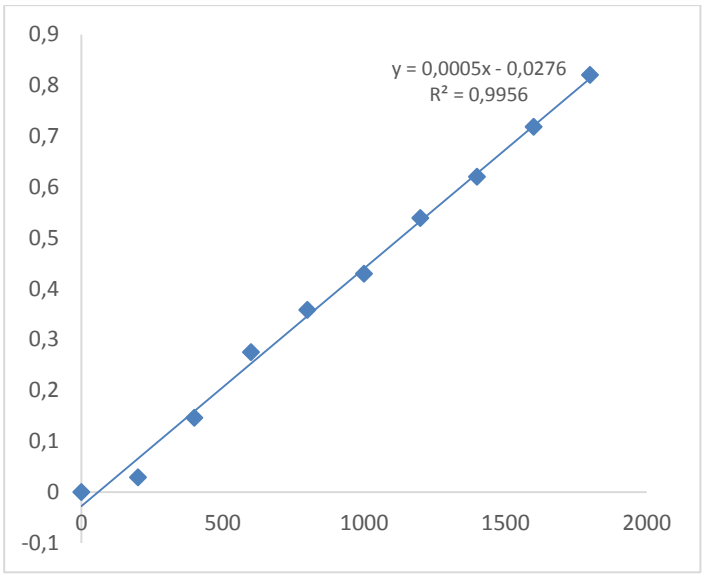

Figure A. 9 : Calibration curve for free fraction of FRAP of cowpea varieties.

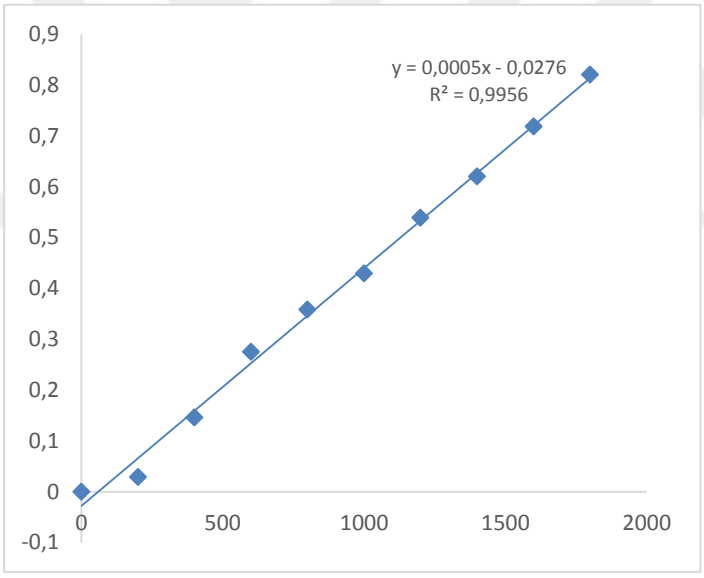

Figure A. 10 : Calibration curve for bound fraction of FRAP of cowpea varieties.

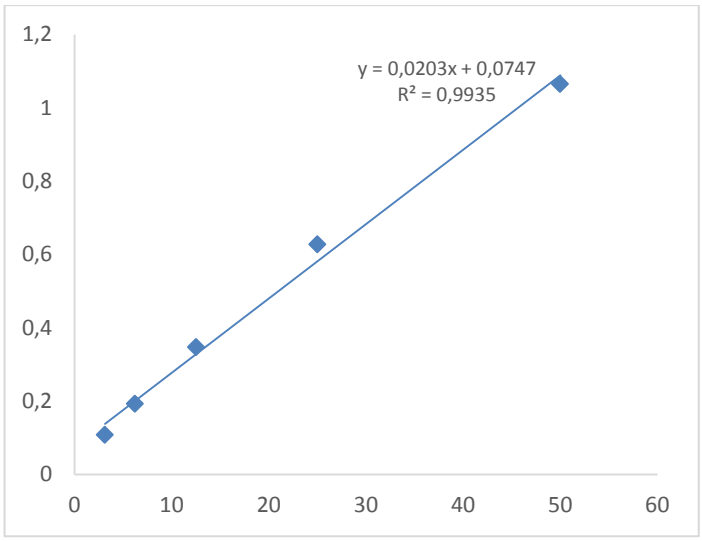

Figure A. 11 : Calibration curve for TPC of processed cowpeas.

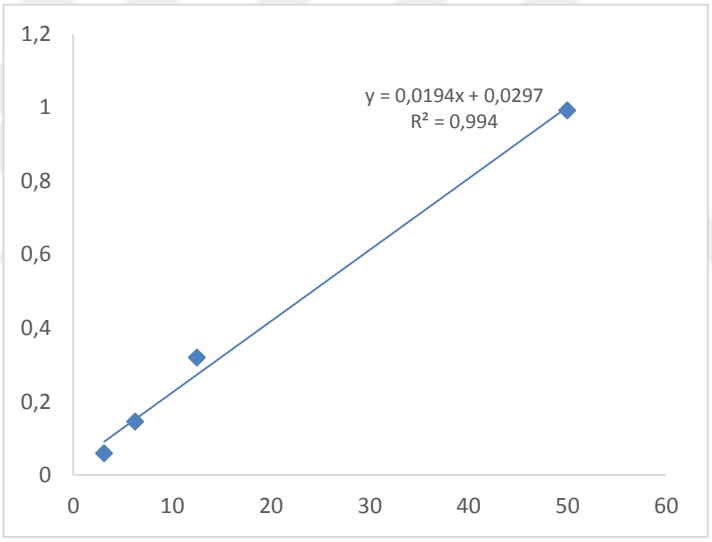

Figure A. 12 : Calibration curve for TFC of processed cowpeas.

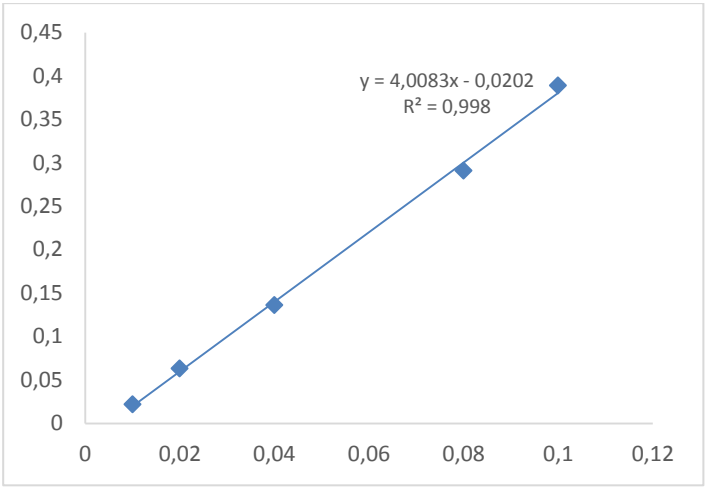

Figure A. 13 : Calibration curve for DPPH of processed cowpeas.

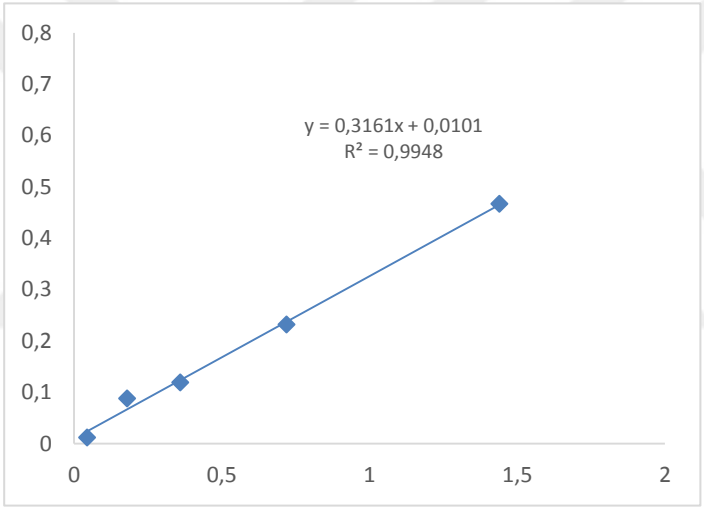

Figure A. 14 : Calibration curve for ABTS of processed cowpeas.

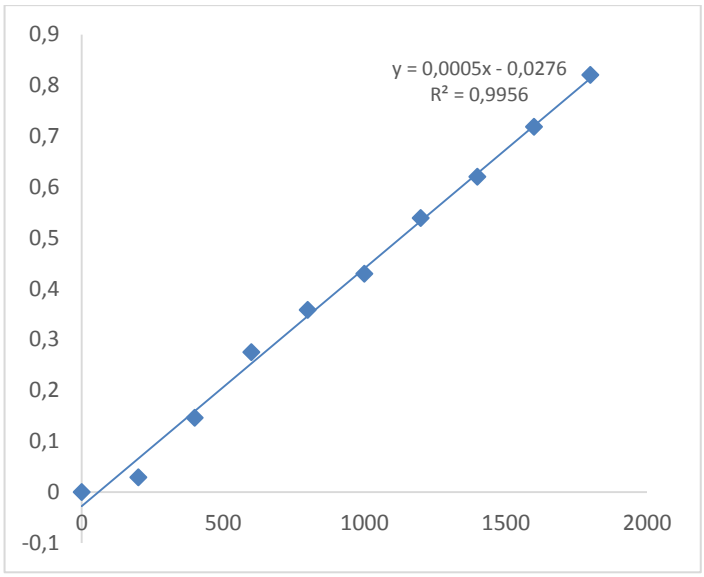

Figure A. 15 : Calibration curve for FRAP of processed cowpeas.

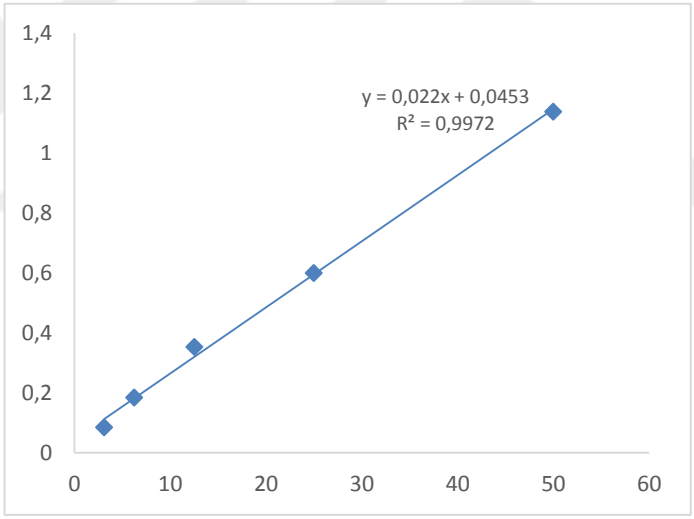

Figure A. 16 : Calibration curve for IN fraction of TPC of processed cowpeas after *in vitro* GI digestion.

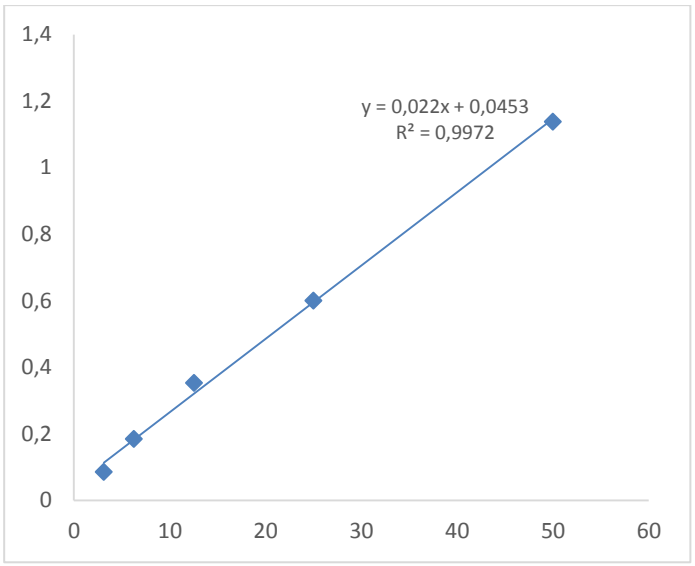

Figure A. 17 : Calibration curve for OUT fraction of TPC of processed cowpeas after *in vitro* GI digestion.

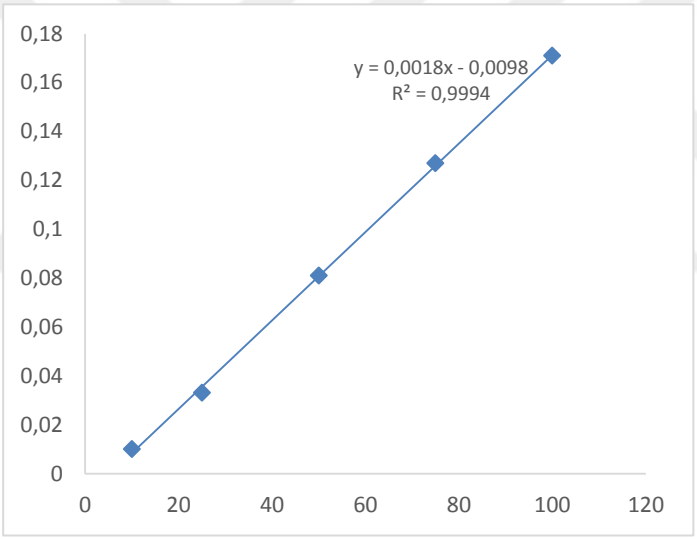

Figure A. 18 : Calibration curve for IN fraction of TFC of processed cowpeas after *in vitro* GI digestion.

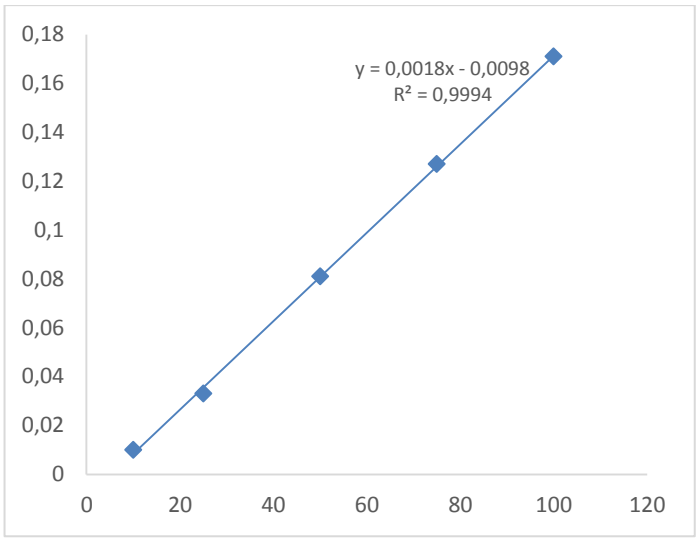

Figure A. 19 : Calibration curve for OUT fraction of TFC of processed cowpeas after *in vitro* GI digestion.

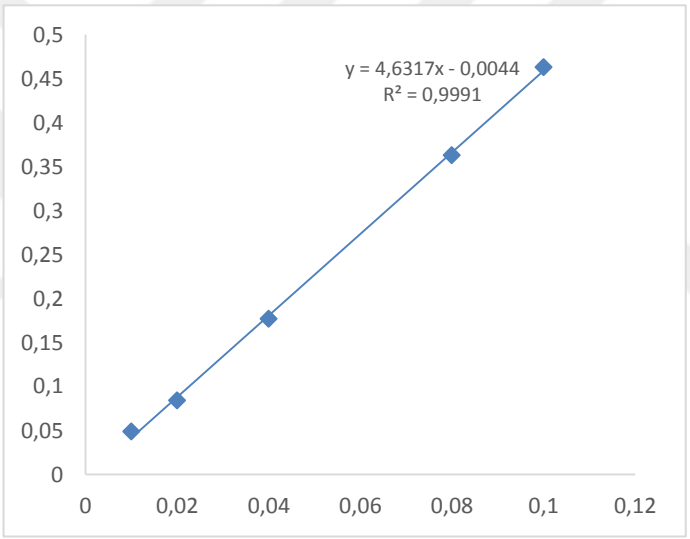

Figure A. 20 : Calibration curve for IN fraction of DPPH of processed cowpeas after *in vitro* GI digestion.

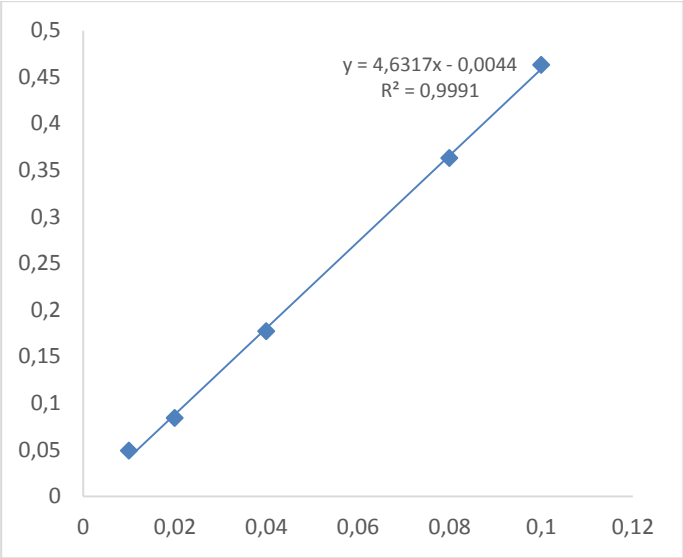

Figure A. 21 : Calibration curve for OUT fraction of DPPH of processed cowpeas after *in vitro* GI digestion.

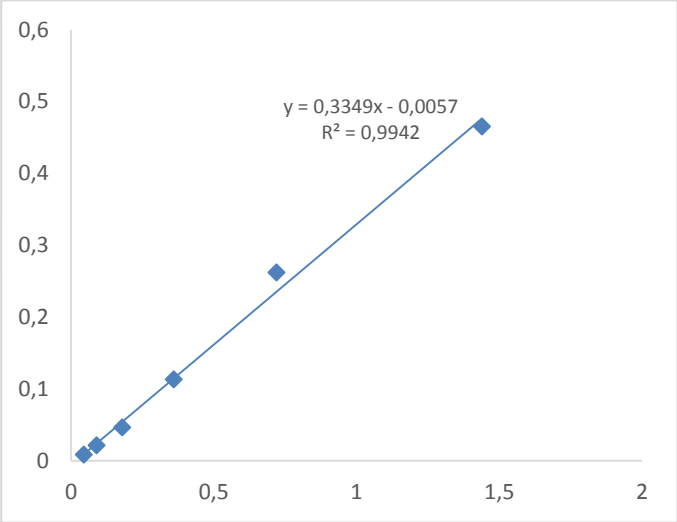

Figure A. 22 : Calibration curve for IN fraction of ABTS of processed cowpeas after *in vitro* GI digestion.

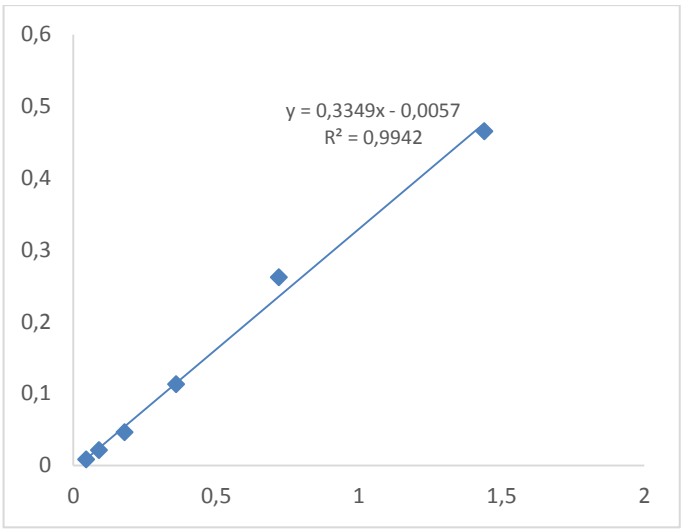

Figure A. 23 : Calibration curve for OUT fraction of ABTS of processed cowpeas after *in vitro* GI digestion.

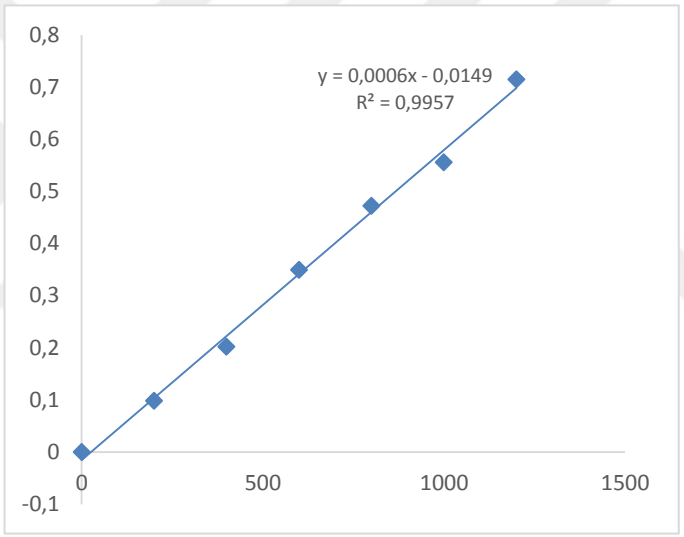

Figure A. 24 : Calibration curve for IN fraction of FRAP of processed cowpeas after *in vitro* GI digestion.

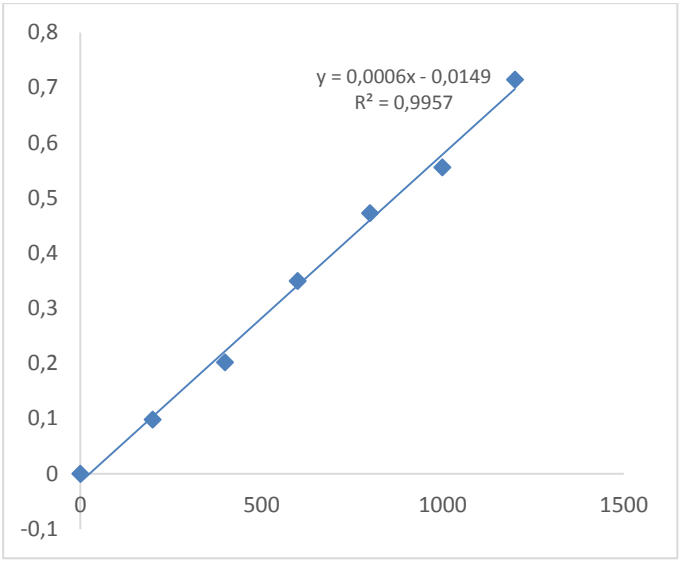

Figure A. 25 : Calibration curve for OUT fraction of FRAP of processed cowpeas after *in vitro* GI digestion.

APPENDIX B

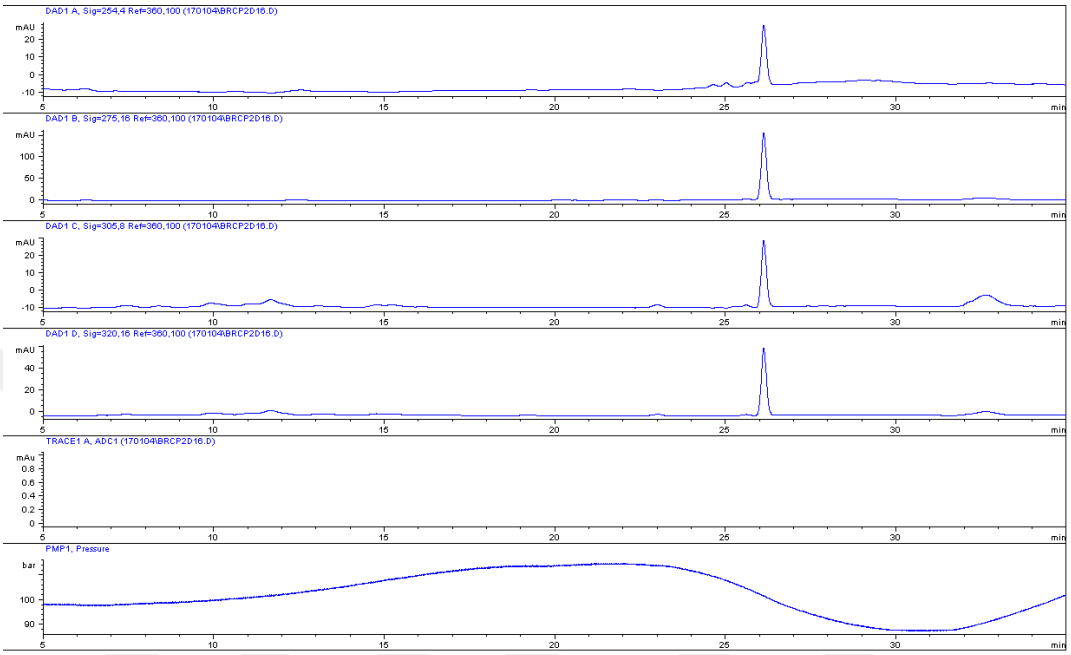

Figure B. 1 : HPLC chromatograms (recorded at 254.4, 275.16, 305.8, 320.16) of initial fraction of raw cowpea.

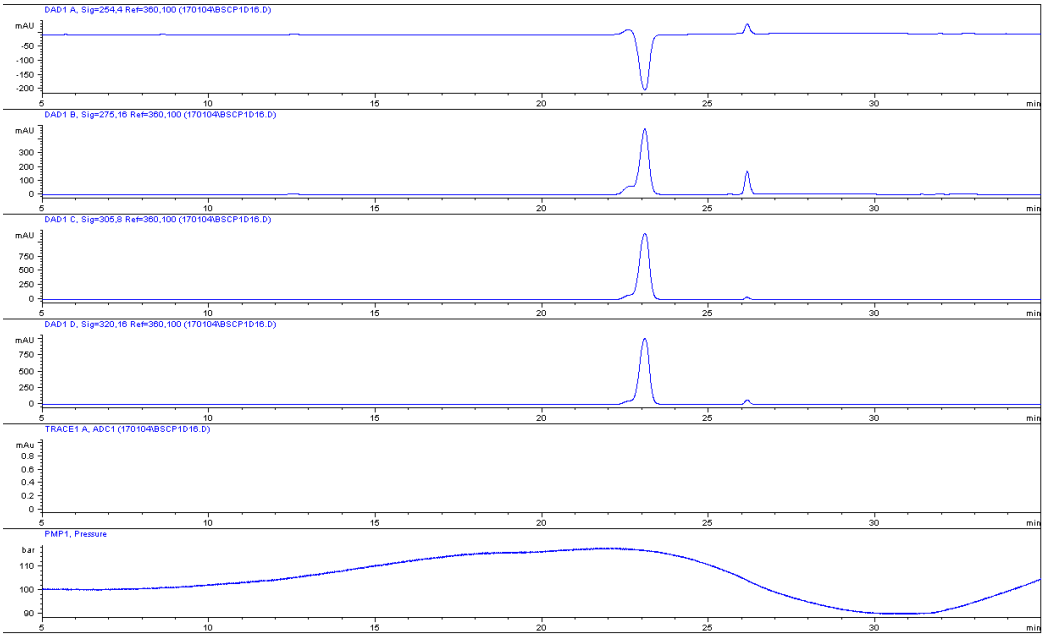

Figure B. 2 : HPLC chromatograms (recorded at 254.4, 275.16, 305.8, 320.16) of initial fraction of soaked cowpea.

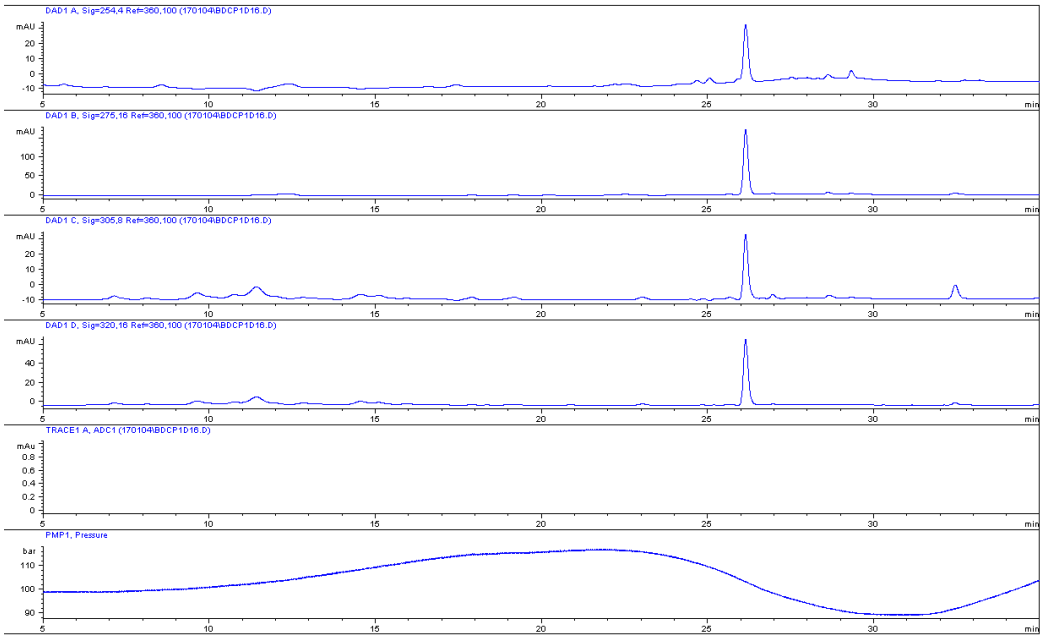

Figure B. 3 : HPLC chromatograms (recorded at 254.4, 275.16, 305.8, 320.16) of initial fraction of dehulled cowpea.

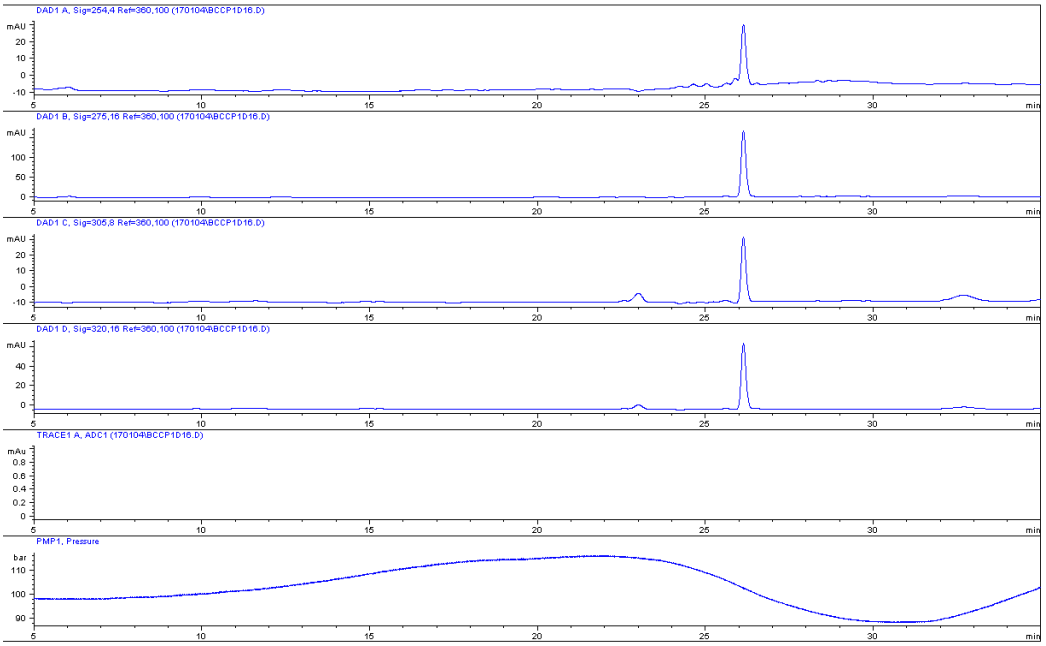

Figure B. 4 : HPLC chromatograms (recorded at 254.4, 275.16, 305.8, 320.16) of initial fraction of cooked cowpea.

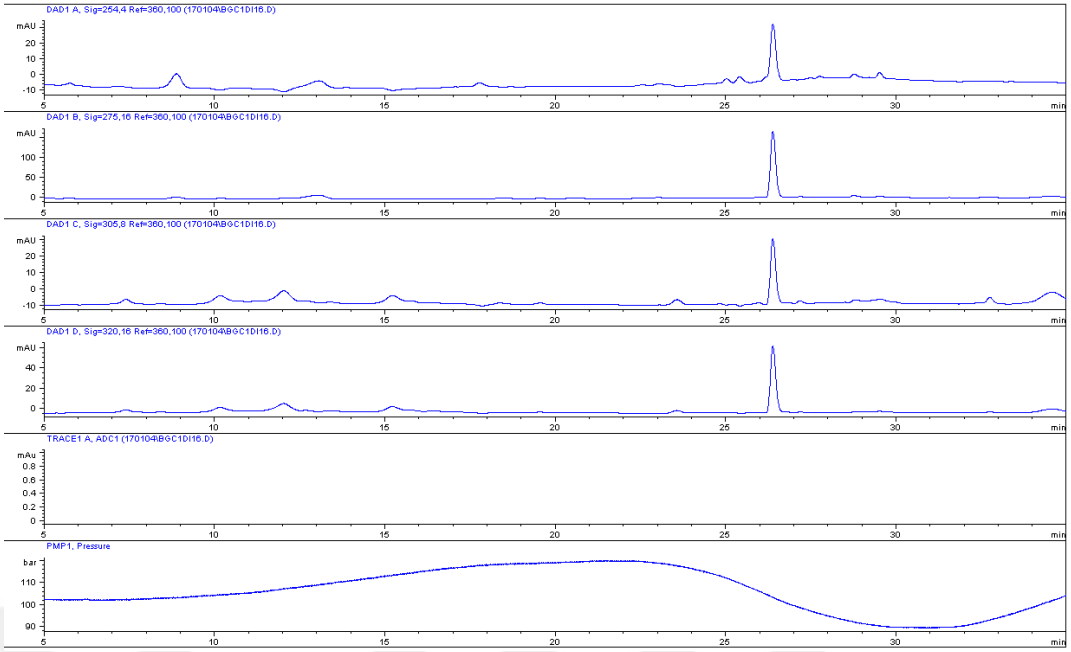

Figure B. 5 : HPLC chromatograms (recorded at 254.4, 275.16, 305.8, 320.16) of initial fraction of germinated cowpea.

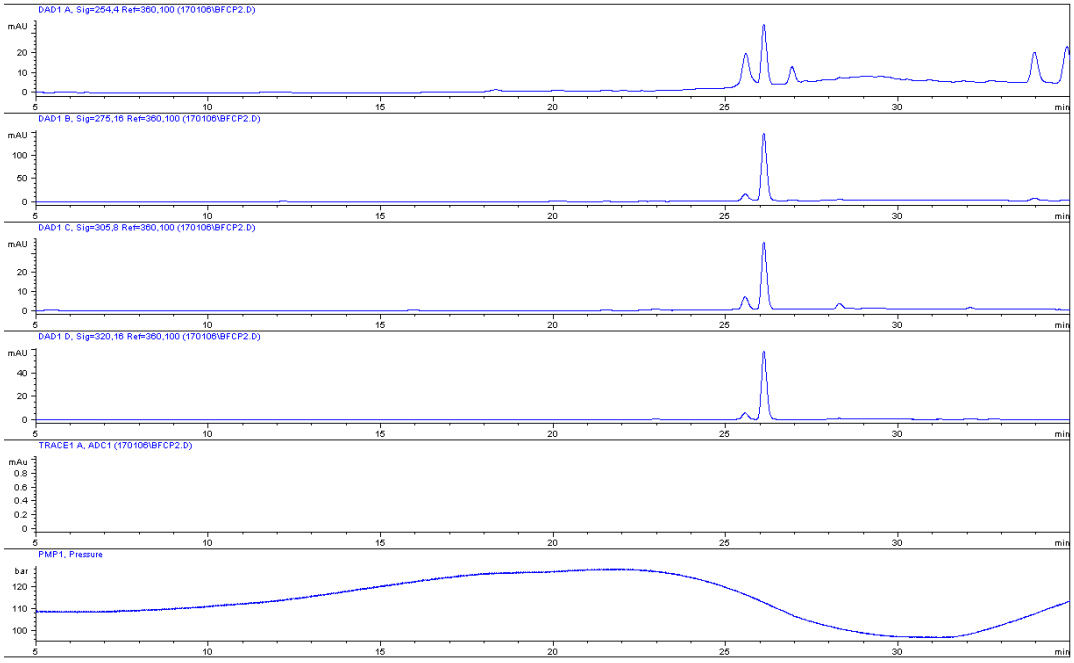

Figure B. 6 : HPLC chromatograms (recorded at 254.4, 275.16, 305.8, 320.16) of initial fraction of fermented cowpea.

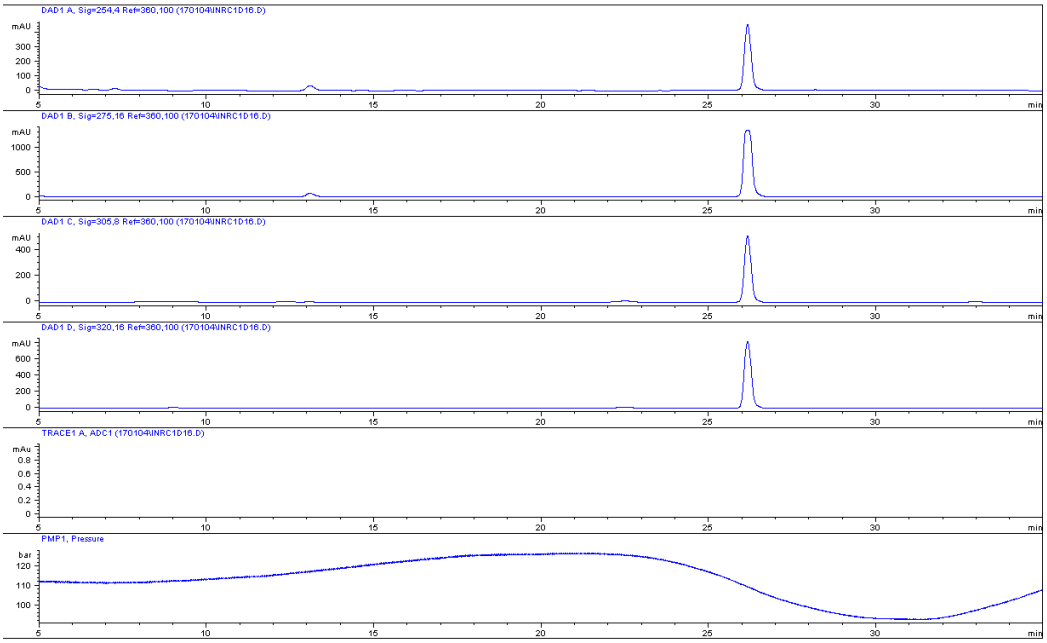

Figure B. 7 : HPLC chromatograms (recorded at 254.4, 275.16, 305.8, 320.16) of IN fraction of raw cowpea.

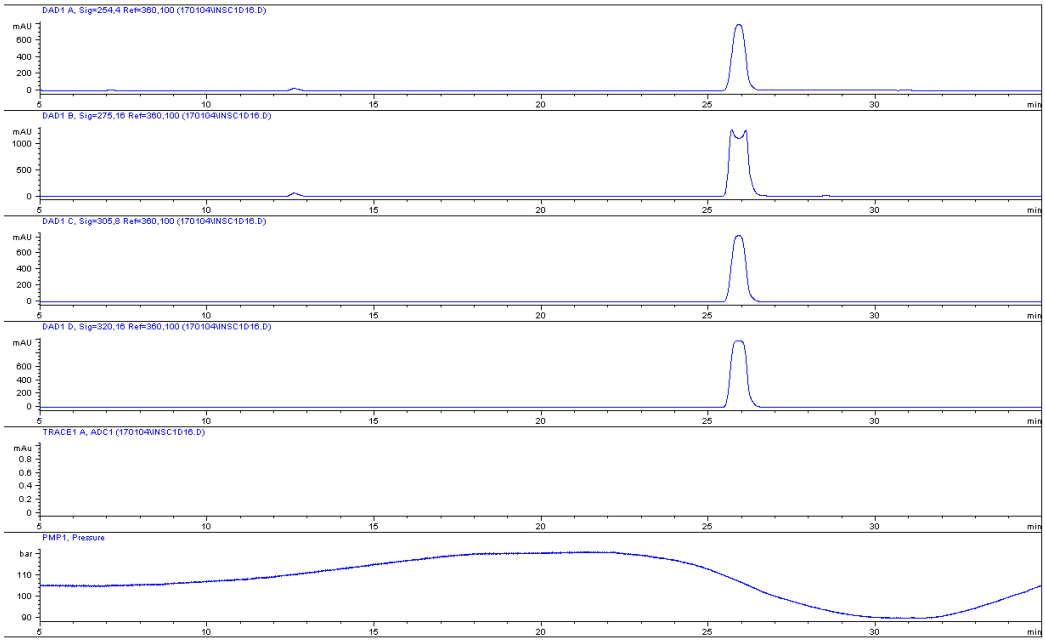

Figure B. 8 : HPLC chromatograms (recorded at 254.4, 275.16, 305.8, 320.16) of IN fraction of soaked cowpea.

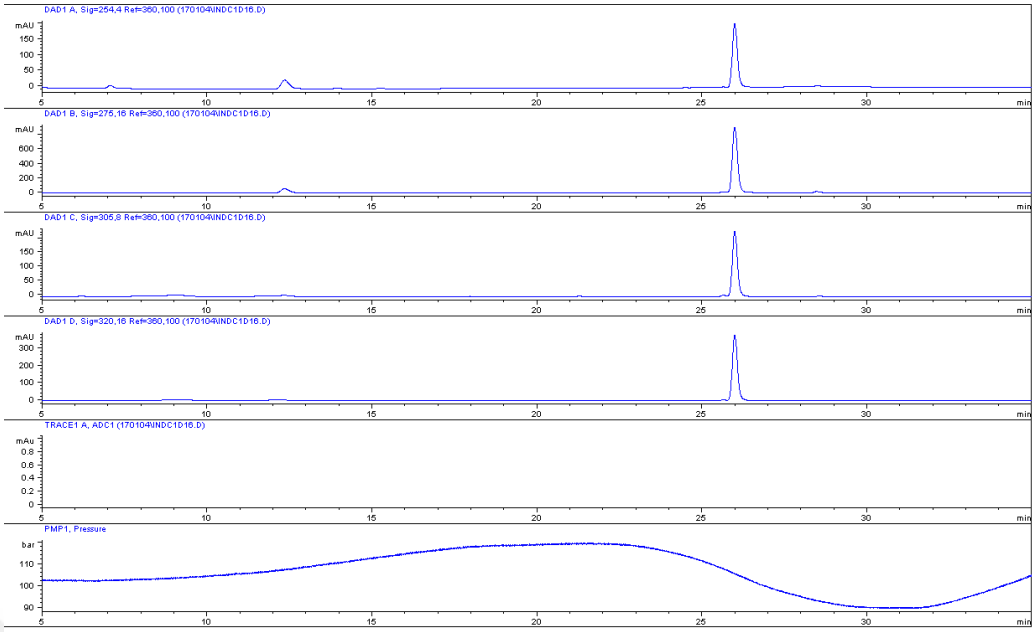

Figure B. 9 : HPLC chromatograms (recorded at 254.4, 275.16, 305.8, 320.16) of IN fraction of dehulled cowpea**.**

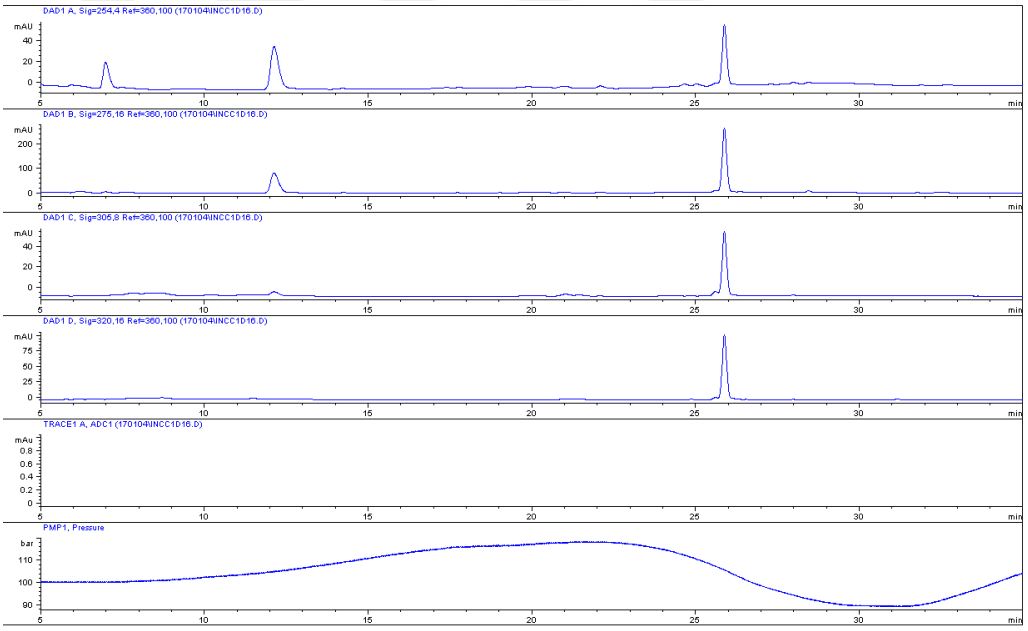

Figure B. 10 : HPLC chromatograms (recorded at 254.4, 275.16, 305.8, 320.16) of IN fraction of cooked cowpea.

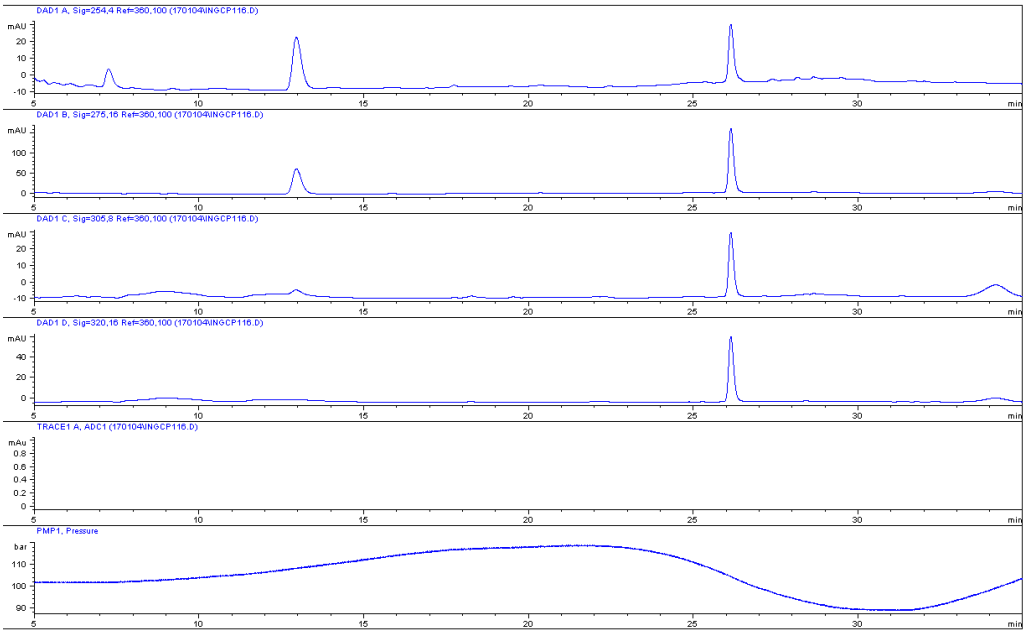

Figure B. 11 : HPLC chromatograms (recorded at 254.4, 275.16, 305.8, 320.16) of IN fraction of germinated cowpea.

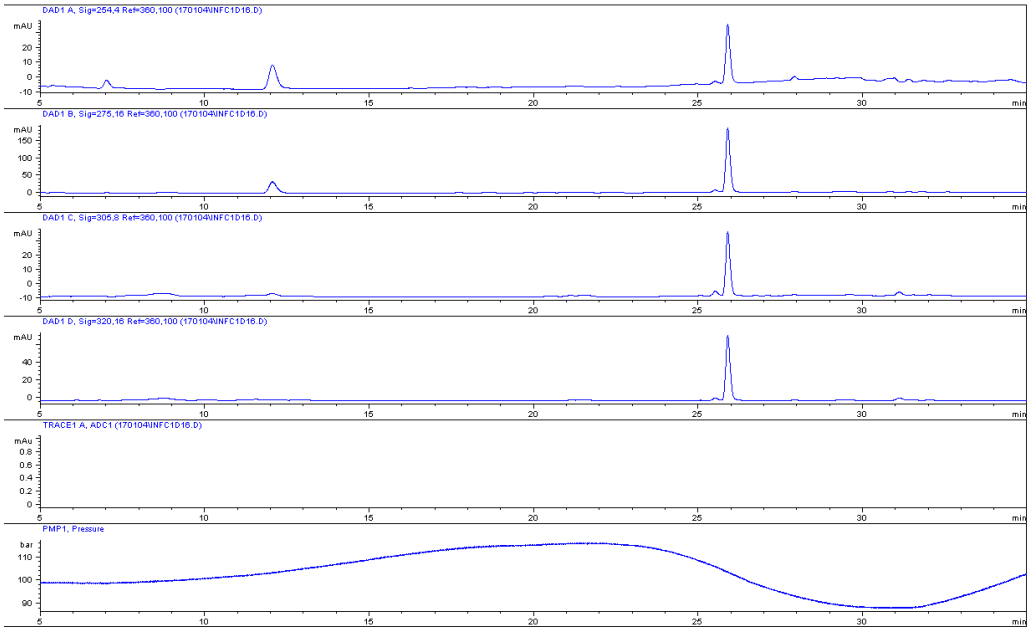

Figure B. 12 : HPLC chromatograms (recorded at 254.4, 275.16, 305.8, 320.16) of IN fraction of fermented cowpea.

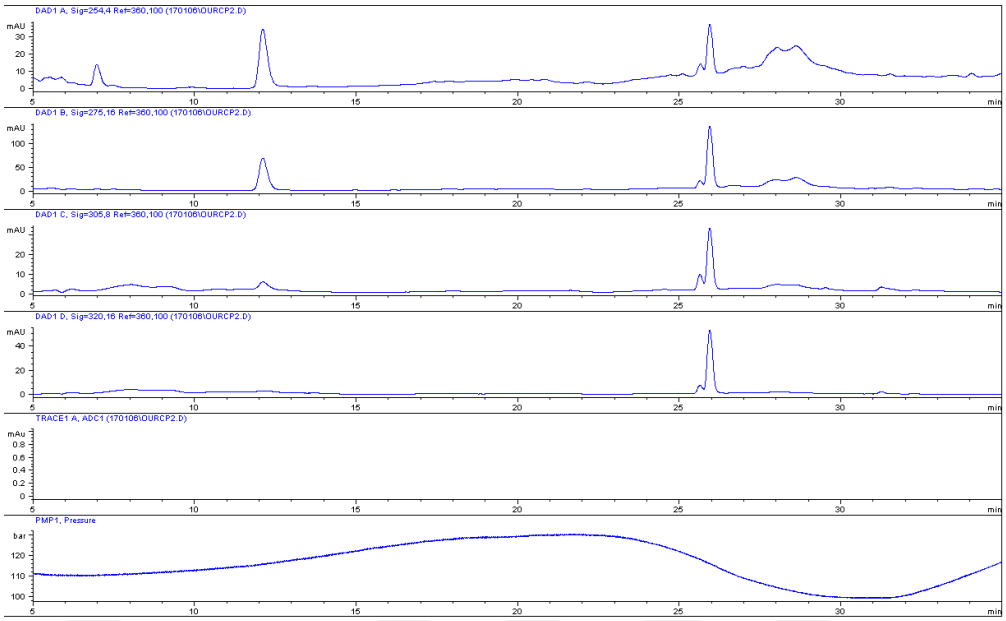

Figure B. 13 : HPLC chromatograms (recorded at 254.4, 275.16, 305.8, 320.16) of OUT fraction of raw cowpea.

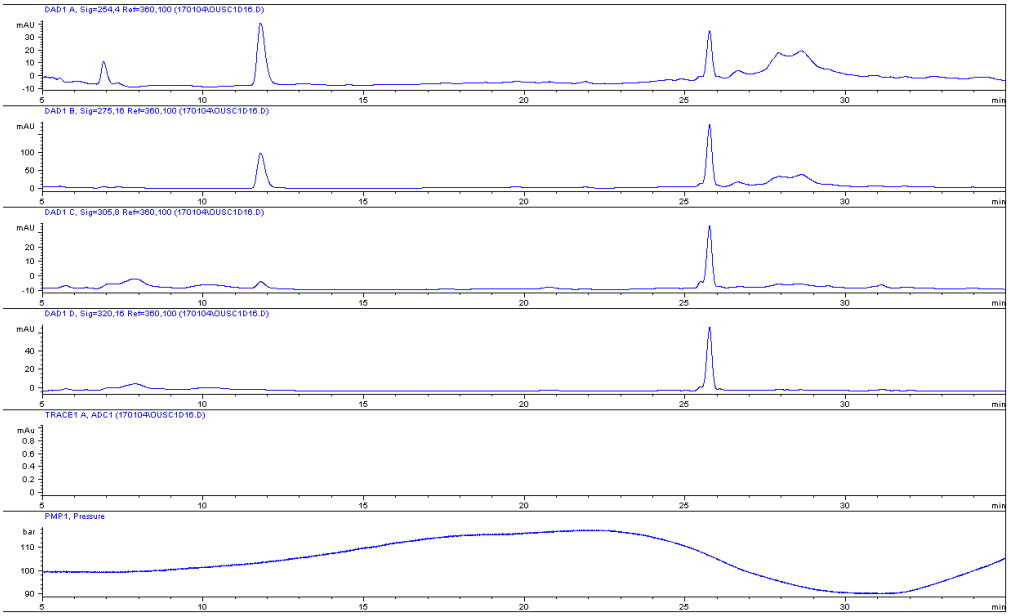

Figure B. 14 : HPLC chromatograms (recorded at 254.4, 275.16, 305.8, 320.16) of OUT fraction of soaked cowpea.

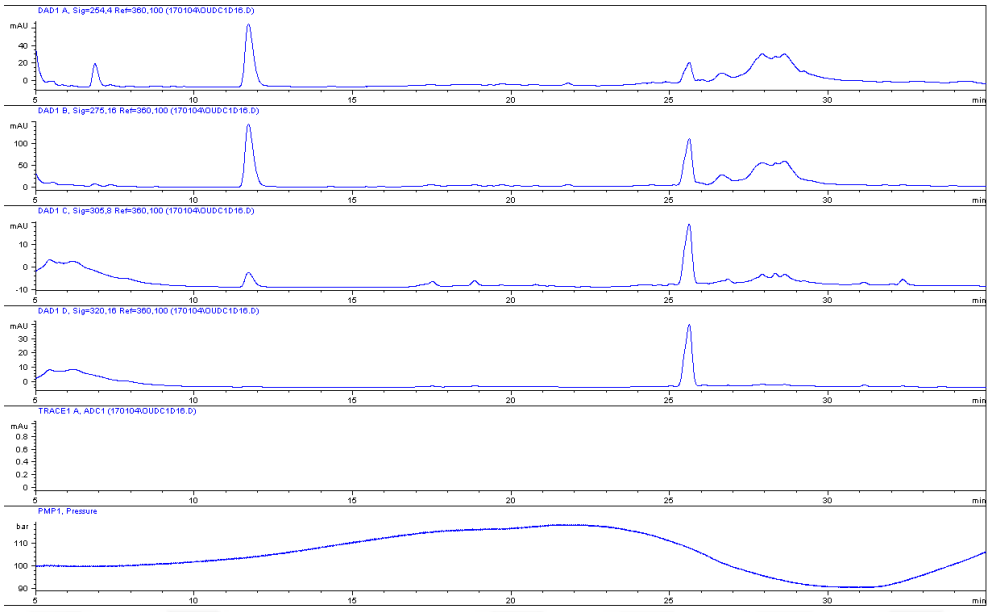

Figure B. 15 : HPLC chromatograms (recorded at 254.4, 275.16, 305.8, 320.16) of OUT fraction of dehulled cowpea.

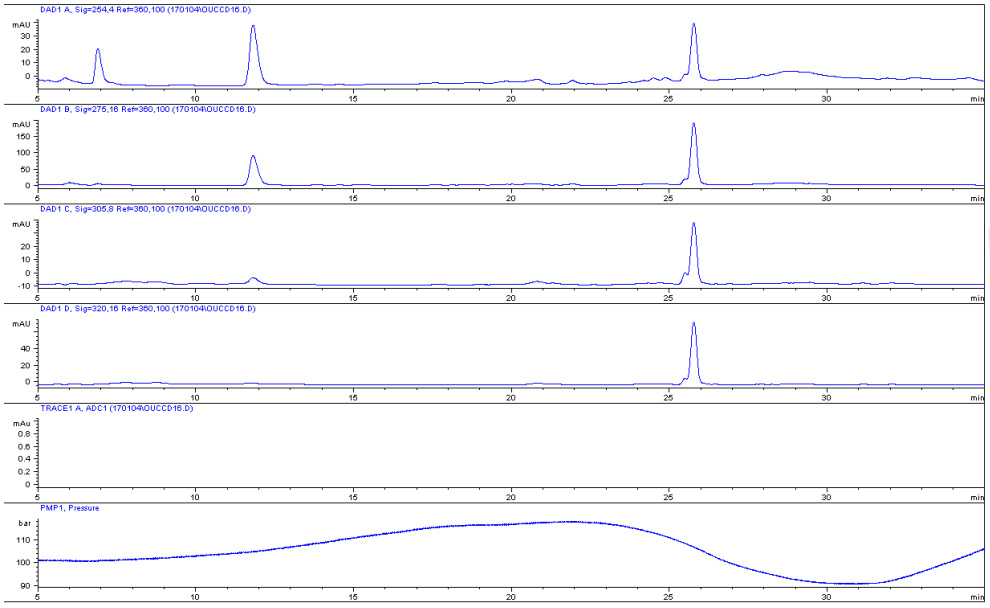

Figure B. 16 : HPLC chromatograms (recorded at 254.4, 275.16, 305.8, 320.16) of OUT fraction of cooked cowpea.

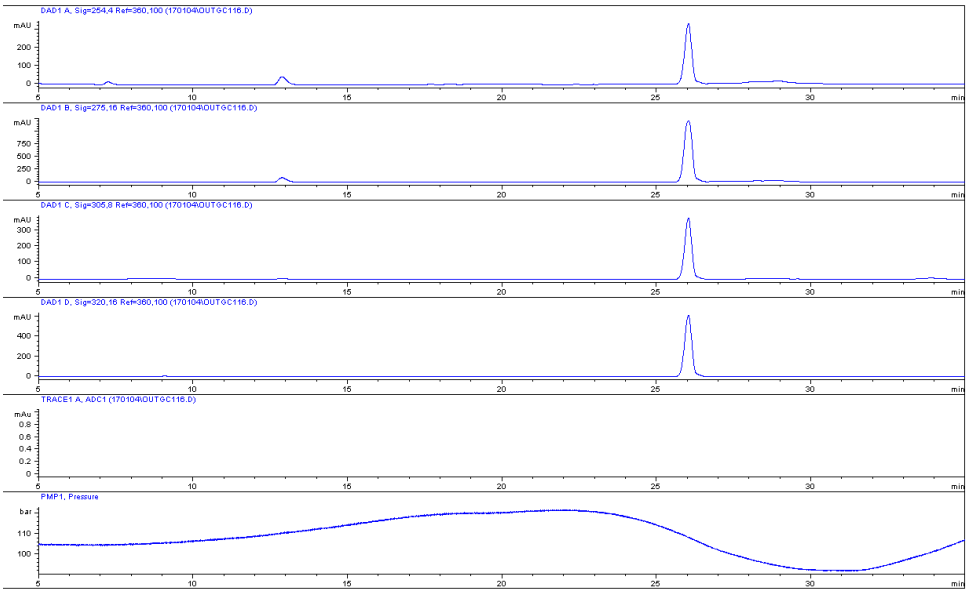

Figure B. 17 : HPLC chromatograms (recorded at 254.4, 275.16, 305.8, 320.16) of OUT fraction of germinated cowpea.

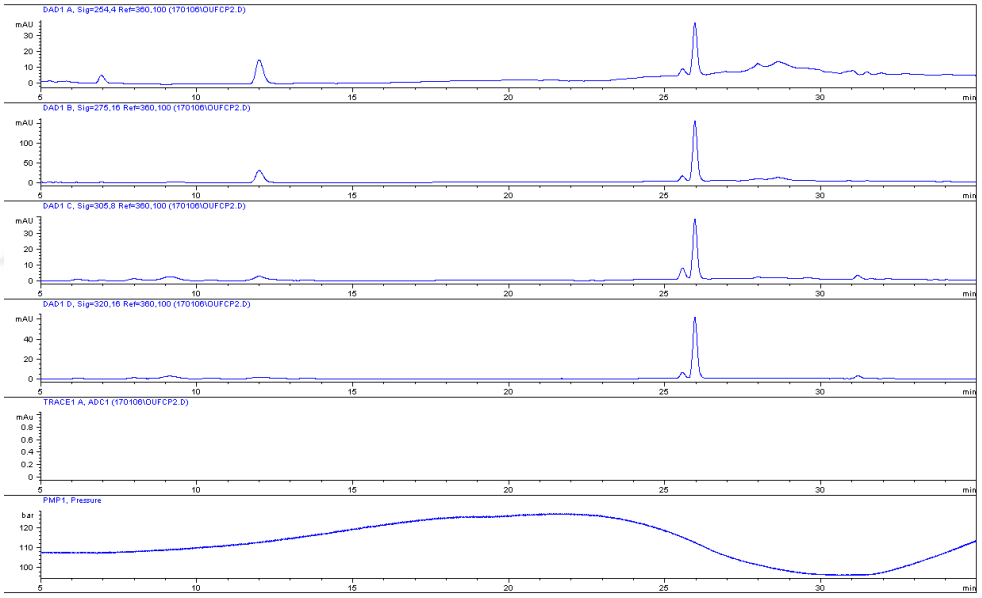

Figure B. 18 : HPLC chromatograms (recorded at 254.4, 275.16, 305.8, 320.16) of OUT fraction of fermented cowpea

APPENDIX C

Table C. 1 : Statistical analysis results of cowpea varieties.

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	TPC(free)	1,453[a]	13	,112	21,073	,000
	TPC(bound)	39,066[b]	13	3,005	7,376	,000
	TFC(free)	2,344[c]	13	,180	59,357	,000
	TFC(bound)	244,111[d]	13	18,778	4,131	,006
	DPPH(free)	27,372[e]	13	2,106	35,905	,000
	DPPH(bound)	174,569[f]	13	13,428	1,380	,279
	ABTS(free)	877,862[g]	13	67,528	81,489	,000
	ABTS(bound)	24927,191[h]	13	1917,476	5,620	,001
	FRAP(free)	75,359[i]	13	5,797	3,156	,021
	FRAP(bound)	38639,526[j]	13	2972,271	1,273	,329
Intercept	TPC(free)	13,366	1	13,366	2519,376	,000
	TPC(bound)	1585,004	1	1585,004	3890,234	,000
	TFC(free)	19,293	1	19,293	6350,286	,000
	TFC(bound)	1692,966	1	1692,966	372,417	,000
	DPPH(free)	107,910	1	107,910	1840,122	,000
	DPPH(bound)	1045,702	1	1045,702	107,456	,000
	ABTS(free)	6325,611	1	6325,611	7633,382	,000
	ABTS(bound)	585743,805	1	585743,805	1716,883	,000
	FRAP(free)	88223,211	1	88223,211	48028,256	,000
	FRAP(bound)	7602192,058	1	7602192,058	3256,243	,000
Varieties	TPC(free)	,867	6	,145	27,247	,000
	TPC(bound)	22,977	6	3,830	9,399	,000
	TFC(free)	1,942	6	,324	106,520	,000
	TFC(bound)	168,487	6	28,081	6,177	,002
	DPPH(free)	18,734	6	3,122	53,242	,000
	DPPH(bound)	144,652	6	24,109	2,477	,076
	ABTS(free)	383,477	6	63,913	77,126	,000
	ABTS(bound)	17048,564	6	2841,427	8,329	,001
	FRAP(free)	45,072	6	7,512	4,089	,014
	FRAP(bound)	20606,800	6	3434,467	1,471	,258
Type	TPC(free)	,095	1	,095	17,837	,001
	TPC(bound)	4,967	1	4,967	12,191	,004
	TFC(free)	,035	1	,035	11,624	,004

	TFC(bound)	64,531	1	64,531	14,195	,002
	DPPH(free)	5,658	1	5,658	96,486	,000
	DPPH(bound)	3,407	1	3,407	,350	,564
	ABTS(free)	363,918	1	363,918	439,155	,000
	ABTS(bound)	6588,287	1	6588,287	19,311	,001
	FRAP(free)	19,962	1	19,962	10,867	,005
	FRAP(bound)	7459,304	1	7459,304	3,195	,096
Varieties * Type	TPC(free)	,491	6	,082	15,439	,000
	TPC(bound)	11,122	6	1,854	4,549	,009
	TFC(free)	,367	6	,061	20,149	,000
	TFC(bound)	11,094	6	1,849	,407	,862
	DPPH(free)	2,980	6	,497	8,470	,001
	DPPH(bound)	26,511	6	4,418	,454	,831
	ABTS(free)	130,467	6	21,744	26,240	,000
	ABTS(bound)	1290,340	6	215,057	,630	,704
	FRAP(free)	10,326	6	1,721	,937	,499
	FRAP(bound)	10573,422	6	1762,237	,755	,616
Error	TPC(free)	,074	14	,005		
	TPC(bound)	5,704	14	,407		
	TFC(free)	,043	14	,003		
	TFC(bound)	63,642	14	4,546		
	DPPH(free)	,821	14	,059		
	DPPH(bound)	136,240	14	9,731		
	ABTS(free)	11,601	14	,829		
	ABTS(bound)	4776,338	14	341,167		
	FRAP(free)	25,717	14	1,837		
	FRAP(bound)	32685,119	14	2334,651		
Total	TPC(free)	14,894	28			
	TPC(bound)	1629,774	28			
	TFC(free)	21,680	28			
	TFC(bound)	2000,720	28			
	DPPH(free)	136,103	28			
	DPPH(bound)	1356,512	28			
	ABTS(free)	7215,075	28			
	ABTS(bound)	615447,335	28			
	FRAP(free)	88324,286	28			
	FRAP(bound)	7673516,703	28			
Corrected Total	TPC(free)	1,528	27			
	TPC(bound)	44,770	27			
	TFC(free)	2,387	27			
	TFC(bound)	307,754	27			

	DPPH(free)	28,193	27			
	DPPH(bound)	310,810	27			
	ABTS(free)	889,464	27			
	ABTS(bound)	29703,530	27			
	FRAP(free)	101,076	27			
	FRAP(bound)	71324,645	27			

Table C. 2 : Statistical analysis results of processed cowpea.

		Sum of Squares	df	Mean Square	F	Sig.
TPC	Between Groups	2,254	5	,451	29,199	,000
	Within Groups	,093	6	,015		
	Total	2,347	11			
TFC	Between Groups	,468	5	,094	15,529	,001
	Within Groups	,048	8	,006		
	Total	,517	13			
DPPH	Between Groups	297,281	5	59,456	1031,598	,000
	Within Groups	,461	8	,058		
	Total	297,743	13			
ABTS	Between Groups	906,299	5	181,260	10,559	,002
	Within Groups	137,329	8	17,166		
	Total	1043,628	13			
FRAP	Between Groups	468,691	5	93,738	4,734	,026
	Within Groups	158,397	8	19,800		
	Total	627,088	13			

Table C. 3 : Statistical analysis results of *in vitro* protein digestibility.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	30,667	5	6,133	74,452	,000
Within Groups	,494	6	,082		
Total	31,162	11			

Table C. 4 : Statistical analysis results of trypsin inhibitor activity.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3041,631	5	608,326	38040,196	,000
Within Groups	,096	6	,016		
Total	3041,727	11			

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