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REVIEW

MOTH BEAN A MARVEL LEGUME - A MINI REVIEW

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ABSTRACT

Traditionally legumes are used as dietary staple which provides proteinaceous foods. Moth bean is one of the drought resistance pulse. It is rich in protein, carbohydrate and also significant amount of anti-nutritional factors. Anti-nutrients are eliminated by thermal and non-thermal processing method. Soaking and germination is one of the traditional method. It is most effective in reducing the anti-nutrients. It increases the starch and protein digestibility and also bioavailability of minerals and vitamins. It helps to improve immunity in body and also strength of the muscle. Sprouted moth bean increases antioxidants properties. It is rich in anti-oxidants like phenols, carotenoids and flavonoids. These antioxidants reduces the risk of non-communicable diseases such as diabetes, cardiovascular diseases and certain cancers.

KEYWORDS:
Nutritional composition, soaking, germination, anti-nutrients and health benefits

INTRODUCTION

Legumes are produced and widely consumed throughout the world particularly in tropical and subtropical areas of Africa, Asia and Latin America. Food legumes are crops of the family Leguminosae also called Fabacae. They are mainly grown for their edible seeds, and thus are also named grain legumes. Grain legumes are used as pulse (dhal) with cereals, grown in both tropical and temperate regions of the globe. Legumes constitutes an important source of protein, B complex vitamins and minerals. India is a major legume producing country accounting for about 27-28 per cent of world legume production. Area under legume cultivation in India is 228.47 lakh hectares yielding an annual production of 130.70 lakh tonnes [1].

Legumes become very important as food and fodder however; it also plays an important role in maintaining or even improving soil fertility through its ability to fix atmospheric nitrogen. Legumes are considered as poor man's meat. They are generally good sources of carbohydrates (viz. dietary fibre) and are rich in proteins (18–25%) some vitamins (thiamine, riboflavin, niacin) and certain minerals [2, 3]. They enhance the protein content of cereal-based diets and may improve the nutritional status of the cereal-based diets. Cereal proteins are deficient in certain essential amino acids, particularly lysine [4]. On the other hand, legumes have been reported to contain adequate amounts of lysine, but are deficient in S-containing amino acids (methionine, cystine and cysteine) [5]. Cereals supply sufficient sulphur containing amino acid to supplement the deficiency in pulse proteins.

In the developed countries, legumes have an increasing use in dietetic formulations in the treatment and prevention of non-communicable diseases such as diabetes, cardiovascular diseases, cancer of colon, and lowering of blood cholesterol levels [6], which indicates their possible therapeutic value in humans. Nowadays, legumes are essential raw material for the modern food industry in the production of protein concentrates, fats and starches, and functional food ingredients (protein isolates, protein hydro lysates, dietary fibres, lecithin and isoflavones) [7].

Dry grain legumes contain several anti-nutritional factors, such as a galactosides, trypsin and chymotrypsin inhibitors, phytates and lectins [8], which delay the availability of nutrients and thus limit their consumption. Removal of these factors is therefore essential for improving the nutritional quality of legumes and, subsequently, to increase their potential as human food. Some simple and inexpensive processing techniques, such as soaking and cooking, are highly efficient for the reduction of these anti-nutritional factors and for improving legume organoleptic quality.

Soaking is an integral part of a number of treatments, such as cooking, canning, germination, and fermentation. It consists of hydration of the seeds in water, usually until they reach maximum weight, with or without discarding of the soaking medium, and the results obtained depend on factors such as legume genus, species and variety, process duration, temperature, pH, salinity of the soaking media, and also the storage conditions undergone before processing. Numerous studies have indicated that soaking could reduce the levels of total sugars, a-galactosides, minerals, phytic acid and proteolytic enzyme inhibitors [9,10,11], which can be partly or totally

solubilised and eliminated with the discarded soaking solution.

Germination is generally preceded by soaking the legume seeds in water. Some of the reserve materials of the seeds are degraded and used partly for respiration and partly for synthesis of new cell constituents of the developing embryo during germination, therefore, this process causes important changes in the biochemical, nutritional and sensory characteristics of legumes [12]. These changes depend on the type of legume and the sprouting conditions, such as time, temperature, the presence or absence of light during the sprouting process or the composition of the soaking and rinsing media [3]. It is known that germination increases the level of amino acids [13, 14], reduces the amount of anti-nutritional factors such as oligosaccharides and trypsin inhibitor activity and increases the levels of some vitamins and minerals. Germination of legume seeds for human consumption has been a common practice in the Orient for centuries where, today, sprouted legumes constitute a good proportion of the total consumption of food legumes.

Vigna aconitifolia (Jacq.) Maréchal (Moth bean) is a hot weather legume, belonging to the family Fabaceae. The seeds and sprouts of this plant are rich in protein and other elements, which make them an excellent supplement to cereal-based diets [15]. It is one of the most drought resistant pulses, is widely grown under rain fed conditions in semi-arid and arid zones of India. Moth bean is a good source of protein, carbohydrate, and also contains significant amounts of antinutritional factors such as phytate, tannins, trypsin inhibitor, phenols etc. [16]. Starch is the major component of moth bean. Before consumption, Vigna aconitifolia is soaked overnight and then cooked; this pre-soaking activity and cooking may affect the nutritional quality of the beans [17]. It can improve the nutritional quality and also help inactive some heat labile anti-nutritional compounds thereby increasing the bioavailability of the legumes as well as permit the digestion and assimilation of proteins and starch [18]. It is often consumed as cooked whole seed, split peas or sprouts [19].

Taxonomical Classification

Kingdom : Plantae
Sub kingdom : Vascular
Order : Fabales
Family : Fabaceae
Sub family : Faboideae
Tribe : Phaseoleae
Genus : Vigna
Species : *V. aconitifolia (Jacq.) Marechal*
Synonyms : Phaseolus aconitifolius Jacq

Source: Singh and Ansari [20]

Distribution and Production: Pulses are major sources of proteins among vegetarians in India, and usually complements the staple cereals in the diets with proteins, essential amino acids, vitamins and minerals. Pulses can be grown on wide range of soil and climatic conditions. They play important role in crop rotation, mixed and inter-cropping, maintaining soil fertility through nitrogen fixation and release of soil-bound phosphorus, thus contributing significantly to sustainability of the farming systems [23, 24].

In general, pulses are mostly grown in two seasons:

(1) The warmer, rainy season or Kharif (June-October), and

(2) The cool, dry season or Rabi (October-April). Chickpea, lentil, and dry peas are grown in the Rabi season, while pigeon pea, urd bean, mung bean, moth bean and cowpea are grown during the kharif season.

TABLE 1
Physical properties of moth bean

Physical parameters	Moth bean			Reference
	Raw	Soaked	Germinated	
Colour	Yellow brown	Yellow brown	White brown	Deshmukh and Pawar [21]
Shape	Oblong rectangular	Oblong	Oblong	
Wt. of 1000 seed (g)	32.70	63.80	60.00	
Density (g/ml)	1.54	0.92	1.74	
Width (mm)	5.13	4.97	6.06	
Length (mm)	5.10	6.31	8.12	
100 kernel weight (g)	3.27	6.38	6.00	Mankotia and Modgil [22]
Length (mm)	5.13	7.17	8.30	
Width (mm)	5.15	5.97	6.18	
Density (g/ml)	1.64	0.94	1.84	

India ranks first in the world in terms of pulse production (25% of total worlds production). Madhya Pradesh, Maharashtra, Uttar Pradesh, Andhra Pradesh, Karnataka and Rajasthan are the major states growing pulses in India. These six states contribute 80% of total pulse production and area [25].

Vigna aconitifolia is inhabitant in North-western desert regions of Indo-Pak subcontinent. In India moth bean is grown in the arid and semi-arid regions, particularly in north western states. Major moth bean growing states are Rajasthan, Maharashtra, Gujarat, Jammu and Kashmir, Punjab, Haryana and Uttar Pradesh. Rajasthan being major moth bean growing state contributes almost 86% of the area under moth bean cultivation in the country [26].

A number of pulse crops are cultivated in India on an area of about 23-24 million hectares. In India, moth bean is grown on 1.5 million hectares producing annually about 0.4 million tonnes of seed, which is traded and consumed within the country. Worldwide moth bean is grown on about 2 million hectare. At the country level, area and production of moth bean have been fluctuating probably due to rainfall intensity. However, area, production and productivity of moth bean at the country level appear to have been settled to 13.52 lakh hectares, 2.42 lakh tonnes and 215.26 kg per hectare [27].

From 2002 to 2007, during this period, the area coverage and production of moth bean were about 12.42 lakh hectares and 2.97 lakh tonnes respectively. Rajasthan was ranked highest both in area (92.43 %) and production (90.24%). Maharashtra stood second in area (3.62%) followed by Gujarat (3.54%), while in production Gujarat stood second with 5.39% followed by Maharashtra 4.04%. The yield was recorded above the National average in the states of Gujarat (364 kg/hectare) and by Maharashtra (267 kg/hectare). From 2007 to 2012, during this period, the area and production of moth bean were about 14.06 lakh hectares and 4.27 lakh tonnes respectively. Rajasthan occupied first position in area is 94.66% and contributed to 92.82% of total production followed by Gujarat with 2.65% of area and 3.79% of the total production and Maharashtra with 1.97% area and 2.25% of production share in the country. The yield was observed below the National average in Rajasthan (297 kg/hectare), which was the major producing state. From 2012 to 2015, a total of 9.26 lakh hectares and 2.77 lakh tonnes of moth bean production was recorded in the country. Area and production of moth bean was the highest in Rajasthan contributing 96.75% of area and 94.49% of production followed by Gujarat (2.38% area and 3.6% production). The yield observed in Rajasthan was about 292 kg/hectare [28].

Nutritional Composition: Legumes are a good source of several essential nutrients and it is suggested especially for vegetarian population in the world to balance the diet. Moth bean is an excellent supplement to cereal diet especially for people who are suffering from malnutrition.

Vigna aconitifolia seeds (moth bean) are devoid of known toxicants and could serve as a good source of nutrients notably carbohydrates, proteins, ascorbic acid, vitamin A, potassium, sodium and flavonoids [29]. Moth bean have a soluble sugars and also dietary fibre [30]. In diet we take high dietary fibres which helps to reduce the blood cholesterol level, constipation and obesity. In plant kingdom legumes are the good sources of proteins and also rich in essential amino acids [31]. Moth beans are rich sources of protein it's about 21.9% [32]. And also higher quantities of Threonine in moth bean [94]. Moth bean are rich sources of vitamins and minerals. Vitamins play an important role in energy metabolism and fatty acids metabolic path way during metabolism in the human body [33].

TABLE 2
Nutritional composition of moth bean

Nutrients	Amount
Moisture (g)	8.14±0.49
Protein (g)	19.75±0.38
Ash (g)	3.14±0.18
Total fat (g)	1.76±0.09
Dietary fibre (g)	15.12±0.49
Carbohydrate (g)	52.09±0.96
Energy (kcal)	1291±16
Total folate (µg)	349±10.8
Vitamin K (µg)	22.75±1.22
Carotenoid (µg)	622±72.9
Calcium (mg)	154±17.0
Iron (mg)	7.90±0.17

Source: Longvah et al., [34]

TABLE 3
Nutritional composition of raw, soaked and sprouted moth bean

Nutrient	Moth bean			Reference
	Raw	Soaked	Sprouted	
Moisture	8.4 ± 2.0	10.0 ± 2.0	10.6 ± 1.2	Deshmukh and Pawar [21]
Fat	1.13 ± 0.5	1.21 ± 0.5	1.09 ± 0.03	
Carbohydrate	60.23 ± 0.5	58.76 ± 0.5	56.05 ± 0.20	
Protein	21.3 ± 1.43	21.09 ± 1.3	23.82 ± 0.45	
Ash	3.4 ± 1.0	3.2 ± 1.0	3.2 ± 0.15	
Crude fibre	4.3 ± 0.3	4.1 ± 0.3	3.78 ± 0.52	
Iron	9.8 ± 1.0	9.94 ± 1.0	10.4 ± 0.6	
Vitamin C	3.7 ± 0.2	7.4 ± 0.2	12.3 ± 1.2	
Non protein nitrogen	0.70	0.54	0.62	Mankotia and Modgil [22]
True protein	17.51	17.75	18.60	
Energy (Kcal)	406.59	397.41	346.10	
Starch	50.25	46.88	41.25	
Trypsin inhibitors activity	122.10 ± 0.01	111.10 ± 0.02	102.92 ± 0.02	Salve et al., [35]

Antinutritional Factors: Anti-nutrients are the chemical compounds which have been evolved by plants for their own defence, generally anti-nutrients reduce the utilization of nutrients especially protein, vitamins and minerals, thus preventing their bioavailability when food is consumed [36]. Anti-nutritional factors can be eliminated by processing method such as dehulling, milling, soaking, cooking, pressure cooking, roasting, germination and fermentation.

Presence of antinutritional factors is one of the main drawbacks limiting the nutritional and food qualities of the legumes. Phytates, widely distributed in food grains, lowers the bioavailability of minerals and inhibits several proteolytic enzymes and amylases [37]. Trypsin inhibitors are protease inhibitors in raw legume seeds. They reduce the biological activity of trypsin. Trypsin is an enzyme involved in the breakdown of many different proteins in humans and other animals. As a result, protease inhibitors that interfere with its activity can have a nutritional effect. Trypsin inhibitors inhibit pancreatic enzyme function, hinder protein digestion and absorption [38]. Tannins are located primarily in the seed coats of cereals and legumes. They cause a decrease in the digestibility of proteins and carbohydrates as a result of the formation of insoluble enzyme-resistant complexes with tannins [39]. Tannins are heat stable and decrease protein digestibility in animals and humans, probably by both making protein partially unavailable or inhibiting digestive enzymes and increasing faecal nitrogen [40]. Lectins are glycoproteins widely distributed in legumes and some certain oil seeds which possess an affinity for specific sugar molecules and are characterized by their ability to combine with carbohydrate membrane receptors. Lectins are carbohydrate binding proteins and importantly, the carbohydrate moieties of the glycoproteins that embedded on the surface of most animal cells [38]. **Soaking:** Soaking is one of the traditional methods of processing legume in India. Soaking is an integral part of a number of treatments, such as cooking, canning, germination, and fermentation. It consists of hydration of the seeds in water, usually until they reach maximum weight. Soaking can reduce the levels of total sugars, α-galactosides, minerals, phytic acid and proteolytic enzyme inhibitors [54]. Soaking improves nutrient bioavailability, cooking quality and degrades the antinutritional factors in pulses [55]. Soaking improves the starch digestibility, availability of minerals (zinc and iron) and dietary fibre component such as cellulose, hemicellulose, lignin and pectin contents which vary in different type of pulse seeds. Soaking tends to destroy the anti-nutritional factors like trypsin inhibitor, phytates, tannins, saponins and total phenols etc. [56].

Effect of Soaking on Nutritive Value: Soaking usually is a part of legume processing methods, because it eases quicker cooking [57]. During soaking there is some metabolic processes which must be inferred and light plays a certain role in the process. Legumes are soaked with and without light it effects on carbohydrates and soluble vitamins during the soaking process. Carbohydrate fraction of glucose and fructose are increased with soaking with light and it not present in raw seeds. Raffinose was completely eliminated in presence of light but was still present after soaking in the dark. There is no effect of light during the soaking process on the vitamin content [58]. Soaking is an attractive method for removing anti nutritional content and it also enhances release of enzymes [59]. Phytates are water soluble so legumes when soaked in water for overnight

showed considerable removal of phytates in water in addition to that it also enhances the naturally occurring phytase [60]. Legume seeds contain about 2-3 times more protein as compared to cereals, but the protein digestibility of legumes is very poor [61]. This has been generally attributed to the presence of protease inhibitors and polyphenols [62, 63]. In soaking it effects on lose of phytic acids and polyphenols, etc., and it helps to improve the protein digestibility and starch digestibility in a human diet.

Germination: Germination is nothing but sprouting of legume seeds that is induced by soaking in water. As soon as the seed is hydrated, chemical changes occur, which results in partial breakdown of storage components and synthesis of new substances such as vitamins [31]. Germination causes important

TABLE 4
Anti-nutrient content of moth bean (mg/100g)

Anti-nutrients	Amount	Reference
Alkaloid	2.03 ± 0.02	Opera et al.,[29]
Tannin	2.89 ± 0.01	
Flavonoid	1.31 ± 0.01	
Polyphenol	3.08 ± 0.07	Bravo et al., [30]
Trypsin inhibitor activity	122.10 ± 0.01	Salve et al., [35]

TABLE 5
Benefits of processing by reducing the anti-nutrients

Processing	Reduction of anti-nutrients	Benefits	Reference
Soaking	Activity of phytase increased which reduced the phytate component present in the grains	Endogenous or Exogenous phytase enzymes could enhance the in vitro digestibility of minerals such as zinc and iron by 2 to 23%.	Ogbonna et al., and Vashisth et al., [41,42]
Soaking and Cooking	Greatly decreased the phytic content in legume grains		Vadivei and Biesalki, [43]
Germination	Tannin and phytic acid reduction	Increase the bioavailability of several minerals, which led to increase nutritional value of food products	Ogbonna et al., and Oghbaei and Prakash, [41,44]
Fermentation	Lowers the phytic acid, tannins and polyphenols	It may increase the amount of soluble iron, zinc and calcium	Simwaka et al., and Gupta et al., [45,46]
Milling	Removes anti nutritional factors (phytic acid, lectins, tannin) which are present in the bran of grains	Improvement of starch and protein digestibility	Gupta et al., and Chowdhary and punia, [46,47]
Roasting	Decreased the trypsin inhibitors activity significant in soybean	Improve the protein digestibility	Vagadia et all., [48]
Cooking	Reduces the lectins and saponins	Improve the nutritional quality of legumes	Maphasa and Jideani,[49]
Pressure cooking	Reduces the tannin content in black gram	Improve the protein digestibility	Shah,[50]
Boiling	Legumes are boiled in water at 100°C for few minutes to eliminate heat liable anti-nutrients	This process enhance the sensory properties of legumes grains	Bishnoi and Khetarpaul, Khalil and Mansour, [51,52]
Extrusion	Reduction of trypsin, chymotrypsin, α-amylase inhibition and hemagglutinin activity	Starch and protein digestion	Alonso et al., [53]

changes in the biochemical, nutritional and sensory characteristics of legumes. These changes depend on the type of legume and the sprouting conditions such as time, temperature, the presence or absence of light during the sprouting process [64]. Germinated seeds contain appreciable amount of ascorbic acids, but are not found in dry grains [65]

Germination plays a significant role in the reduction of antinutritional factors, including phenolic compounds, phytic acid, trypsin inhibitors and oligosaccharides [66]. Sprouted grains break down phytate, a form of phytic acid that normally decreases absorption of vitamins and minerals in the body. So, sprouted grains have more available nutrients than matured grains. Sprouted Mothbeans analysed after six days had significant increase in both nutrient content and antioxidant properties [67]

Effect of Germination on Nutritive Value: Germination is one of the most common process for improving the nutritional quality of pulses [68]. It involves changes in the nutritional, biochemical and sensory characteristics of the food [69]. It increases the nutritional digestibility, reducing the level or activities of anti-nutritional compounds and boosting the content of free amino acids and available carbohydrates and improves functionality [70, 11].

Carbohydrates: Germination triggers enzymatic activity in sprouting seeds, leading to the breakdown of carbohydrate in to simpler forms [71]. In germination the increased activity of α-amylases consequently increases the digestibility of starch [44]. During germination the starch content was decreased accompanied by an apparent increase in both of reducing and non-reducing sugar [72]. The content of the reducing sugars in cereals and legumes was not significantly affected during the first 12hr of germination. However, after 12hr the content of reducing sugars increased 20 times suggesting increased enzymatic hydrolysis of starch. This happens due to the action of α-amylases which is activated during germination leading to hydrolysis of carbohydrates, changes of taste and digestibility of carbohydrates [73].

Proteins: In germination the protein showed slight reduction in their content, while variations were observed in the amounts of amino acids. The process of germinations leads to an increase in most of the essential amino acids [72]. Sprouting grains cause increased activities of hydrolytic enzymes like lipase, improvement of total proteins, essential amino acids, total sugars, fat, B-group vitamins, protein and starch digestibility. In turn the decrease in phytates and protease inhibitors, there is an increase in amino acid lysine and increase in proteolytic activity which leads to hydrolysis of prolamines and increased lysine [74].

Vitamins: Germination increases various vitamins present in cereals and legumes such as tocopherols, riboflavin, niacin and thiamine [75]. During germination water soluble vitamins are lost. Germination carried out in the presence and absence of light had no significant effect on the thiamine and riboflavin contents in germinated lentils, while the niacin content was higher when germination was conducted in the light. When germination was carried out for 10 days in dark and with daily rinsing, maximal increases of the riboflavin and available niacin contents with minimal decreases in the thiamine content were observed [64]. The process of germination in vitamin B12 values increased in all the pulses and the increase was maximum in most of the pulses on the 4th day of germination [76]. Vitamin C content was detected in germination of the seeds but vitamins were not detected in raw seeds [77].

Minerals: Germination improves the nutritional quality of foods by increasing their nutrient content and digestibility. The mineral content of legumes is high, but the bioavailability is poor due to the presence of phytate, which is a main inhibitor of iron and zinc absorption. Some legumes also contain considerable amounts of Fe-binding polyphenols inhibiting Fe absorption. Efficient removal of phytates and polyphenols can be obtained by enzymatic degradation during food processing like soaking, germination and fermentation. Once phytate is degraded, legumes would become good sources of Fe and Zn as the content of these minerals is high [78].

Anti-nutrients: Germination plays a significant role in the reduction of anti-nutritional factors, including phenolic compound, phytic acid, trypsin inhibitors and oligosaccharides [66]. Germination is a traditional process is an ancient and popular practice known to cause important changes in seeds which improve nutritional quality by increasing certain vitamins, minerals and some essential amino acids and by decreasing several anti-nutritional factors [79]. Most of the anti- nutrients are bind to protein and form complex with enzymes, rendering them unavailable or inactive for digestion [80]. [32] Examined about the changes in phytate phosphorus and other minerals during germination of moth bean and horse gram. The phytate phosphorus was continuous decreases and also significant amount of calcium was leached out in both legumes. A slight increase in magnesium content was noticed in both legumes in 30hr and 48hr germinated seeds. [81] Investigated about the trypsin inhibitor from moth bean for thermal stability and changes during germination and cooking. The application of dry heat did not inactivate the inhibitor. However, autoclaving at 120 °C at 15 pounds pressure destroyed inhibitor activity completely. The extracted inhibitor lost 70% activity in 60 min when incubated at 100 °C. Soaking of moth

bean seeds for 8hr decreased trypsin inhibitor activity by 20%. The germination of seeds for 24hr resulted in 70% reduction in inhibitor activity. No activity was detected in 48hr germinated seeds. Germination (for 24hr) followed by cooking of moth bean seeds destroyed the trypsin inhibitor completely. [82] Studied the effect of germination of soaked seeds on starch and protein digestibility of four varieties (Jwala, RMO 225, and RMO 257) and one local variety of moth bean. Germination of soaked (12hr) moth bean for 60hr improved the starch digestibility of all the varieties by 84–88%. Local and RMO 257 sprouts showed similar increments (84%) in starch digestibility whereas, maximum enhancements were noticed in RMO 225 (88%) and Jwala (85%) sprouts. The increase in starch digestibility during germination may be caused by the pre-digestion of starch molecules by amylolytic enzymes. Amylases and phosphorylases may become active during germination and catalyze amylolysis. Thus the resulting increase in concentration of oligosaccharides may contribute towards better starch digestibility. The hydrolysis of seed proteins, protease inhibitors, phytic acid and polyphenols during germination may account for considerably increased protein digestibility in legumes.

Health Benefit: Malnutrition is currently wide spread in the world and the most serious one is protein calorie malnutrition (PCM). PCM is a widespread problem throughout the world, especially among children in the developing countries; and has both health and economic consequences. It is a major nutritional syndrome affecting more than 170 million preschool children and nursing mothers in developing Afro-Asian countries where provision of adequate proteins of animal origin is difficult and expensive.

Legumes used as a dietary staple provides proteinaceous foods to all category of people, because of affordability it is used in diet since early times and today. It improves the nutritional status of undernourished as well as over nourished individuals, and to reduce risk for chronic diseases. Legumes are the major contributors of protein and calories in Afro-

TABLE 6
Changes in nutrient content on germination

Nutrients	Enzymes	Outcomes	Benefits	Reference
Carbohydrate	Activation of hydrolytic and amylolytic enzymes	Decrease in starch and increase in simple sugars (it depends on germination hour)	Improves digestibility	Oghbaei and Prakash, [44]
	α and β amylases	Increase of total sugar, reducing sugar and non-reducing sugar	Improves digestibility and changes in taste	Jorgenson, [83]
	Activation of amylases and phosphorylases	Increase in oligosaccharides	Improves starch digestibility	Negi et al., [82]
Protein	Enzymatic hydrolysis of protein	Degradation of the anti-nutrients such as phytic acid, polyphenols and protease inhibitors and availability crude protein and essential amino acid	Improving the protein digestibility	Chitra et al.,; Mwikya et al., and Lee et al., [84,85,86]
	Increase in proteolytic action of enzyme	Break down of complex protein molecules in to simpler units of amino acid	Increasing essential amino acids needed for human consumption	Hegazi, [72]
Vitamins	Enzymatic hydrolysis of starch by amylases and diastases	Increase availability of glucose for biosynthesis of vitamin C	Improves nutritional and antioxidant properties	Desai et al., [87]
Minerals	Increase phytase activity	Reduces phytic acid which binds minerals and increase mineral availability such as zinc and iron	Absorption of minerals	Luo et al., [88]
	Activation of endogenous phytase	Break down of phytates, liberating inorganic phosphate	Availability of minerals in human body	Shimelis and Rakshit, [66]

Asian diets 89, 90]. The nutritional benefits of pulses are well recognized by government and health organizations globally and are recommended as part of healthy eating. Health organizations focused on diabetes, heart disease and cancer promote pulse consumption as part of healthy diets for reducing the risk of these chronic diseases [91].

Dietary fibre: It promotes health and prevents chronic diseases such as diabetes, obesity, coronary heart disease, stroke, hypertension and certain gastrointestinal disorders. High intake of dietary fibre improves serum lipid concentrations, lowers the blood pressure, improves blood glucose control in diabetes and also improves the immune function [92]. When intake of diet is high in fibre it protects from some gastrointestinal diseases such as colon cancer and large bowel cancers through mechanisms such as altering bile acid metabolism, increasing faecal bulk or decreasing gut transit time. Fibres in legumes keep human intestinal system healthy. They keep the bowels healthy and improves constipation and combat against colon cancer and intestinal disorders [93, 94].

Diabetes mellitus: When diets are rich in legumes it might decrease the risk of diabetes by improving blood glucose control, decreasing insulin secretion. Legumes contain anthocyanins which may lower blood glucose by improving insulin resistance, protecting β cells, increasing secretion of insulin and reducing digestion of sugars in the small intestine [95]. Legumes reduces the risk of developing diabetes because of their high-fibre, low fat content and low glycaemic indices [96]. Epidemiological studies strongly support the suggestion that high intakes of wholegrain foods protect against the development of type 2 diabetes mellitus. People who consume three or more servings of wholegrain foods per day are less likely to develop type 2 diabetes mellitus than low consumers with a 20–30% risk reduction [97].

Cardiovascular diseases: Legumes contains phytochemicals such as carotenoids and tocopherols which may prevent the risk of cardiovascular diseases. It has been reported in several studies that the consumption of leguminous foods has reduced the risk of cardiovascular diseases and coronary heart diseases [98]. Epidemiological evidence showed a 22% reduction in coronary heart disease and 11% reduction in cardiovascular disease with consumption of legumes four times or more per week compared with once a week [99]. Legumes contain low glycaemic index valves and are less likely to raise blood glucose and insulin levels, which may also decrease cardiovascular diseases [98].

Non-nutritive bioactive compounds: A variety of phytochemicals are increasingly being recognized for their potential benefits for human health, which includes polyphenolic compounds, lectins, phytates and trypsin inhibitors, among others. Lignans and isoflavones have anticarcinogenic, weak oestrogenic and antioxidant properties. Phenolic compounds, including tannins found mainly in the seed coat, have antioxidant activity. Phytoestrogens in pulses may play a role in the prevention of hormone-related cancers, such as breast and prostate cancer [97]. The occurrence of bioactive phenolic compounds and their antioxidant activity/radical-scavenging capacity make pulses a very useful food for daily inclusion in the human diet [100]. Polyphenols present in lentils not only contributes to the antioxidant activities, but they also inhibits glucosidase and lipase, contributing to controlling obesity and blood glucose levels in humans [73]. Moreover, polyphenols also function as anti-allergic, antimicrobial, anticancer and anti-inflammatory agents [101]. When phytochemicals containing foods are consumed in sufficient quantities, this might help to reduce tumour risk and could potentially account for a protective effect [98].

Antioxidant activity: The human body has a complex system of natural enzymatic and non-enzymatic antioxidant defences which counteract the harmful effects of free radicals and other oxidants. Free radicals are responsible for causing a large number of diseases including cancer, cardiovascular disease, neural disorders, Alzheimer's disease, alcohol induced liver disease and atherosclerosis [102,103,104]. The antioxidant activity and free radical-scavenging potential of phenolics of raw and dry heated moth bean (Vigna aconitifolia) (Jacq.) Marechal seed extracts were studied. The raw seeds consists maximum levels of total phenolics (6.54%) and tannins (1.91%) than the dry heated seeds [105]. Sprouting of moth beans increases the health benefits by increasing its free radical scavenging abilities higher than raw seeds [106].

Anti-inflammatory: Inflammation is a defense response of our body to hazardous stimuli such as allergens and/or injury to the tissues; on the other hand, uncontrolled inflammatory response is the main cause of a vast continuum of disorders including allergies, cardiovascular dysfunctions, metabolic syndrome, cancer, and autoimmune diseases imposing a huge economic burden on individuals and consequently on the society. Thus, we need to apply natural anti-inflammatory factors within medication therapy to achieve increased pharmacological response and the lowest degree of unwanted side effects [107,108].

Legumes have both protective and therapeutic effects and this has been well documented [96]. Legumes are excellent foods to increase dietary fibre consumption and most individuals can incorporate legumes into their diet. It is a health promoting diet

becomes important to meet the daily dietary recommendations to improve the nutritional status of undernourished as well as over nourished. On the other hand legume based diets could reduce risk for chronic diseases such as cardiovascular disease, diabetes mellitus, cancer and osteoporosis. Recommended intakes of legumes were the amount of cooked pulses recommended (100 - 200 g/day) should reflect a balance between desirable metabolic effects and possible dietary compliance. Whereas 25 g soy protein per day may be required to obtain a significant hypocholesterolaemic effect, intake of significantly smaller amounts (some soy foods weekly) may provide distinct health benefits. Another study investigated about the health benefits of low glycaemic index foods, such as pulses [109]. Nutritional factors potentially play a crucial role in health and disease. A low-fat, high-carbohydrate diet is often recommended as a part of a healthy lifestyle. Historical works have shown that carbohydrate foods differ in their ability to affect post-ingestive glycaemia. The glycaemic index concept allows a ranking of carbohydrate-rich foods in terms of their blood glucose raising potential. Pulses are very low glycaemic index foods. Numerous studies have documented the health benefits that can be obtained by selecting foods of low glycaemic index. These benefits are of crucial importance in the dietary treatment of diabetes mellitus. Glycaemic control is improved as well as several metabolic parameters, such as blood lipids. The results of human studies have been confirmed by animal experiments in the field of diabetes. Diets with low glycaemic index value improve the prevention of coronary heart disease in diabetic and healthy subjects. In obese or overweight individuals, low-glycaemic index meals increase satiety and facilitate the control of food intake. Selecting low glycaemic index foods has also demonstrated benefits for healthy persons in terms of postprandial glucose and lipid metabolism. Several public health organizations have recently integrated consideration of the glycaemic index in their nutritional recommendations for patients with metabolic diseases and for the general population.

Studied the potential health benefits of legumes as a good source of dietary fibre has been studied [110]. Dietary fibre has been shown to have important health implications in the prevention of risks of chronic diseases. Six to ten local legumes were studied as follows: cowpeas, mung beans, pole sitao, chickpeas, green peas, groundnuts, pigeon peas, kidney beans, lima beans and soybeans. Thus, authors reported that mineral availability from legumes differs and may be attributed to their mineral content, mineral–mineral interaction and from their phytic and tannic acid content; legumes are considered low-GI foods and have shown potential hypocholesterolaemic effects. *In vitro* binding of bile acids by kidney bean (Phaseolus vulgaris), black gram (Vigna mungo), bengal gram (Cicer arietinum) and moth bean (Phaseolus aconitifolins) has been studied [111]. Considering cholestyramine as 100% bound, the relative *in vitro* bile acid binding for kidney bean, black gram, bengal gram and moth bean on equal protein basis was 12%, 15%, 35% and 13%, respectively. Their data suggested that of all four kinds of beans tested, bile acid binding may be related to the anionic, cationic, physical and chemical structure, composition, metabolites, or their interaction with active binding sites. Further animal studies were in progress by authors to validate relationship of in vitro bile acid binding of various beans observed herein to lipid, cholesterol-lowering and atherosclerosis amelioration.

CONCLUSION

Legumes forms part of most traditional diets and usually legume and pulse consumption has beneficial role to play with regard to health. Moth bean is one of the inexpensive sources of supplementary protein in Indian diets. It is one of the most drought resistant pulses, becomes a good source of protein and carbohydrate and a significant amount of antinutritional factors such as phytate, tannins, trypsin inhibitor, phenols etc. are present. Simple processing methods like soaking and germination has an effect on the nutritional quality. Thus, improves the bioavailability of minerals and vitamins, and increases the nutrient digestibility in a diet. It is rich in antioxidants like phenols, carotenoids and flavonoids. These antioxidants reduces the risk of non-communicable diseases like diabetes, obesity, cardiovascular diseases and certain cancers. Sprouting of moth beans increases the health benefits by increasing its free radical scavenging abilities compared to raw seeds. Thus, Moth bean seeds and sprouts are being used in a traditional diet with very high nutritional value.

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A COMPARATIVE INVESTIGATION ON THE CORRELATION BETWEEN THE TOTAL PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY OF DAPHNE GNIDIUM'S LEAVES STEMS FLOWERS AND FRUITS

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ABSTRACT

This study aims to optimize solvents' effects on the yield's crudes extraction and the concentration of both phenolic and flavonoid compounds in leaves, stems, fruits and flowers of daphne gnidium native Morocco. The first step was designed to study the phytochemical screening constituents of daphne gnidium's parts (leaves, stems, flowers and fruits) which revealed the presence of important families: alkaloids, tannins, flavonoids, sterols and mucilage moreover those parts showed the absence of antraquinons and saponins. Followed by extracting the daphne gnidium's essential oil and determinate its chemical composition by gas chromatography-mass spectrometry (GC/MS), which revealed that the phytol (45.75%) is the major compound. The second step was devoted to evaluate the pure solvents'; which are water, ethanol, ethyl-acetate, dichloromethane and hexane; effect on yields extraction and the concentration of phenolic content and on the antioxidant capacity, also the correlation between the TPC and the TAC. Moreover, depending on the extraction method, it was noted that for each part of daphne gnidium the water revealed the higher yields' extraction, the higher TPC and TFC and the greatest TAC, as against ethanol, ethyl-acetate, dichloromethane and hexane that showed the lowest yield's extraction, lowest concentration of phenolic and flavonoid compounds also the lowest TAC value. As a result, the values of yields extraction, the TAC, the TPC and the TFC are increasing when the solvent used polarity increases too. Also, the results show a significant positive correlation between TAC and the total phenolic and flavonoid content. These results revealed the powerful capacity of daphne gnidium's crudes extracts can be a natural resource of antioxidants.

KEYWORDS:
Daphne Gnidium, Phytol, Flavonoids, Antioxidant Activity

INTRODUCTION

A long time ago aromatic medicinal plants were used due to their richness of antimicrobial and antioxidant compounds, which are devoted as a treatment of some humans and animals diseases [1]. Alkaloid and phenolic compounds are the major families of bioactive molecules responsible of a plant's curing effect [2]. Phenolic compounds are the main secondary metabolites of aromatics plants which have the greatest antioxidant capacity [3].

The daphne family gathers a large number of species around the world. Among the daphne species, this study was interested by the gnidium, which is native Morocco. This work has the aim of valorize leaves, flowers, stems and fruits of daphne gnidium's secondary metabolites also highlight the factors affecting yields' phenolic concentration and antioxidant activity such as solvents used during the extraction, and the correlation between the TFC, TPC and the TAC.

MATERIALS AND METHODS

Vegetal material. The vegetal material we are working on is the daphne gnidium which belongs to Thymlaeaceae's family, Magniliopsida's class and Daphne's type. Diverse parts of daphne gnidium which are leaves, stems, flowers and fruits were harvested on April, June and July of 2019 from Meknes Morocco. Each sample was well dried separately for 15 days under the shadow, later reduced and stored to fine powder for different extractions type.

Extraction of essential oil. The extraction of the essential oil was executed by hydro distillation in a Clevenger device. Three distillations were executed by boiling for an hour and a half 200 g of fresh vegetal material with 1L of water in a 2L tank which is 60 cm high. This tank has a column coming out of it which is connected to a refrigerant. The essential oil that we got was then stocked in a dark place in a temperature of 4°C with the presence of anhydrous

sodium sulphate. Afterwards it was diluted in methanol (1% v/v) before doing the CG and CG/SM analysis. [4].

Chemical composition. GC–MS:

The identification of chemical components was carried out using mass spectrometer interfaced with a gas chromatograph in the common analysis center faculty of sciences Meknes, GC-MS: Agilent technologies 7890 B, equipped with Agilent 19091S-433 fused silica capillary HP-5MS column (5% phenyl Methylpolysiloxane, 30 m, 250 µm; film thickness 0.25 µm), coupled to mass spectrometer Agilent technologies 5977 A MSD (ion source βγ0°C, 70ev) GC oven initial temperature was 70 ◦C during β min. and programmed to β50 ◦C at a rate of 10 ◦C/min and β50 ◦C during 45 min under the following operation conditions: vector gas: Helium. The injector temperature was 250°C; split ratio of 80/1 was injected; helium was used as the carrier gas at 3L/min. Identification of components was done by comparison of the case registry number (Cas) and MS with the corresponding database (NIST library) and with mass spectral literature [5].

Phytochemical screening. Qualitative tests based on coloration change or precipitation reactions admit to identify chemical family Alkaloids, Tannins, Flavonoids, Saponins, Sterols tri-terpenes, anthrancenics and Mucilage in each part of plant: leaves and stems [6].

Optimization of extraction factors: a)The best solvent extraction

The crudes extracts were obtained from vegetal powder of daphne gnidium's parts and six systems of pure solvents. Depending of the extractions methods used, the solvents used have a different increased polarity according the following ordre hexane then dichloromethane, ethyl-acetate, ethanol and water.

b)Preparation of extracts:

Soxhelt method: A mass of 30 g of leaves or stems of Adenocarpus bacquei was extracted with of different solvents (300 mL) hexane, dichloromethane, ethyl acetate, and ethanol. Using the Soxhelt device until the solvent turned colorless again. Then, the mixtures were filtered and the solvents were evaporated by rotary evaporator to dryness. And yields were calculated. [7]

Infusion: The aqueous extracts obtained by adding boiling distillated water to 30g of leaves and stems during 6h. Then the mixture was filtered and the solvents were evaporated by rotary evaporator to dryness.

Extracting rending: R %= (M1/M0) ×100

M1: mass of extract (g).

M0: masse of vegetal material (g).

Instrument: All spectrophotometric data were treated using SHIMADZU UVmin-1240 UV-VIS spectrophotometer. Glass cuvette (1cm× 1cm× 4.5 cm).

Statistical analysis: The results are mentioned as mean ± standard deviation of three replicates. Statistical analyzes were performed using Microsoft Excel followed by one way-ANOVA. Pearson's table of correlation coefficients was used to determinate the interrelationships among TFC, TPC and antioxidant activities, Values were performed statistically significantly when $P<0.05$.

Determination of phenolic compounds contents. a) Total phenolic content

The total phenolic content was determined by the Folin-ciocalteu method [8]. Briefly 0.3mL at 1mg/mL of each crude extract was mixed with 1.5mL of Folin-ciocalteu's reagent (diluted by distilled water to 1:10 v/v) and 1.2 mL solution at 7.5 % of sodium carbonate (Na_2CO_3). The mixture was incubated at dark for 2 hours then the absorbance was read with spectrophotometer at 765nm. The TPC was calculated using regression equation from the calibration curve using gallic acid standard, and the data are results of three replicates ± standard deviation. The total phenolic content was expressed in mg of Gallic acid equivalents per gram of dry weight (mg GAE/g).

b) Total flavonoid content

The Total flavonoid content was determined by the technique of Olajire A and Azeez L [9]. 1ml (1mg/ml) solution of each extract was added to 2,4mL of distilled water and 0,3mL of 5% $NaNO_2$ (Sodium Nitrate), after 5 minutes we added 0,3mL of 10% $AlCl_3$. The mixture allowed standing for 6 minutes incubate at dark, finally 1mL of NaOH (Sodium Hydroxide) (1M) was added and this mixture was up to 10ml with distilled water. After agitation, the Absorbance was read immediately at 510 nm with a spectrophotometer. The values are results of three replicates and the data are expressed in term of milligram equivalent of Quercitin per gram of crude extract (mg QE/g) ± standard deviation. These results were calculated from calibration of quercetin calibration.

Antioxidant activity: Total antioxidant activity (TAC):

This procedure is a spectroscopic method to quantify the total antioxidant activity, which is based on reducing in an acid environment Mo (VI) that is a colorless solution to Mo (V) which is green. This activity was calculated using the method described by PRIETO et al. [10] NUR ALAM. [11]

TABLE 1
Chemical composition of Daphne Gnidium's essential oil

RT	Percentage	Nomenclature	Chemical structure	Chemical Formula
25.92	0 ,25	β-Ionone		$C_{13}H_{20}O$
31.72	0,58	α-Bisabolol		$C_{15}H_{26}O$
35.99	2,85	Phthalic acid, hex-3-yl-isobutyl ester		$C_{18}H_{26}O_4$
36.41	45,57	Phytol		$C_{20}H_{40}O$
36.65	0,64	Pentadecanoic acid		$C_{15}H_{30}O_2$
37.70	0,65	Farnesyl acetone		$C_{18}H_{30}O$
38.23	1,03	Methyl hexadecanoate		$C_{17}H_{34}O_2$
38.90	1,24	Isophytol		$C_{20}H_{40}O$
39.33	22,84	Hexadecanoic acid		$C_{16}H_{32}O_2$
39.89	0,26	Ethyl hexadecanoate		$C_{18}H_{36}O_2$
40.49	0,30	2,6,10,14-Hexadecatetraen-1-ol, 3,7,11,15-tetramethyl-, acetate, (E,E,E)-		$C_{22}H_{36}O_2$

40.98	0,75	Cubebol		$C_{15}H_{26}O$
41.14	1,20	(E)-10-Heptadecen-8-ynoic acid methyl ester		$C_{18}H_{30}O_2$
42.00	1,52	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)		$C_{19}H_{32}O_2$
42.30	0,24	Octadecane, 3-ethyl-5-(2-ethylbutyl)		$C_{26}H_{54}$
42.94	11,34	9,12,15-Octadecatrienoic acid, (Z,Z,Z)		$C_{18}H_{30}O_2$
43.03	1,13	cis-Vaccenic acid		$C_{18}H_{34}O_2$
43.55	0,17	Ethyl 9,12,15-octadeca-trienoate		$C_{20}H_{34}O_2$
		Total of compounds: 92,56%		

RT: Retention time of each compound.

Briefly, 0,1ml of sample (1mg/ml) mixed with 1ml of reagents (0,6 M sulfuric acid, 28 mM sodium phosphate and 4 mM of ammonium molybdenum).The tube is incubated at 95°C during 90min.after cooling, the solution's absorbance is measured at 695 nm. Each measure was repeated three times. Ascorbic acid (AA) was used as reference and results were expressed in milligram equivalent of AA per gram of crude extract (mg AA/g).

RESULTS AND DISCUSSION

Extraction of essential oil. Extraction of daphne gnidium's essential oil using the Clevenger device shows a rending equals to 0.017 ± 0.001 % after tree hydrodistillation.

Chemical composition. Analyzing the essential oil of Daphne Gnidium by gas chromatography coupled to mass spectrum (see Table 1) furthermore revealed that the major compound was the phytol (45.75%).

Phytochemical screening. The phytochemical screening's results (see Table 2) of daphne gnidium's leaves, stems, flowers and fruits showed the great presence of Alkaloids, flavonoids, tannins, Antraquinons combined, sterols and triterpenes, mucilage, oses and holosides, while saponins antraquinons free were absent.

Impact of extractions solvents on crude extracts: The results in Table 3 showed that yields of crude extracts prepared from each parts of daphne gnidium using different pure solvents were differ significantly ($p<0.05$). Yet, the water showed the best yield's crudes extractions from leaves, stems, flowers and fruits of daphne gnidium with an average of 20.94% pursued with ethanol (11.85%) then ethyl acetate (6.96%) and dichloromethane (4.90%) while the hexane revealed the lowest yield of extraction with 2.62%. Those discoveries indicated that yields of crude extracts could be affected by the solvents' polarities as mentioned in previous study [12] also it could be explained by the difference in solubility of different compounds in different solvent.

Solvents' effect on total phenolic (TPC) and flavonoid content (TFC). The TPC was determined by Folin-Ciocalteu's method. The concentrations of total phenolic compounds were obtained from the calibration curve y= 0.0095x+0.0629, with R^2 = 0,998. The results were expressed by mg GAE/g of crude extract. The TFC were obeyed Olajire A and Azeez [10] protocol. The concentration were obtained from calibration curve y = 0.0046x + 0.0326, with R^2 = 0,999. The TFC values were expressed by mg QE/g of crude extract.

TABLE 2
Results of phytochemical screening of daphne gnidium (leaves, stems, fruits and flowers).

Phytoconstituent		Test performed	Leaves	Stems	Flowers	Fruits
Alkaloids		Mayer's Test	+++	+++	+++	+++
		Wanger's Test	+++	+++	+++	+++
Tannins Catechics		Stiansy reaction	+++	+++	-	-
Tannins Gallics		Lead acetate	+++	--	+++	++
Flavonoids		Shinoda's test	+++	+++	++	+
Antraquinons free		Borntrager's Test	--	--	--	--
Antraquinons combined	O-heteroside	Modified Borntrager's	++	++	++	++
	Heteroside Genins		--	++	++	++
	C-Heteroside		--	++	++	++
Sterols and Triterpenes		Liberman-burchard	++	++	++	++
Saponins		Foam index :positive >100	--	--	-	-
Mucilage		Alcohol 95%	++	++	+++	+++
Oses and holosides		Alcohol saturated with Thymol	++	++	+++	+++

High concentration (+++); moderate concentration (++); low concentration (+); absence (--)

TABLE 3
Yields (%) of crude extraction from different parts of daphne gnidium.

	Yield				
	Water	Ethanol	Ethyl acetate	Dichloromethane	Hexane
Leaves	32.05±1.07	8 .74± 0.09	9.00± 0.04	5.67± 0.09	2.47± 0.04
Stems	22.40± 0.2	10.09± 0.15	4.91± 0.08	3.1± 0.08	2.66± 0.07
Flowers	18.8±0.02	15.9 ± 0.08	5 .45±0.03	4 .00±0.04	2.85±0.05
Fruits	10.5±0.05	12.7±0.08	8.5±0.00	6.85±0.00	2.5±0.04
Means yields %	20.94	11.85	6.96	4.90	2.62

The values are the mean average of three replications for each solvents ± standard deviation. Values are significantly different (p<0.05)

TABLE 4
Total phenolic and flavonoid contents of daphne gnidium's extracts.

Bioactive compounds		Water	Ethanol	Ethyl Acetate	Dichloromethane	Hexane
Plant's parts						
TPC (mg GAE/g)	Leaves	403.23±1.70	288.53±1.0	260.85±0.40	240.62±1.08	207.27±0.45
	Stems	303.58±0.94	190.85±0.96	184.11±0.33	183.82±0.96	172.85±0.41
	Flowers	263.06±0.90	183.27±0.52	175.27±0.73	168.82±0.90	134.64±0.52
	Fruits	274.32±1.12	251.37±0.97	206.64±0.81	196.82±0.54	150.85±0.45
TFC (mg QE/g)	Leaves	271.39±1.8	187.82±1.06	98.13±0.90	90.8±0.91	86.01±0.36
	Stems	200.13±0.95	106.21±0.90	90.65±0.53	86.23±0.40	79.52±0.25
	Flowers	199.00±1.09	108.13±1.60	98.52±0.17	80.87±0.45	64.65±0.44
	Fruits	82.26±0.53	34.00±1.20	20.82±0.92	15.89±0.64	10.60±0.25

The values are the mean average of three replications for each solvents ± standard deviation. Values are significantly different (p<0.05)

The results of TPC and TFC of leaves, stems, flowers and fruits' crude extractions from daphne gnidium showed a difference between the plant parts' of phenolic and flavonoid content(Table 4), this distinction could be attributed to different solvents polarities used during extractions. [13, 14]., in the other hand, this distinction revealed that daphne gnidium is plenty of phenolic compounds. The TPC and TFC of all the crudes extracts showed a significant difference (p<0.05).

Daphne gnidium's leaves contained the higher concentration of phenolic compounds (403.23±1.70to 207.273±0.45 mg of GAE/g of crude extract), followed by stems (303.58±0.94to 172.85±0.41mg of GAE/g) pursued by Fruits (274.32±1.12to 150.85±0.45mg of GAE/g) then flowers (263.06±0.90to 134.64±0.52mg of GAE/g), for each part the TPC concentrations decrease in the following order: water > ethanol > ethyl acetate > dichloromethane > hexane, which is the same order

of polarities decreasing.These results further revealed the influence of solvent's polarity used during the extraction of TPC.The results were supported by previous studies [15,16], which are confirmed that extraction solvents play an important role in TPC's yields extracted. For example, Berbouchi et al [17] reported that water was the best solvent extraction for TPC from *Pistachia Lentiscus L*, while Do et al [12] found that absolute ethanol was the best extraction solvent for TPC from *Limnophila aromatic*. The differences could be reported to the various polarities of each solvents, which selectively extract different hydrophobic and hydrophilic phenolic compound in sapmles.

According to results, the greater total flavonoid concentration (see Table 4) showed by leaves (271.39±1.8 to 86.01±0.36mg of QE /g), followed by stems (200.13±0.95to 79.52±0.25mg of QE /g) then flowers (199.00±1.09to 64.65±0.44mg of QE /g), while fruits showed the lowest concentration (82.26±0.53to 10.60±0.25mg of QE /g). For each part of daphne gnidium water revealed the greatest content of flavonoid followed by ethanol then ethyl acetate, dichloromethane and hexane, which is the same order of polarity decreasing.

Based on results of crudes extracts' concentration of TPC and TFC, the water and ethanol, have the higher polarities and solubility, are more effective in extraction process than ethyl acetate, dichloromethane and hexane which have the lowest polarities. It may be concluded that phenolic concentration, in crude extracts, increases when the solvent's polarity used for extraction increases too, diver studies [18], showed that the higher polar solvent is more effective in extraction of phenolic compounds

Solvent's effect on total Antioxidant activity of daphne gnidium. The phosphomolybdenum assay was used to determine total antioxidant activity of daphne gnidium, which is expressed in mg AA/g of crude extract. Table 5 showed that the extraction's solvent significantly affected antioxidant capacity of daphne gnidium's leaves, stems, flowers and fruits ($p<0.05$). For each part of daphne gnidium, water revealed the greatest TAC, followed by ethanol then ethyl acetate and dichloromethane while hexane showed the weakest total antioxidant activity. The TAC values decreased in the same order of solvents', used for extraction, polarities decreasing. The results shows a various solvents' impact on antioxidant activity of daphne gnidium's different part, these differences could be explained by the variation of extractable bioactive compounds by different solvents. Each bioactive group contributed with different antioxidant capacity.

Correlation. The addiction of antioxidant activities was calculated, during this study, through the TAC assays that showed a positive correlation with the TPC and the TFC of daphne gnidium's crudes extracts.

Each bioactive group contributed with a different antioxidant capacity as these groups founded to have a positive correlation with antioxidant capacity. The phenolic compounds had a strong correlation with the antioxidant activity ($R^2 = 0.905$) (see Figure 1), followed by flavonoids ($R^2 = 0.679$) (see Figure 2). These results revealed that antioxidant capacity of daphne gnidium's leaves, stems, fruits and flowers was mainly contributed by phenolic and flavonoids compounds, which is supported by previous studies that showed the influence of antioxidant activity by phenolic and flavonoids. As a result of this work, different part of an aromatic plant's phenolic content may be the major contributor of the antioxidant activities, which is similar to previous studies [17], [19].

TABLE 5
Total antioxidant capacity (TAC) of leaves, stems, flowers and fruits of daphne gnidium:

TAC	Water	Ethanol	Ethyl acetate	Dichloromethane	Hexane
Leaves	189.481±2.50	118.37±1.54	96.222±0.32	65.02±0.64	33.259±0.84
Stems	164.667±1.5	50.169±0.94	44.296±0.43	40.65±0.57	21.334±0.35
Flowers	110.556±1.3	55.778±0.71	22.814±0.31	18.621±0.9	10.222±0.33
Fruits	97.259±1.24	60.962±0.63	53.185±0.21	34.785±0.82	27.111±0.43

The values are the mean average of three replications for each solvents ± standard deviation. Values are significantly different ($p<0.05$)

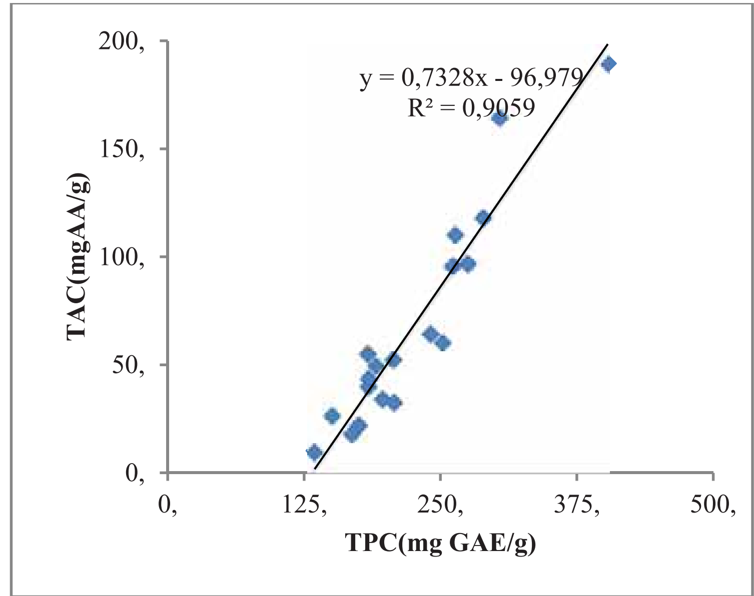

FIGURE 1
Linear dependency between the Total Antioxidant Activity and TPC.

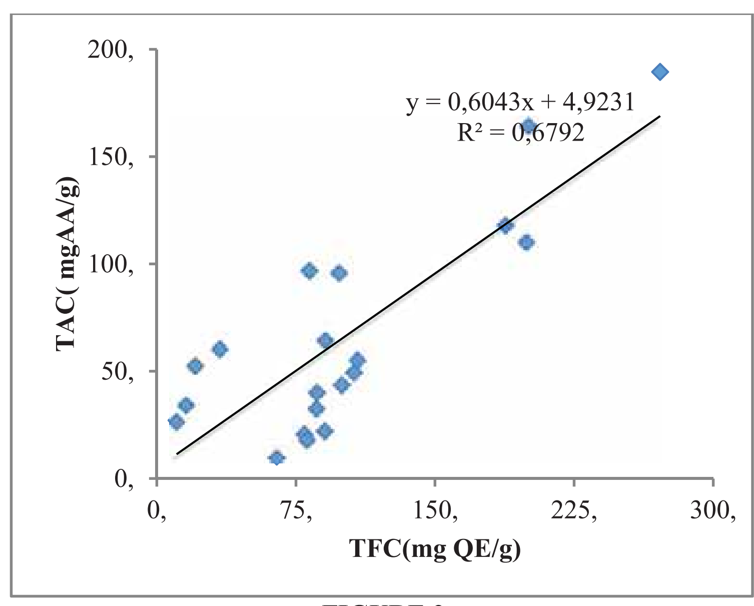

FIGURE 2
Linear dependency between the Total Antioxidant Activity and TFC.

CONCLUSIONS

This work aimed to study the screening phytochemical, to evaluate the total phenolic, flavonoids compounds and the total antioxidant activity of leaves, stems, flowers and fruits of daphne gnidium collected from Morocco. The results proved that the pure solvent of extraction had a big influence significantly ($p<0.05$) on extraction bioactive compounds and antioxidant activity from daphne gnidium's parts. Absolute hexane, dichloromethane and ethyl acetate were not effective, whereas water and pure ethanol were solvents yielding high crude extracts and high phenolic and flavonoids compounds for each part of daphne gnidium. Among these two solvents water was found to have the highest TFC, TPC and the greatest antioxidant capacity. The results of this work revealed that daphne gnidium's part, when a proper extraction solvent is released, could serve as a natural antioxidant against oxidative stress.

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Data availability. The authors confirm all data underlying the finding in this study are fully available without restriction

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QUALITATIVE AND QUANTITATIVE ANALYSIS AND ANTIFUNGAL EFFECTS OF *SATUREJA CALAMINTHA* SSP. (*NEPETA*) BRIQ FROM EASTERN ALGERIA

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ABSTRACT

The aim of this study is to carry out a qualitative and quantitative analysis of the biomolecules of *Satureja calamintha* harvested in the region of Skikda (Algeria), as well as the evaluation of the antifungal activity of the aqueous and methanolic extract against five ubiquitous molds belonging to the genera of *Aspergillus* (*A. flavus, A. parasiticus* and *A. ochraceus*) and *Penicillium* (*Penicillium chrysogenum* and *Penicillium aurantiogriseum*) producing mycotoxins isolated from traditional food (Couscous and Hror) marketed in the Bechar region. The identification of the different components by phytochemical tests based on different reactions, the total contents of polyphenols, flavonoids and tannins of the two extracts were determined by spectrophotometric assay, and the evaluation of the antifungal activity was carried out according to the technique of poisoned foods. The phytochemical analysis carried out revealed the presence of eight major chemical groups (terpenes, flavonoids, coumarins, steroids, phenolics compounds, saponins and tannins). The total phenol contents were 5.37±0.03 mg/g and 10.70±0.06 mg/g gallic acid equivalent for the methanolic and aqueous extracts respectively. The evaluation of the antifungal activity of the extracts (60 mg/ml) shows that the methanolic extract had a higher activity (>69.04% ±2.06) against all the molds tested compared to the aqueous extract (>29.76% ±2.06), while AMB was potent with an inhibition rate greater than 84.05 % ±6.64. We can assume that *Satureja calamintha* exhibits antifungal potency that may be useful in treating fungal infections, storage fungi and food spoilage in the food industry field.

KEYWORDS:
Antifungal activity, *Aspergillus*, molds, *Penicillium*, *Satureja*

INTRODUCTION

Food security is the major concern in the majority of countries. Governments around the world are working with scientists to ensure that all people have physical, social and economic access to sufficient and nutritious food [1]. Unfortunately, despite all efforts, contamination of food is inevitable at different stages of the food chain (in the field and during storage) in tropical and subtropical countries, where varying climatic conditions, poor agricultural practices and their status socioeconomic providing an appropriate environment for the accumulation and proliferation of harmful molds [2]. These make food products unfit for consumption and become a threat to food security [3].

Mycotoxins are natural products of very low molecular weight produced as secondary metabolites by certain filamentous fungi. Over the years, the contamination of food by these substances constitutes a real public health problem at the global level because they present a real threat to humans and animals through their wide range of toxic effects: carcinogens, mutagens, teratogens and immunosuppressant's [4]. The Food and Agriculture Organization of the United Nations (FAO) estimates that around 25% of grains are contaminated with mycotoxins [5].

Several strategies have been used to control fungal growth as well as limit human and animal exposure to mycotoxins [6]. These techniques are undoubtedly very effective, but they have several drawbacks such as the development of several resistant pathogens, the reduction in the nutritional value of foods and often require very expensive equipment [7]. Moreover, these strategies cause catastrophic consequences on the environment due to their long period of degradation, which affects the natural ecological balance [8].

For this, scientific research has opted for the use of biomolecules to deal with this dangerous contamination. In general, nature constitutes a source of therapeutic agents, of which medicinal plants represent a precious resource in secondary metabolites

[9]. Pharmacologists confirm that these phytochemicals have played a vital role in the treatment of various diseases thus used in food preservation since the beginning of humanity [10].

Satureja calamintha ssp. *Nepeta* (L.) Briq. (Syn: *Calamintha nepeta*) is a perennial, pubescent, aromatic, 40 to 80 cm tall, with greyish green leaves and purple petiolate white flowers [11]. Many studies have been made on this plant that prove their antibacterial, antifungal, antioxidant and anti-inflammatory, excluding the authors suggest the presence of essential oils [11-17] and phenolic compounds [15,18-20].

The objective of the present study is to evaluate the antifungal activity of *S. calamintha nepeta* against five species of molds synthesizing mycotoxins with the aim of developing a herbal formulation to reduce or inhibit the proliferation of toxigenic molds in food.

MATERIALS AND METHODS

Plant material. The plant material (Figure 1) was harvested in November 2019 from the mountains of the Skikda region located in the eastern region of Algeria (Figure 2). The plant was cleaned thoroughly with tap water and sterile distilled water to remove dust and other inert material. The parts of the plant were dried in the shade at room temperature for 3 weeks, crushed and stored for further analysis.

FIGURE 1
***Satureja calamintha* subsp. *nepeta* (L.) Briq**

FIGURE 2
Geographical location of the Skikda region [21]

Phytochemical screening. The preparation of the extracts was carried out according to the protocol described by Nemlin and Brunel [22]. A quantity of 20 g of powder was macerated 3 times with 60 ml of diethyl ether for ten minutes. The extracts were filtered through Whatman filter paper and concentrated to 25 ml. The filtrate was labeled as an extract of diethyl ether. The dry residue was then macerated in methanol using the same protocol above. The resulting extract was labeled as a methanolic extract. Adding a further 5 g of plant material to the dry residue, the total was extracted by infusion into 50 ml of distilled water. After stirring for 15 minutes, the extract was filtered through Whatman paper and labeled as an aqueous extract. The three extracts were used for phytochemical screening with the aim of qualitatively detecting the various chemical compounds existing in the plant according to the methods described in the litterature [23-25].

Terpenoids: 5 ml of extract was mixed with 2 ml of chloroform and 3 ml of H_2SO_4. the presence of terpenoids is indicated by a reddish-brown color at the interface

Tannins: 0.5 ml of 5% ferric chloride is added to 5 mg of plant extract. The development of a dark bluish black color indicates the presence of tannins.

Coumarin: 5 ml of methanolic extract were evaporated to dryness, and then a volume of 1 to 2 ml of hot water was added to the dry residue. The volume was divided into two, one of which was treated with 0.5 NH_4OH (10%) and the other served as a control. the presence of coumarins was indicated by intense UV fluorescence (λ = 265 nm or 365 nm).

Reducing sugars: 2 ml of plant extract was mixed with equal volumes of Fehling's solution A and B. The development of a red precipitate indicates the presence of free reducing sugars.

Steroid: 10 ml of chloroform is added to 1 ml of extract, then an equal volume of concentrated H_2SO_4 acid is added to the peripherals of the test tube. The appearance of a yellow color with green fluorescence in the H_2SO_4 layer indicates the presence of the steroid.

Phenolic compounds: 0.5 g of plant extract is dissolved in water, then mixed with a few drops of a 5% $FeCl_3$ solution. The appearance of a dark green color indicated the presence of phenolic compounds.

Saponins: 0.5 mg of each extract was taken into a test tube, then a few drops of 5% sodium bicarbonate solution were added. The mixture was stirred vigorously and kept for 3 minutes. The presence of saponins was indicated by the formation of honeycomb like moss.

Flavonoids: The presence of flavonoids was confirmed by the appearance of a yellow color in a solution of 1 ml of plant extract mixed with a few drops of a 1% $AlCl_3$ solution.

Alkaloids: Wagner test: 10 mg of each extract were taken, then a few drops of Wagner's reagent were added. The formation of a reddish-brown precipitate indicates the presence of alkaloids.

Preparation of the aqueous extract. The aqueous extract was prepared according to the method described by Majhenic et al [26] and adopted by Bougandoura and Bendimerad [20]. A quantity of 10 g of plant material was refluxed for 2 hours in 150 ml of distilled water, then filtered with whatman paper to remove particles and the resulting filtrate was evaporated to dryness under reduced pressure at 65°C using a rotary evaporator (Buchi Rotavapor R-200).

Preparation of the methanolic extract. The methanolic extract was prepared according to the method described by Sharma [27] with some minor modifications. 25 g of the powdered plant material was macerated with 100 ml of methanol for 72 hours at room temperature with occasional stirring, and then filtered through whatman paper to remove particles. The resulting filtrate was concentrated by evaporation to dryness under reduced pressure at 65°C using a rotary evaporator (Buchi Rotavapor R-200).

Determination of extraction yield. The yield of the two extracts was calculated according to the formula described by Rahmoun et al [28]:

Yield (%) = Where, *m0*: mass in grams of the resulting dry extract and *m1*: mass in grams of the plant material to be treated.

Determination of total phenolic compounds. The total polyphenol content of the aqueous and methanolic extract of *S. calamintha nepta* was measured using a spectrophotometer. According to the colorimetric method using the Folin-Ciocalteu reagent, the protocol carried out in this work is that described by Geremu et al [29]. A volume of 0.5 ml of each extract is introduced into glass hemolysis tubes, adding 2.5 ml of Folin-Ciocalteu reagent diluted ten times with 2 ml of 7.5% sodium carbonate. The tubes were covered with aluminum foil and left to stand for 30 min at room temperature. The absorbance reading was determined at 765 nm using a UV / Vis spectrophotometer (Zuzi 4211/50). Three replicates were performed for each test sample. A calibration curve was performed in parallel under the same operating conditions using gallic acid at different concentrations. The results were expressed as milligrams of gallic acid equivalent per gram of dry extract (mg EAG/g E).

Determination of total flavonoids. The quantification of the flavonoids was carried out according to the protocol described by Ali-Rachedi et al [30]. A volume of 400µl of extract was added to 120µl of 5% $NaNO_2$. After 5 minutes, 120 µl of 10% $AlCl_3$ was added. After 6 minutes, a volume of 800 µl of 1 M NaOH was added to the medium. The absorbance reading was determined with a spectrophotometer (Zuzi 4211/50) at 765 nm. A calibration curve was performed in parallel under the same operating conditions using quercetin at different concentrations. Results were expressed as milligram equivalent of quercetin per gram of dry extract (mg EQ/g E).

Determination of total tannins. The tannin content was determined according to the method described by Ramya and Dhamotharan [31]. A volume of 1 ml of the extract was mixed with 0.5 ml of Folin-Ciocalteau reagent, followed by the addition of 1 ml of saturated Na_2CO_3 solution and 8 ml of distilled water. The mixture was vortexed, then incubated for 30 minutes at room temperature. The absorbance reading was determined with a spectrophotometer (Zuzi 4211/50) at 725 nm. A calibration curve was performed in parallel under the same operating conditions using tannic acid at different concentrations. The results were expressed in milligrams equivalent of tannic acid per gram of dry extract (mg TAE/g E).

Origin of fungal strains. A collection of seven pathogenic filamentous fungi (*Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus ochraceus*, *Penicillium chrysogenum* and *Penicillium aurantiogriseum*) was used in this study. These fungal strains were isolated from traditional food marketed in the city of Bechar, Algeria. These molds were identified by the "Single Spore" technique described by Pitt and Hocking [32]. The choice of these molds was based on their degree of toxicity and their ability to synthesize mycotoxins, which makes the contamination of food by its molds a real public health problem which really requires very effective control, means to eliminate them or more or less minimize them to the maximum of any type of food whatever human or animal.

Antifungal activity. The antifungal activity of the extracts against the different strains was evaluated according to the technique of poisoned foods described by Amadioha [33]. First, dimethyl sulfoxide (DMSO) is the most widely used solvent by the majority of authors, who have shown that it has no remarkable antifungal power. For this reason, our crude extracts (aqueous and methanolic) were dissolved in DMSO at different concentrations (60, 40, 20, 10, 5 and 2.5 mg / ml). The extracts were filtered through a 0.45 µm membrane filter. A volume of 1 ml of each concentration was aseptically poured into test tubes followed by the addition of 19 ml of PDA acidified with 25% lactic acid molten at 45°C and was thoroughly shaken for good dispersion of the extract in the culture medium. The whole was poured into glass Petri dishes (90 mm). Each Petri dish was inoculated in the center with a mycelial disc (6 mm in diameter) taken from the periphery of young colonies (72 hours) of the fungal strains tested in this study and incubated at 25°C for 7 days. The commercial fungicide amphotericin B (AMB) was used as a positive control and DMSO was used as a negative control. Three repetitions of each test were performed. The average diameter of the fungal colonies was measured on the 7th day of incubation and the percentage inhibition of mycelial growth was calculated according to the formula proposed by Sztejnbergetal et al [34] and adopted by Pinto et al by [35]:

Statistical analysis. All experiments were spotted three times. Ms Excel 2007 was used to express the values as the mean ± deviation.

RESULTS

Phytochemical screening. The phytochemical study allowed us to characterize the different families of chemical compounds existing in the plant studied. This screening revealed the presence of terpenes, tannins, coumarins, steroids, phenolics compounds, saponins, flavonoids, while, reducing sugars, alkaloids were not detected. The results of the phytochemical tests have been summarized in Table 1.

Yield. After the step of extracting the raw extracts, the calculation of the yields is very important because it gives us the possibility of having an idea on the quantity necessary to take before each in-depth study on the studied plant. The yield results showed that the aqueous extract (19.57%) is more superior to that of the methanolic extract (9.64%).

Total content of polyphenols, flavonoids and tannins. The total polyphenol contents were determined by colorimetric assay using the Folin-Ciocalteu reagent, and the results are expressed in mg GAE/mg of extract with reference to the established calibration curve (Figure 3). The total flavonoids are determined by the aluminum trichloride reagent, and results are expressed as mg QE/mg extract based on the standard curve established with quercetin (Figure 4). The total tannins were determined using the Folin-Ciocalteu reagent, and the results are expressed in mg TAE/mg of extract by referring to the established calibration curve (Figure 5). The results of the polyphenols, flavonoids and tannins contents of the extracts of *Sateruja calamintha nepeta* are shown in Table 2.

TABLE 1
Phytochemicals detected in extracts of *Satureja calamintha nepeta*

Phytochemicals	Diethyl ether extract	Methanolic extract	Water extract
Terpenoïds	-	+	+
Tannins	+	+	+
Comarins	-	+	-
Reducing sugar	-	-	-
Steroids	+	+	+
Phenolics compounds	+	+	+
Saponines	+	+	+
Flavonoids	-	+	+
Alkaloids	-	-	-

Key : + = present ; - = absent.

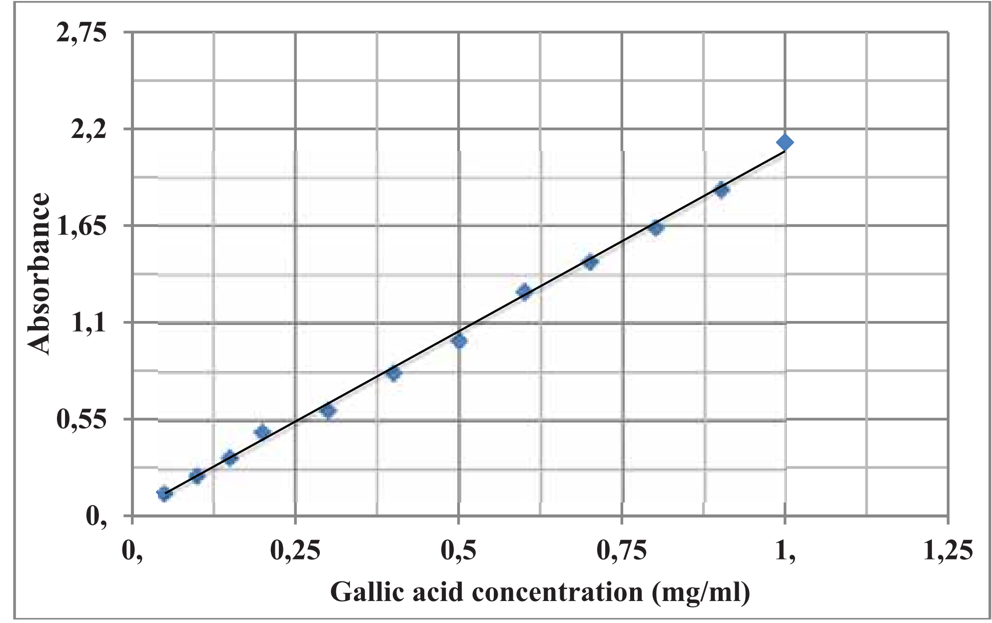

FIGURE 3
Calibration curve for standard gallic acid

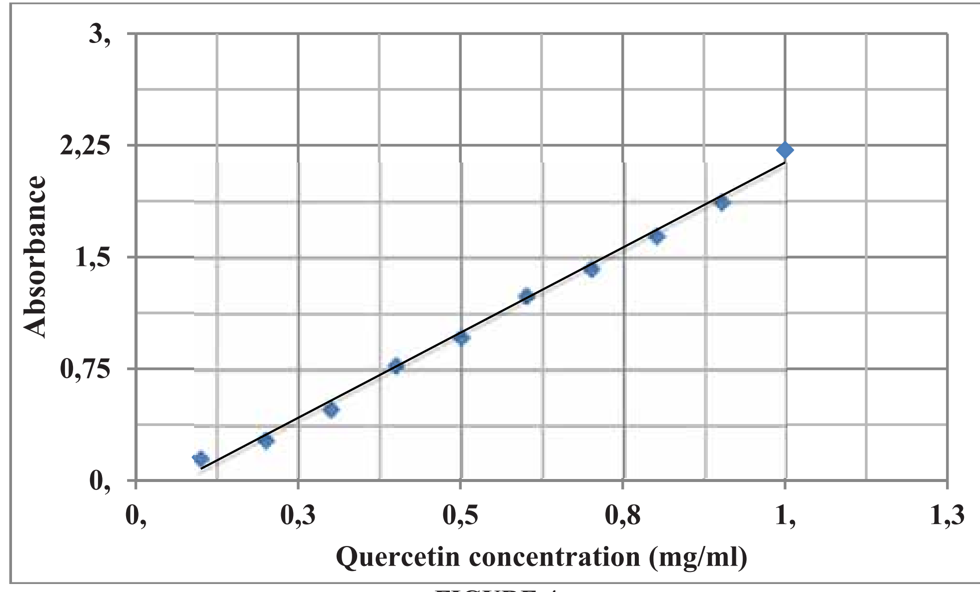

FIGURE 4
Calibration curve for standard quercetin

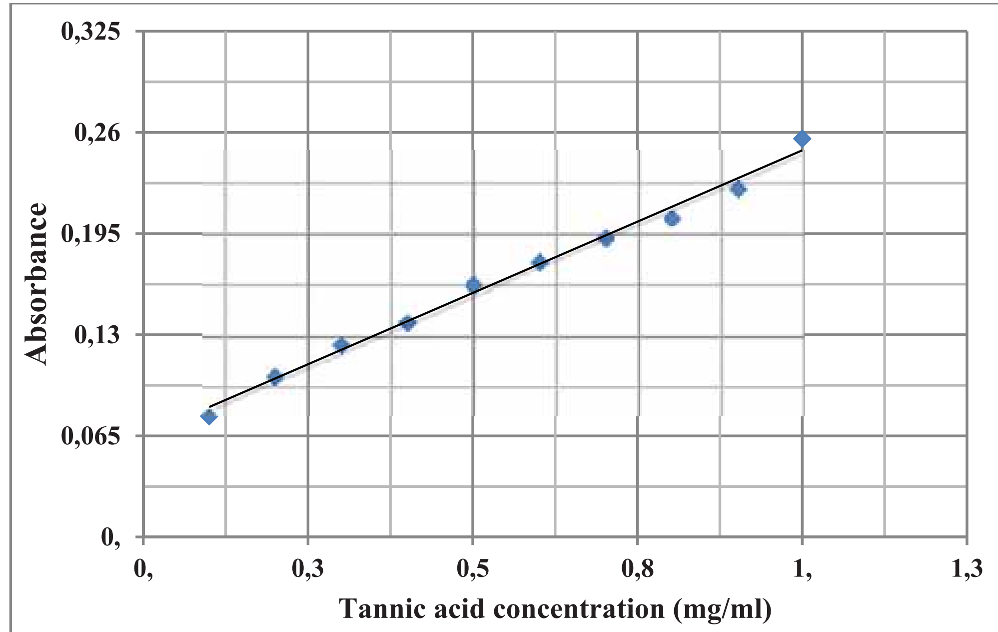

FIGURE 5
Calibration curve for standard tannic acid

TABLE 2
Results of the determination of total polyphenols total, total flavonoids and total tannins in extracts of *Sateruja calamintha*

Extract	Total phenol content (mg GAE/g E)	Total flavonoids content (mg QE/g E)	Total tannin content (mg TAE/g E)
Methanolic	5.37 ±0.03	3.19±0.01	0.58±0.16
Water	10.70 ±0.06	4.11±0.02	2.23±0.27

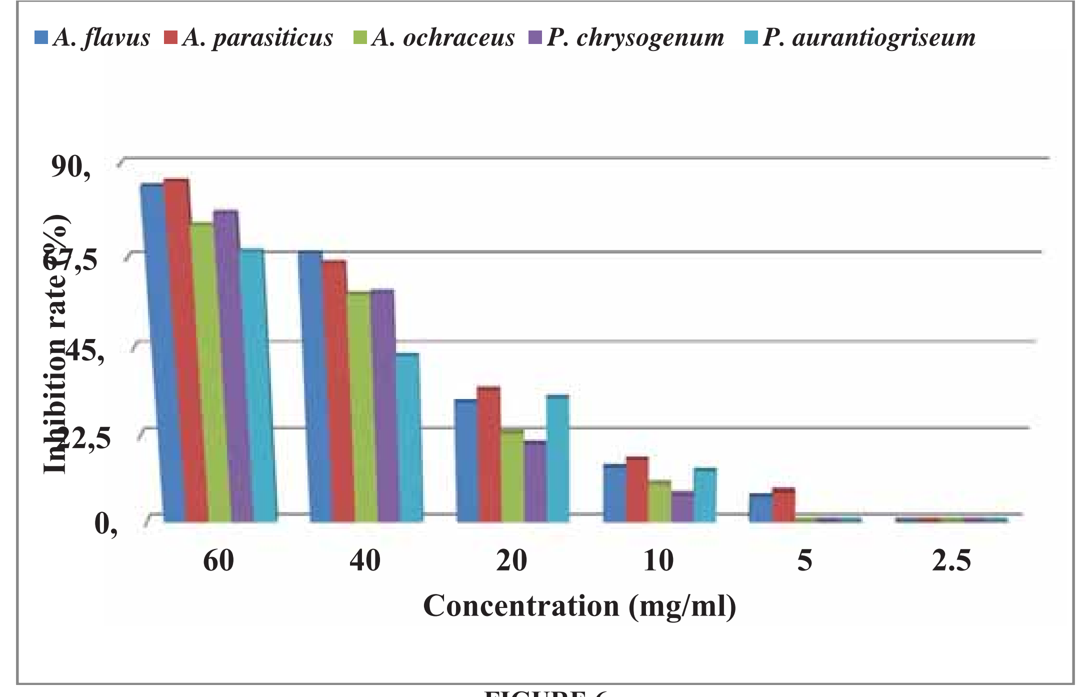

FIGURE 6
Inhibition rate (%) of the methanolic extract on the tested molds

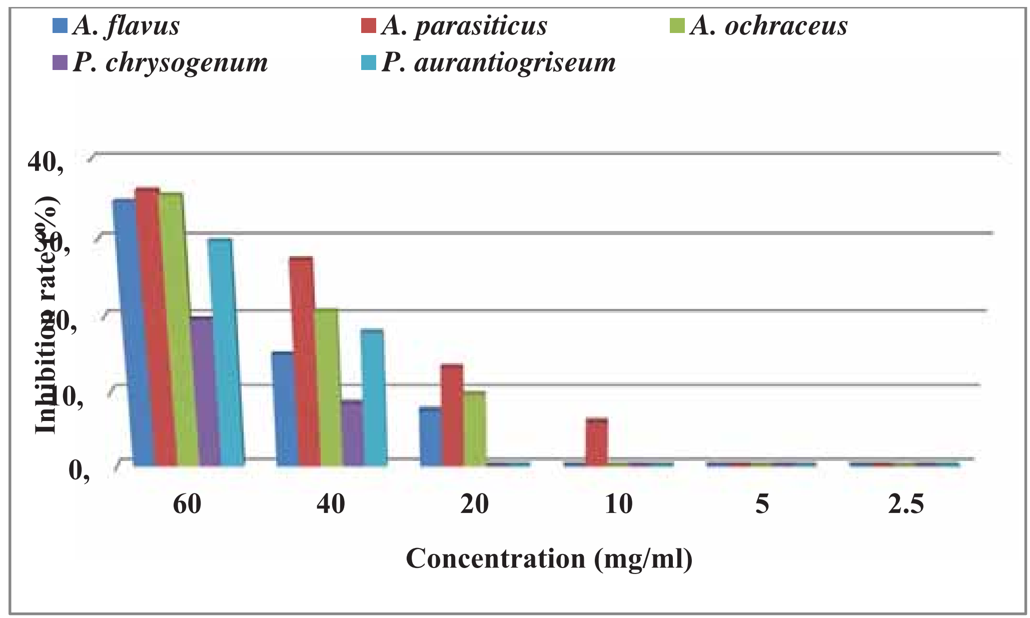

FIGURE 7
Inhibition rate (%) of the aqueous extract on the tested molds

Antifungal activity. As seen in Figures 6 and 7, the results of antifungal tests of methanolic extracts (60 mg/ml) showed a high degree of inhibition against all molds: *A. flavus* (84.5%), *A. parasiticus* (85.84%), *A. ochraceus* (75.55%), *P. chrysogenum* (78.43%) and *P. aurantiogriseum* (66.66%). The aqueous extract (60 mg / ml) also showed a remarkable rate of inhibition but lower than that of the methanolic extract: *Aspergillus flavus* (34.74%), *Aspergillus parasiticus* (36.07%), *Aspergillus ochraceus* (35.55%), *Penicillium chrysogenum* (19.6%) and *Penicillium aurantiogriseum* (24.63%).

As seen in Tables 3 and 4, the concentrations of 2.5 and 5 mg/ml of the methanolic extract had no effect on the molds studied, except a slight inhibition with 5 mg / ml on the growth of *A. flavus* (6.57%) and *A. parasiticus* (7.76%). While the concentrations of 2.5, 5 and 10 mg/ml of the aqueous extract did not promote any inhibition, except with 10 mg / ml gave a slight inhibition on *A. parasiticus* (5.93%).

The minimum inhibitory concentration was examined at different concentrations of *S. calamintha*. For the methanolic extract the lowest values were recorded against the strains *A. flavus* (5 mg/ml) and *P. chrysogenum* (10 mg/ml) and for the aqueous extract the lowest value was recorded against *A. parasiticus* (10 mg/ml) (Tables 3, 4). The results also

TABLE 3
Inhibition rate (%) of different concentrations of the methanol extract of *Satureja calamintha*

Concentration (mg/ml)	*A. flavus*	*A. parasiticus*	*A. ochraceus*	*P. chrysogenum*	*P. aurantio-griseum*
60	84,5±1,40	85,84±1,58	75,55±2,22	78,43±1,96	69.04±2.06
40	68,54±2,15	66,21±1,58	58,51±1,28	60,13±4,52	42.87±3.57
20	30,98±1,40	34,24±2,37	22,96±3,39	20,26±1,13	32.14±3.57
10	14,08±2,09	15,98±2,09	9,62±1,28	7,18±1,13	13.09±2.06
5	6,57±1.40	7,76±2,09	0	-	-
2,5	-	-	-	-	-
DMSO	-	-	-	-	-
AMB	90,14±1,40	93,15±1,36	92,59±1,28	94,77±2,26	84,05±6,64

- : No inhibition

TABLE 4
Inhibition rate (%) of the various concentrations of the aqueous extract of *Satureja calamintha*

Concentration (mg/ml)	*A. flavus*	*A. parasiticus*	*A. ochraceus*	*P. chrysogenum*	*P. auranti-ogriseum*
60	34,74±0,81	36,07±2,85	35,55±2,22	19,6±1.96	29.76±2,06
40	15,02±0,81	27,39±1,36	20,7±1,28	8,49±1.13	17.85±0.00
20	7,51±0,81	13,24±2,85	9,62±1,28	-	-
10	-	5,93±2,09	-	-	-
5	-	-	-	-	-
2,5	-	-	-	-	-
DMSO	-	-	-	-	-
AMB	90.14±1.40	91,78±1.36	92.59±1.28	94.77±2.26	84.05±6.64

- : No inhibition

show that 60 mg / ml amphotericin B was hyperactive with a higher inhibition rate (≥ 84.05%) than the extracts of *S. calamintha* exploited against the five strains: *A. flavus, A parasiticus, A. ochraceus, P. chrysogenum and P. aurantiogriseum*, which showed high sensitivity to AMB (Tables 3, 4).

DISCUSSION

According to Labiod [15], the yield of methanolic extract of *S. calamintha nepta* collected in the regions of Annaba and Jijel is 14.6% and 12% respectively. This yield is higher than that found in our study, this may be due to the difference in the extraction technique used because the extraction by solvents at high temperature made it possible to obtain higher yields of dry extracts [37]. In addition, our results are in harmony with the study conducted by Bougandoura and Bendimerad [20] on *S. calamintha* collected in the town of Tlemcen. On the other hand, the study carried out by Hayani et al [37], shows that *S. calamintha* from the Ouazzane region in Morocco has a much higher yield compared to that of our plant.

Phytochemical screening on plant extracts present an essential stage since they reveal the presence of constituents known by their physiological activities and their medicinal interests [38]. Phytochemical tests performed by Hayani et al [37] revealed the presence of flavonoids, tannins, along with the absence of alkaloids, which is in agreement with our results.

On the other hand, the results of qualitative analysis of phenolic compounds show that the aqueous (AE) and methanolic (ME) extract of *S. calamintha* are very rich in polyphenols (10.70 ± 0.06 mg GAE/gAE and 5.37 ± 0.03 mg QE/gME) in flavonoids (4.11 ± 0.02 mg QE/gAE and 3.19 ± 0.01 mg QE/gME). The work carried out by Bougandoura and Bendimerad [19], shows that the quantity of polyphenols (12.6 ± 0.775 GAE/gAE and 2.968 ± 0.809 GAE/gME) and of flavonoids (3.131 ± 0.154 mg QE/gAE and 1.280 ± 0.077 mg QE/gME) was almost similar compared to our study. On the other hand Hayani et al [37], reported that the content of polyphenols in the aqueous extract (20.42 ± 0.004 mg GAE/g) and in organic fractions (varies between 15.26 ± 0.0075 mg QE/g and 33.48 ± 0.0235 mg QE/g) were much higher than those found in our study, which shows that the Moroccan flora is much richer in bioactive compounds. The content of bioactive substance can be influenced by several parameters such as the method and conditions of extraction, chemical composition, climate and period of maturity of the plant at harvest time and storage conditions [39].

The results of the antifungal tests of the methanolic extract showed a high degree of inhibition against all molds compared to the aqueous extract, this confirms the previous results according to which the methanolic extract of *S. calamintha* from the Tlemcen region showed a higher antifungal power than that of the aqueous extract against three pathogenic molds: *Aspergillus flavus* (87.5%), *Fusarium oxysporum* (66.66%) and *Cladosporium herbarum* (67.94%) [20]. These results can also be explained by the richness of the methanolic extract in flavonoids which are considered to be bioactive compounds with strong antifungal activity. According to Hernández et al [40], during an experiment carried out on *Satureja parvifolia* collected in South America (Argentina), alcohol favored better extraction of flavonoids.

The difference in the structure of the phenolic compounds of the two extracts may also have contributed to these results. In fact, many studies have revealed the relationship between the chemical structure of phenolic compounds and their antimicrobial power. According to Chabot et al [41], flavonoids without a hydroxyl group on the B ring, exhibited a higher antimicrobial activity than those with the-OH group. On the other hand, Nishino et al [42] and Mori et al [43] showed that the flavonoids most substituted by the free hydroxyl group are mainly involved in the antimicrobial activity. These conflicting results require further, more in-depth research on the structure-activity relationship to understand the mechanism truly responsible for the antimicrobial effects.

The reason for this difference in activity between the two extracts can be explained by the choice of the extraction solvent, knowing that the bioactive compounds that cause the antimicrobial activity dissolve more easily in organic solvents compared to aqueous solvents.

CONCLUSION

The antifungal properties of the tested extracts of *S. calamintha* is probably due to the presence of secondary metabolites such as flavonoids, phenolic compounds, saponins, steroids, terpenoids and tannins. Our results suggest that extracts from this plant could be used as an alternative source of antifungal agents against fungal infections which subsequently cause the production of mycotoxins which are harmful to humans and animals. Further, more in-depth research is more than necessary to discover the true bioactive molecules responsible for antifungal activities.

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CHEMICAL COMPOSITIONS ANTIOXIDANT ANGIOTENSIN CONVERTING ENZYME AND CARBOHYDRATE HYDROLYZING ENZYME INHIBITORY PROPERTIES OF MIRACLE BERRY SEED (SYNSEPALUM DULCIFICUM DANIELL) FLOUR

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ABSTRACT

Miracle berry fruit pulp is used as a sweetening and medicinal agents. The fruits were collected from Dagbolu village near Iwo Township, Osun State, Nigeria. The undefatted (UMF), defatted (DMF), ethanolic extract (MSE) and protein isolate (MPI) of miracle seed were evaluated for proximate, phytochemicals and amino acid profiles, while MSE and MPI were evaluated for antioxidants and enzymes (angiotensin-1-converting, α-amylase and α-glucosidase) inhibitory activities. The protein content of the flour samples varied from 8.49 to 81.04 g/100g for UMF and MPI, respectively. The in-vitro digestibility of starch, protein and extract of the seed were 67.6%, 65.1% and 52.9%, respectively. Arginine/lysine ratio, essential, branched chain and aromatic amino acids (g/100g protein) varied from 1.10 - 2.04, 30.67 - 38.29, 7.97 - 11.64 and 12.77 - 16.22, respectively. Phytochemicals were lower than critical levels, while phenolic profile had phloridzin (2537.03 mg/100g) as the most abundant followed by isoquercitrin (206.052 mg/100g) and quercetin (155.985 mg/100g), respectively. In-vitro antioxidant activity (IC50) (µg/mL) of MPI and MSE against DPPH, ABTS, iron chelation and OH free radicals varied from 94.61 - 113.8, 94.61 - 113.9, 94.59 - 103.2 and 91.99 - 94.56, respectively. The IC50 of MPI and MSE on α-amylase and α-glucosidase enzyme activities ranged from 77.97 - 82.02 and 76.3 - 79.3 µg/mL, respectively, while that of angiotensin-1-converting enzyme inhibitory activity were 72.3% and 87.8%, respectively. Study established that MSE exhibited higher antioxidant, and inhibitory activity on α-amylase, α-glucosidase and angiotensin-1-converting enzyme. Hence, MSE may be suitable as antidiabetic and antihypertensive agent.

KEYWORDS:
Miracle berry seed, Bioactive compounds, Blood glucose reducing, Antioxidant activity, Antihypertensive activity

INTRODUCTION

Miracle berry plant (Synsepalum dulcificum (Schumach. & Thonn.) Daniell) is a tropical evergreen shrub or small tree indigenous to tropical West and West-Central tropical Africa [1]. The plant is Sapotaceae family and commonly known as miracle fruit, miracle berry, miraculous berry, etc. [2]; and it is called Agbayun (Yoruba, Nigeria), asowa (Twi, Ghana) and ele (Ewe, Ghana) [3]. The fruit has a relatively large elongate ovoid shape seed, which is encapsulated by a translucent edible pulp that changes colour into bright red upon ripens and the pulp is covered by a thin skin [4]. The plant has been discovered since the 18th century [5] due to its remarkable property of glycoprotein known as miraculin, which help to modify a sour taste into sweet taste [4]. The fruit pulp is a natural food sweetener and it could be possibly be used to sweeten foods particularly for diabetic patient and dieters due to its characteristic of being a low-calorie sweetness enhancer, hence, limiting sugar and energy intake [3, 6]. Besides, miracle berry fruit contains antioxidant, which study has confirmed to possess medicinal activity against chronic diseases, such as cardiovascular diseases, obesity, diabetes and certain cancers [7].

Antioxidants are chemicals with the ability to neutralize harmful reactive free radicals in the body cells that may cause oxidative stress [8]. Oxidative stress is caused by the imbalance between the formation and neutralization of free radicals, and it has been implicated to be the main risk factor responsible for various human diseases such as diabetes, hypertension and other degenerative diseases [9, 10]. Antioxidants act as a defense mechanism against reactive oxygen species formation that are naturally produced in man or as a result of environmental factors like radiation, bacterial and viral toxin and lifestyle like smoking, alcohol, psychological, or emotional stress [11]. However, human cells have several ways of inhibiting the production of free radicals and to manage oxidative stress, which include enzymic activities (superoxide dismutase, catalase, glutathione reductase) and non-enzymic (food supplements)

[12]. Previous study has revealed that whenever the protective roles of enzymatic antioxidants (catalase, superoxide dismutase, and glutathione) are disrupted due to various pathological processes, there is a need to complement their activities with nonenzymatic antioxidant agents either in form of synthetic antioxidant or food-based antioxidant supplements. However, epidemiological study has implicated synthetic antioxidant agents with many side effects [13], but present studies are advocating for plant-based food supplements with non-side effects as alternate to synthetic antioxidant supplements [10,14].

This study was aimed to determine nutritional composition, antioxidant and inhibitory activities of miracle berry seed bioactive components on carbohydrate hydrolyzing enzyme and angiotensin-converting enzyme. The results obtained would increase the utilisation of miracle berry seed bioactive components as ingredient in functional foods and nutraceutical supplements.

MATERIALS AND METHODS

Source of raw materials. The miracle berry seeds were collected from Dagbolu village near Iwo Township (7038´N, 4011´E), Osun State, Nigeria. The seed was identified at the Herbarium Unit of the Department of Crop Production and Pest Management, Federal University of Technology, Akure, Nigeria.

Processing of Miracle Berry Seed Flour. Processing of Miracle Berry Seed flour (UMF). The miracle berry seeds were manually dehulled, washed with distilled water, drained and dried in a hot-air oven (Plus11 Sanyo Gallenkamp PLC, Loughborough, Leicestershire, UK) at 60 oC for 20 h. After oven dried, the seeds were milled (Laboratory blender, Model KM 901D; Kenwood Electronic, Hertfordshire, UK) and sieved (60 mm mesh sieve, British Standard) to obtain whole miracle berry seed flour (MSF). The flour was stored at room temperature (~27 oC) until analysis.

Processing of Defatted Miracle Berry Seed Flour (DMF). The whole miracle berry seed flour (250g) was defatted using n-hexane as solvent in a Soxhlet apparatus for 12 h. The defatted flour was oven dried using a hot-air oven (Plus11 Sanyo Gallenkamp PLC, Loughborough, Leicestershire, UK) at 40 oC for 2 h to allow the solvent to escape. The defatted flour was sieved with a 60 mm wire mesh sieve (British Standard). The defatted flour was stored at room temperature (~27 oC) until analysis.

Preparation of Miracle Berry Seed Protein Isolate (MPI). The miracle berry seed protein isolate was prepared as described by Dawodu and Abdulsalam [15]. Defatted miracle berry seed flour was dispersed in distilled water (water: flour ratio, 1:25 (w/v)), the mixture was maintained at alkaline medium of pH 11.0 using 1 M NaOH to solubilize the proteins and the temperature of the mixture with continuous stirring was maintained at 37 ^{o}C for 2 h. After, the slurry was centrifuged at 15,000 x g for 20 min at 4 °C. The residue was discarded, while the supernatant was adjusted to pH 4.0 (isoelectric point) with 1 M HCl to precipitate the proteins. The precipitated protein was washed twice times with distilled, centrifuged at 15,000 xg for 20 min at 4 °C, re-dissolved in water, neutralized to pH 7 with 0.1 N NaOH, and then freeze-dried. The miracle berry seed protein isolate was stored for further analysis.

PLATE 1
Picture of miracle berry seeds

Preparation of Miracle Berry Seed Ethanolic Extract (MSE). The defatted seed flour (500 g) was dispersed in 2.5 L of 95% ethanol, and extracted exhaustively via marceration for 48 h using modified method of Sunday and Uguru [16]. After marceration, the mixture was filtered with Muslin cloth and Whatman No. 1 filter paper (Qualitative Circles 150 mm Cat No. 1001 150); and the filtrate was concentrated (Rotary evaporator; Model 349/2, Corning Limited) at 35 ºC for 24 h. The ethanol in the concentrated filtrate was allowed to evaporate at room temperature and the dried extract sample was stored (~27 ºC) until required for use.

Chemical Analyses of Miracle Berry Seed Flour Samples. Proximate composition. The proximate composition [moisture (AOAC 929.02), protein (AOAC 975.17), fat (AOAC 973.22), crude fibre (AOAC 962.09C), and ash (AOAC 922.02)] of milled miracle berry seed flour samples was determined using the standard method of AOAC [17]. Total carbohydrate was calculated by difference. The total energy content was obtained using Atwater conversion factors [18].

Total energy (kcal/100 g)= (4×% protein)+(9×% fat)+(4×% carbohydrate)

Determination of *in-vitro* starch and protein digestibility. The *in-vitro* starch digestibility of the miracle seed samples was determined using the method described by Singh et al. [19]. Each of the flour samples (50 mg) was mixed with 1 mL of 0.2 M phosphate buffer (pH 6.9). Thereafter, 0.5 mL of pancreatic alpha amylase (100 unit/mg) was added to the mixture and incubated at 37°C for 2 h. After incubation, 2 mL of 3, 5-DNS reagent was added, and then the mixture was heated for in a water bath at 100 ºC for 10 min. After heating, 1.0 mL of 40% potassium: sodium Tartarate solution was added to the mixture and then allowed to cool at room temperature (25°C). After cooling, the mixture was made up to 25 mL with distilled water, filtered and then measured the absorbance at 550 nm. A blank was run simultaneously. A standard curve was prepared using maltose. Values were expressed as mg maltose released per 100 mg of sample.

The *in-vitro* protein digestibility of the flour samples was determined by enzymatic method of Ogodo et al. [20]. Each of the flour samples (1 g) was prepared in triplicate and digested with 1 mg pepsin in 15 mL of 0.1 M HCl at 37°C for 2 h. The reaction was terminated by the addition of 15 mL of 10% trichloro-acetic acid (TCA). The mixture was filtered through Whatman No. 1 filter paper. Nitrogen in the TCA soluble fraction was determined using the micro-kjeldahl method. Protein digestibility of the sample was calculated by the following formula

Determination of amino Acids composition of miracle berry seed samples. The amino acid was determined as described by Mansouri et al. [21]. The defatted miracle seed flour sample (500 mg) was dissolved in 10 mL of 6N hydrochloric acid containing 0.1% of phenol. The mixture sample was hydrolyzed under nitrogen at 110 °C for 24 h., and allowed to cool. After cooling, the pH was adjusted to 2.2 using NaOH, and 0.5 mL of norleucine (Sigma-Aldrich, St. Louis, MO, USA) at 50 mM/mL was added as an internal standard. The mixture sample was filtered through a 0.2 mm filter and 20 mL of the filtrate were analyzed by high performance liquid chromatography (HPLC Biochrom Plus amino acid analyzer, Pharmacia, Cambridge, UK) equipped with sodium oxidized column, cation exchange resin, followed by post-column derivatisation of the amino acids to ninhydrin and spectro-photometric detection at 570 nm, except for proline, which was detected at 440 nm.

The tryptophan was determined as described by the modified method of Hartcamp et al. [22]. The defatted flour sample was mixed with a papain solution, incubated at 60 ±2 °C for 18 h. After incubation, the mixture was cooled and centrifuged at 2500 xg for 15 m, and 1 mL of hydrolyzate was mixed with a solution of ferric chloride and sulfuric acid. The mixture was re-incubated at 60 ±2 °C for 10 h, allowed to cool and the reading was taken at 560 nm to calculate on a standard curve the amount of tryptophan, relative to the protein.

Quantitative Determination of Phytochemical Constituents of the Miracle Seed Flour. Determination of Tannin Content. Tannin content of the sample was determined as described by the method of Fagbemi et al. [23] with slight modification. The sample was measured (1.0 g) and dispersed in distilled water (10 mL), vortex vigorously and centrifuged at 3000 x g for 20 min. The filtrate (2.5 mL) and standard tannic acid solution (2.5 mL) were separately dispersed into 50 mL flask, respectively, and folin-denis reagent (1.0 mL) and saturated Na2CO3 solution (2.5 mL) were poured into each of the volumetric flasks, respectively. Thereafter, the mixture was diluted with distilled water to mark in the volumetric flask (50 mL) and incubated for 60 min. at room temperature. The absorbance was measured at 250 nm in an electronic spectrophotometer (Genway model 6000i). Readings were taken with the reagent blank at zero. The tannin content was calculated.

Where

Ab =Absorbance of test sample, Ast =Absorbance of standard solution, Conc. = Concentration of standard solution, W = Weight of sample used Vf = Total volume of extract Vol. = Volume of extract.

Determination of Flavonoid. Flavonoid was determined by the method described by Boham and Kocipai [24]. The flour sample (5.0 g) was mixed with 50 mL of 80% aqueous methanol in a 250 mL beaker and incubated for 24 h at room

temperature. The mixture was filtered and the supernatant was discarded. The residue was re-extracted (thrice) with 50 mL of ethanol. The combined mixture was filtered with Whatman filter paper number 42 (125 mm), and the filtrate was transferred into a crucible. The filtrate was evaporated to dryness using a water bath, cooled in a desiccator and weighed until constant weight was obtained. The flavonoid was calculated.

Determination of Saponin. The saponin in the flour samples was determined as described by the method of Obadoni and Ochuko [25]. The flour sample (20 g) was poured into a 250 mL conical flask containing 100 mL of 20% aqueous ethanol. The mixture was transferred into a hot water bath maintained at 50 ^{0}C for 3 h with continuous stirring. The residue of the mixture was re-extracted with another 100 mL of 20% aqueous ethanol after filtration. Thereafter, the combined mixture was filtered and concentrated in a water bath at 90 ^{0}C for 30 min. To the concentrated filtrate, 20 mL diethyl ether was added and mixed vigorously to separate the aqueous layer from the ether layer, which was discarded. This purification process was repeated twice. The separated aqueous solution was mixed with 60 mL of n-butanol. The mixture was then washed twice with 10 mL of 5% aqueous sodium chloride, and the sodium chloride layer was separated and discarded, while the left over was re-concentrated in a water bath at 90 ^{0}C for 30 min. The filtrate was then transferred into a crucible, and thereafter oven dried in hot-air oven (Gallenkamp, England) at 60 ^{0}C to a constant weight. The saponin content was calculated.

Determination of Oxalate. The oxalate was determined as described by the modified method of Adeniyi et al. [26]. The sample (2.5 g) was digested with 10 mL 6 M HCl at a temperature of 60 ^{0}C for 60 min. with continuous stirring using a magnetic stirrer, and then filtered. To 5.0 mL of the filtrate, 1.0 mL of 5 M ammonium hydroxide solution was added (to adjust pH) until the colour of the solution changed from salmon pink to a faint yellow colour. Phenolphthalein indicator (2 drops), glacial acetic acid (3 drops) and 5% calcium chloride (5.0 mL) were added to the solution to precipitate insoluble oxalate, and the solution was allowed to incubate for 120 min. at room temperature before centrifuged at 2500 *x g* for 20 min. The precipitate was washed with distilled hot water, 5.0 mL of 3 M tetraoxosulphate (VI) acid was added and incubated in a water bath at 60°C for 20 min. Freshly prepared 0.01 M potassium permanganate ($KMnO_4$) was titrated against 12.5 mL of the filtrate until a faint pink colour, which persisted for about 30 sec. and the volume of $KMnO_4$ used was read from the burette reading. The oxalate content was calculated (mg/g).

Where, V_T = Titre volume (ml).

Determination of total alkaloids: The sample (5 g) was weighed into a 250 mL beaker and 200 mL of 10% acetic acid in ethanol was added, covered and allowed to incubate for 4 h. The mixture was filtered and the filtrate was concentrated in a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the filtrate until the precipitation was completed. The solution was allowed to stand and the precipitated was collected, washed with dilute ammonium hydroxide and then filtered. The residue (alkaloid) was dried and weighed [27].

Determination of Cardiac Gylcosides. Cardiac glycoside content in the sample was evaluated using Buljet's reagent as described by El-Olemy et al. [28]. The sample (1g) was soaked in 10 mL of 70% alcohol for 2h and then filtered. The filtrate was purified using lead acetate and Na_2HPO_4 solution before the addition of freshly prepared Buljet's reagent (containing 95 mL aqueous picric acid + 5ml 10% aqueous NaOH). The difference between the colour intensity (absorbance) of the experimental sample and the blank (distilled water and Buljet's reagent) sample was the concentration of the glycosides.

Determination of Total Terpenoids. Terpenoids was determined as described by Indumathi et al. [29]. The miracle seed flour (2 g) was weighed and soaked in 50 ml of 95% ethanol for 24 h in a conical flask. The extract was filtered and the filtrate extracted with petroleum ether (60-800C) and concentrated to dryness. The dried ether extract was treated as total terpenoids.

Determination of Total Phenols. The phenol in the food samples was determined as described by the method of George et al. [30] with minor modifications. The food sample (2.0 g) was defatted using 100 mL ether in a soxhlet apparatus for 90 min. The defatted food sample (1.0 g) was dispersed in 50 cm^3 boiled ether for 15 min. to extract the phenolic components. Into the extracted filtrate (5.0 mL), distilled water (10.0 mL), 0.1 N ammonium hydroxide solution (2.0 mL), and concentrated amyl alcohol (5.0 mL) were added, and the mixture was left to react for 25 min. for colour development. The optical density was measured at the absorbance of 505 nm. For the preparation of phenol standard curve, 0.20 g of tannic acid was dissolving in distilled water and diluted to 200 mL mark (1 mg/mL). The standard tannin acid solution was measured into five different test tube by varying its concentration between 0.2 – 1.0 mg/mL. The standard tannic acid solution was then pipetted against the solution of NH_3OH (2.0 mL), amyl alcohol (5.0 mL), and distilled water (10.0 mL). The solution was thereafter made up to 100 mL volume and left to react for 25 min. for colour development. The optical density

was measured with spectrophotometer (Cecil 3021 spectrophotometer, Cambridge, United Kingdom) at 505 nm. The amount of phenolic compounds was expressed as mg of gallic acid per g of extract (mgGAE/g).

Determination of polyphenolic profiles. HPLC Name: Agilent 1200 series; detector: Agilent 1260; wavelength: 320 nm; column: chromspher 5, C18; Column temp. 40 ^{0}C; dimension: 5 micrometer, 3mm x 250 mm with Hamlton microliter syringe; injection: 100 µl; flow rate: 0.7 ml/min; pressure 180 x 10^5 Pa; Isocratic elution: 2% Acetic acid in water – methanol mixture (82:18, v/v)

Two stage extraction procedures followed for the effective removal of the phenolic compounds;

Stage 1: 50.0 mg of the sample was extracted with 5 ml of 1 M NaOH for 16 h on a shaker at ambient temperatures as described by Kelley et al. [31] and Provan et al.[32]. After extraction, the sample was centrifuged (5000 x g), rinsed with distilled water, centrifuged again, and the supernatants were combined and placed in a disposable glass test tube and heated at 90 ^{0}C for 2 h to release the conjugated phenolic compounds as supported by Whitehead et al. [33]. The heated extract was cooled, titrated with 4 M HCl to pH <2.0, diluted to 10 ml, with deionized water, and centrifuged to remove the precipitate. The supernatant was saved for subsequent purification and the residue was extracted further in stage 2.

Stage 2: The residue from stage 1 above was extracted with 5 mL of 4 M NaOH, heated to 160 ^{0}C in Teflon as described by Provan et al. (1994) [32]. After cooling, the mixture was filtered. Supernatant was collected and the residue was washed with deionized water. The supernatants were combined and adjusted to pH < 2.0 with 4M HCl. The filtrates were combined for further purification.

Purification of extracted phenolic acids. An aliquot (5-15 mL) of the various supernatants was passed through a conditioned Varian (Varian Assoc., Harbor City CA) Bond Elur PPL (3 mL size with 200 mg packing) solid-phase extraction tube at 5 mL min-1 attached to a Visiprep (Supelco, Bellefronte, PA). The tubes were then placed under a vacuum (-60 kPa) until the resin was thoroughly dried after which the PASs were eluted with 1 mL of ethyl acetate into gas chromatography autosampler vials. The PPL tubes were conditioned by first passing 2 mL of ethyl acetate followed by 2 mL water (pH< 2.0). The phenolic acid standards used were purchased from Aldrich (Aldrich Chemical Co., Milwaukee, WI).

Determination of *in-vitro* Antioxidant Activity of Miracle Berry Seed. DPPH radical scavenging assay. The free radicals scavenging activity of miracle seed samples (i.e., extract, protein isolate & oil) on 2, 2- Diphenyl-1-picryhydrazyl (DPPH) was determined as described by Girgih et al. [34]. Each of the miracle seed samples (10 mg) was added into 1 mL of 0.1 M sodium phosphate buffer at pH 7.0 with 1% (w/v & v/v, respectively) Triton X-100. The DPPH solution (DPPH dissolved in methanol, 100 µM. Conc.) (100 µL) was mixed with 100 µL of the sample in 96-well plate to a final assay concentration of 1 mg/mL. The mixture was then incubated at room temperature in the dark for 30 min, and the absorbance values of the blank (sodium phosphate buffer), Glutathione (positive control) and samples were measured at 517 nm. The DPPH free radical scavenging activity (%) of the samples were calculated using the following equation:

ABTS radical scavenging activity. The radical scavenging activity of miracle berry seed was determined using modified method of Re et al. [35]. The $ABTS^{++}$ solution was prepared by reacting 7.4 mM of ABTS solution with 2.6 mM potassium persulphate solution in equal amounts (pH 7.4, 1:1), and the mixture was incubated at room temperature for 12–16 h in the dark. The blue-green ABTS working solution was freshly prepared and diluted with methanol to an absorbance of 1.1 at 734 nm after 7 min using spectrophotometer (Phoenix-2000V UV-VIS, Biotech Engineering Management Co. Ltd. (UK)). Then, 150 µL of Trolox standard or the sample were vigorously mixed with 2850 µL of the ABTS solution and incubated for 60 min. at room temperature. The decrease of absorbance was monitored spectrophotometrically at 734 nm (UV-spectrophotometer (Phoenix-2000V UV-VIS, Biotech Engineering Management Co. Ltd. (UK)). The percentage inhibition of $ABTS^{.+}$ by the miracle seed samples was calculated and compared with that of BHT and rutin using the following equation.

Ferrous metal irons chelating effect. The metal chelating activity of miracle seed samples were determined according to the method of Girgih et al. [34]. Each of the samples, Glutathione solution (1 mg/dL), 0.05 mL of 2 mM $FeCl_2$, 1.85 mL distilled water and Ferrozine solution (0.1 mL of 5 mM) were thoroughly mixed in a tube. The mixture was incubated at room temperature for 10 min., and the mixture 200 µL was removed and poured into a clear bottom of 96-well plate. Distilled water (1 mL) was used as a blank (control), and the absorbance of the blank and miracle seed sample were measured at 562 nm using a spectrophotometer. The metal chelating activity (%) of the sample was calculated using the following equation:

Hydroxyl radical scavenging Assay: The hydroxyl radical scavenging activity of the miracle seed samples was determined as described by Girgih et al. [34]. The samples, Glutathione (GSH) and 1, 10-phenanthroline (3 mM) was each separately dissolved in in tube containing 0.1 M phosphate buffer

(pH 7.4) solution. Similarly, 0.01% hydrogen peroxide and $FeSO_4$ (3.0 mM) was each poured in a tube containing distilled water separately. The samples, GSH (1 mg/mL) and buffer (blank) was each added (50 µL) to a clear, flat bottom 96-well plate, and thereafter 50 µL of 1, 10-phenanthroline and 50 µL of $FeSO_4$ were added, respectively. To the mixture, 50 µL of hydrogen peroxide was added to initiate the Fenton reaction in the wells, and then the mixture, covered and incubated at 37 °C for 1 h with shaking. The absorbance readings were measured using a spectrophotometer at 536 nm at 10 min intervals for 1 h. The hydroxyl radical scavenging activity was calculated (%) using the reaction rate (DA/min) equation below:

Ferric-reducing antioxidant power (FRAP): The FRAP activity of the miracle berry seed samples was determined using method of Mau et al. [36]. The sample or Glutathione (GSH) was each poured into tube containing 0.2 M phosphate buffer at pH 6.6. The aliquot (250 µL) was then mixed with buffer (250 µL) and 1% potassium ferricyanide (250 µL) solution, and the mixture was vortex thoroughly and then incubated (50 °C) for 20 min. After incubation, 250 µL of 10% trichloroacetic acid (TCA), 50 µL of 0.1% ferric chloride (dissolved in H_2O) and 200 µL of distilled water were added, respectively. The mixture was centrifuged (1000 ×g, 10 min.) and the supernatant (200 µL) was transferred to a clear bottom 96-well plate and the absorbance was measured at 700 nm.

Carbohydrate Hydrolyzing Enzymes Inhibitory activities of Miracle seed bioactive Compounds. Determination of *α*-Amylase inhibition Assay. The alpha-amylase inhibitory activities of the miracle berry seed extract and protein isolate were determined using standard method (Nickavar and Youse, 2009) [37]. Each of the miracle berry seed extract and protein isolate solution was serially diluted (0 - 200 µL) by mixing with 500 µL Sodium phosphate buffer (0.02 mol/dm^3, at pH = 6.9 and 0.006 NaCl as the stabilizer), containing pancreatic alpha-amylase (0.50 mg/mL) of Porcine origin (EC 3.2.1.1). Each of the mixtures was incubated at 37 °C for 5 min, and then 500 µL of starch solution (1 mg/100 mL in 0.02 mol/dm^3 sodium buffer at pH of 6.9 with 0.006 NaCl) was added into the reaction mixtures, and incubated at 37°C for 5 min in a water bath. The reaction was then stopped using 1.0 mL dinitrosalicylic acid (DNSA) and further incubated in boiling water for 5 min. The blank sample had no starch solution and enzyme in it, while the control (reference sample) had all the reagents and the enzyme except the starch solution. Acarbose was used as positive control. When the reaction mixtures were cooled, absorbance was read at 540 nm.

Determination of α – glucosidase inhibition assay. The α-glucosidase inhibition was determined by the modified method Bräunlich et al. [38]. Each of the miracle berry seed extract and protein isolate solution was serially diluted (0 to 200 µL), and were mixed with 100 µL Sodium phosphate buffer (0.1 mol/dm^3, at pH = 6.9) containing alpha-glucosidase (EC 3.2.1.2; 1.0 U/mL). The mixture was incubated at 37 °C for 5 min., and 0.05 mL of para-nitrophenyl-α-D-glucopyranoside (5.0mmole/ dm^3) solution in Sodium phosphate buffer (0.1 mol/dm^3, at pH = 6.9) was added to the reaction mixture and incubated at 37 °C for 5 min. The reaction was terminated by adding 1.0 mL dinitrosalicylic acid (DNSA), and further incubated in boiling water for 5 min., cooled to room temperature and the absorbance was read at 405 nm. The blank sample had no starch solution and enzyme in it, while the control (reference sample) had all the reagents and the enzyme except the starch solution. Acarbose was used as the positive control.

Determination of Angiotensin Converting Enzyme (ACE) inhibition activity. The ACE-inhibitory activity was determined in vitro using spectrophotometric method described by Vermeirssen et al. [39] with slight modifications [40]. This method was based on the liberation of hippuric acid from hippuryl-L-histidyl-L-leucine (Hip-His-Leu) catalyzed by ACE. For the assay, 42.5 µL of the sample solution (2 mg/mL) was pre-incubated at 37 ◦C for 5 min with 10 µL ACE (0.6 mU/mL) enzyme. The mixture, 20 µL of the substrate (5 mM HHL in 10 µM zinc chloride containing 100 mM sodium trizma base and 300 mM NaCl at pH 8.3) was added and incubated (37 ◦C for 60 min). After incubation, 12.5 µL of 5 M HCl. was added to terminate the reaction. The ACE-inhibition (%) was determined by HPLC system with a 486 tunable UV detector. The average value from three determinations at each concentration was used to calculate the ACE-inhibition (%) rate as follows:

Where A is the absorbance (Abs) of HA generated in the presence of ACE-inhibitor, B the Abs of HA generated without ACE-inhibitor and C the Abs of HA generated without ACE (corresponding to HHL autolysis in the course of enzymatic assay). Captopril was used as a positive control for ACE-inhibition.

Statistical analyses. Triplicate data were analysed and results were presented as means (±SE), while differences between means were determined using Analysis of Variance (ANOVA) and Duncan's Multiple Range Test (DMRT), and values were considered as statistically significant at $p < 0.05$ [41].

RESULTS AND DISCUSSION

Proximate Composition of miracle berry seed flour. The proximate composition of miracle berry seed flours is presented in Table 1. The moisture content of miracle seed extract (MSE) had the higher value (9.43 g/100g), while that of defatted miracle seed flour (DMF) had the lowest value (7.21 g/100g). These values were comparatively higher than moringa seed flour (4.70-5.03%) reported by Abiodun et al. [42], but were within the recommended value (<10). The disparity between the moisture content of miracle seed flour and that of Moringa oleifera seed flour could be attributed to the variety, climatic condition and processing method. High moisture content depicts high water activity needed for metabolic activities, hence reduced storage life. The crude fat and protein of the seed flour ranged from 2.31 to 11.59 and 5.40 to 81.04 g/100g, respectively, and these values were significantly ($p<0.05$) higher in undefatted (UMF) and protein isolate (MPI) flour, respectively compared to other flour samples. The protein content of undefatted and defatted seed flour were lower than what Govardhan Singh et al. [43] reported for Moringa oleifera seed flour and Njoku et al. [44] for miracle seed flour. The variation in results could be attributed to climatic conditions, processing methods and seed varieties.

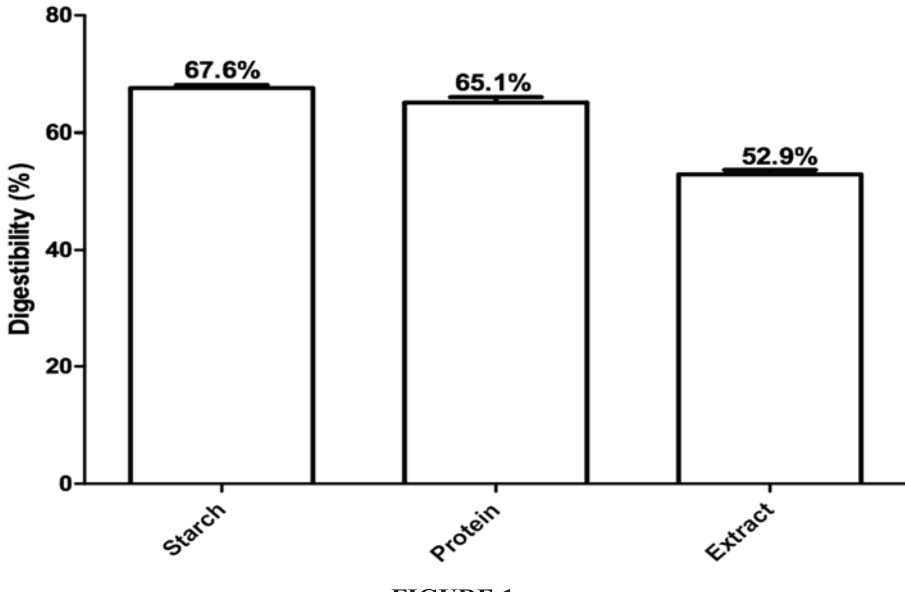


FIGURE 1
In vitro digestibility of starch, protein and extract of miracle berry seed flour. Bars are expressed as means ± standard error of mean (SEM) of triplicate determinations (n=3). Bars with the same letters do not differ significantly while values with different letters are significantly different from one another at p<0.05.

TABLE 1
Proximate Composition (g/100 g) and Calculated Energy Value (Kcal/100 g) of miracle berry seed flour samples

Samples	UMF	DMF	MSE	MPI	*RV
Moisture	$6.87±0.07^{d}$	$7.21±0.17^{c}$	$9.43±0.51^{b}$	$8.69±0.17^{b}$	<10
Ash	$2.28±0.19^{a}$	$2.62±0.00^{a}$	$1.96±0.57^{a}$	$0.74±0.08^{b}$	<3
Fiber	$1.37±0.02^{a}$	$0.39±0.01^{b}$	$0.39±0.00^{b}$	$0.23±0.03^{c}$	10-25
Fat	$11.59±0.21^{a}$	$6.19±0.14^{b}$	$2.64±0.17^{cd}$	$2.31±0.04^{d}$	<5
Protein	$8.49±0.05^{c}$	$11.64±0.25^{b}$	$5.40±0.00^{d}$	$81.04±0.56^{a}$	>14
Carbohydrates	$69.40±0.33^{b}$	$71.96±0.19^{b}$	$79.21±0.66^{a}$	$7.00±0.35^{c}$	64
Energy	$415.87±0.23^{a}$	$390.11±0.15^{b}$	$362.20±0.21^{e}$	$372.95±0.17^{c}$	400-425

Means (±SEM) with different alphabetical superscripts in the same column are significantly different at P<0.05. UMF: undefatted; DMF: defatted; MSE: extract; MPI: Protein isolate.

In-vitro starch and protein digestibility of miracle berry seed flour. The *in-vitro* starch, protein and extract digestibility of miracle berry seed flour are presented in Figure 1. The in-vitro digestibility of the starch, protein and extract of miracle seed were 67.6%, 65.1% and 52.9%, respectively. The in vitro starch digestibility value of the miracle seed flour obtained in this study was high and this could be as a result of its low fiber content. Findings have shown that the amount and nature of crude fiber in foods could influence starch digestibility, for instance, high amount of crude fiber in food may reduce starch digestibility by trapping starch granules within a viscous protein-fiber-starch network (Chinma et al., 2011; Chinma et al., 2012) [45,46]. Hence, the starch in miracle berry seed flour in this present study may not be suitable for the production of functional food for weight reduction and treatment of diabetes.

The *in-vitro* protein digestibility is an important criterion for evaluation of protein quality as well as an indicator for protein bioavailability in foods [46, 47]. The high protein digestibility values obtained in this study could be associated with the unfolding of native protein structure in the miracle berry seed, which facilitates digestibility or presence of soluble protein fraction such as albumin, which is easily hydrolysed by the enzymes. The high bioavailability of miracle seed flour protein that was observed in this study may be an advantage for its inclusion in complementary food formulations for the prevention of protein-energy malnutrition.

Amino Acids Composition of miracle berry seed samples. The amino acid composition of miracle berry seed flour samples is presented in Table 1. The total essential and non-essential amino acid composition of the miracle seed flour samples varied from 30.67 - 38.29 g/100g protein and 52.52 - 54.31 g/100g protein, respectively, and miracle berry seed protein isolate (MPI) was significantly ($p<0.05$) higher in essential amino acid than other flour samples. The essential amino acid index (EAAI) and predicted biological values of the seed flour varied from 48.77 - 76.66% and 43.32 - 71.86%, and were significantly higher in protein isolate than other flour samples. This observation could be attributed to the fact that MPI, a protein isolates, had been isolated from other components of the miracle seed to smaller molecule weight with high biological properties.

The branched chain amino acids (Leucine, Isoleucine, Valine) (BCAA), aromatic amino acids (Phenylalanine, Tyrosine & Tryptophan) (AAA) and arginine/lysine ratio of the miracle seed flour samples varied from 7.97 - 11.64, 12.77 - 16.22 and 1.10 - 2.04, respectively. The miracle seed protein isolates (MPI) had the highest BCAA/AAA ratio (2.04) and BCAA/tyrosine ratio (5.34), while DMF had the least values (1.1 & 2.99, respectively). The BCAA/AAA and BCAA/Tyrosine ratios of MPI were significantly ($p<0.05$) higher than other miracle seed samples. This indicates that miracle seed contain appreciable amount of branched chain amino acids. Recent studies have established that regular intakes of branched amino acids may prevent stunted growth in children [48], facilitate blood glucose utilisation in diabetic patients [49] and prognosis in liver diseases [50].

The arginine/lysine ratio of the seed samples, except in MPI, was higher than recommended values (>1). This indicates that arginine is present in an appreciable amount in the miracle seed; and that exploiting this advantage may be health beneficial particularly in the prevention and treatment of high blood pressure. In human body, arginine plays an important role in the production of nitric oxide, which helps in the relaxation of the arteries to ease flow of blood, and thereby reduces the risk of high blood pressure [51]. High blood pressure or hypertension is a terminal and degenerative disease that is responsible for the high numbers of hospital admissions and mortality rate in both developing and developed countries [52].

Phytochemical composition and polyphenolic compound of miracle berry seed flour samples. The total phenolic compound, flavonoid and saponin in miracle seed samples ranged from 6.36 - 19.41 mg/g, 0.08 - 1.59 mg/g and 95.82-146.36 mg/g, respectively. For phytate, tannin, terpenoid, the concentration ranged from 21.42 - 25.13 mg/g, 1.1 - 1.86 mg/g and 8.68 - 21.79 mg/g; while oxalate, glycoside and alkaloid were 1.08 - 2.61 mg/100g, 6.22 - 21.88 mg/g and 21.31 - 38.21 mg/g, respectively. The phytochemicals in miracle berry seed samples observed in this present study were lower than critical levels, and were similar in composition to that of moringa seed reported by Olajide et al. [53]. Scientific evidences have revealed that phytochemicals in plants, which are commonly used for the prevention and/or treatment of different diseases like diabetes, hypertension, etc. at lower concentration due to their pharmacological effects [54, 55]. In recent time, there was a shift away from the used of synthetic antidiabetic and antihypertensive agents due to their side effects such as flatulence, abdominal distention, and possibly diarrhea [56]. This threat increases the demand for the use of natural bioactive phytochemicals with antipostprandial hyperglycemia activity and antihypertensive with less or without side effects.

The phenolic profile (mg/100g) of the miracle seed flour is presented in Table 3b. The quantitative estimation of the phenolic profile in miracle seed flour showed that phloridzin was present in highest concentration (2537.03 mg/100g) followed by isoquercitrin (quercetin-3-O-b-D-glucopyranoside)

(206.052 mg/100g) and quercetin (3,3′,4′,5,7-pentahydroxyl-flavone) (155.985 mg/100g), respectively. Quite a number of studies have reported that Phloridzin [phloretin (4,2,4,6-tetrahydroxy dihydrochalcone)-20 -o-glucoside] abundantly present in apples and apple food products has antioxidant activity, that is, ability to inhibit formation of free radicals (Bellion et al., 2008) [57]. Besides, evidences have shown that oral administration of Phloridzin reduced postprandial blood glucose levels; hence, it is used as antidiabetic agent [58, 59]. Similarly, studies have shown that quercetin and Isoquercitrin have many pharmacological properties such as antioxidant, neurological, antiviral, anticancer, cardiovascular, antimicrobial, anti-inflammatory, hepatoprotective, protective of the reproductive system and anti-obesity agent [60, 61].

***In-vitro* antioxidant activities of miracle seed flour.** The in-vitro antioxidant activities of miracle berry seed extract and protein isolates were presented in Table 4, respectively. Antioxidant is any substance when present at low concentrations delays, prevents or scavenges free radicals that may cause ageing and degenerative diseases like diabetes, hypertension, etc. [62]. In this study, the in-vitro antioxidant activities of miracle berry seed extract and protein isolates against DPPH, ABTS, iron chelation and OH free radicals were concentration dependents, that is, as the concentration of the samples increase the percentage of inhibition increase. The IC_{50} (the concentration of extract or protein isolate required to inhibit 50% of the oxidant) of MPI and MSE activity against DPPH, ABTS, iron chelation and OH free radicals varied from 94.61 - 113.8 µg/mL, 94.61 - 113.9 µg/mL, 94.59 - 103.2 µg/mL and 91.99 - 94.56 µg/mL, respectively. However, it was observed that the antioxidant activity of MPI was stronger than that of extract, except against OH free radicals. This finding could be attributed to the acidic and hydrophobic amino acids in the protein isolate with potential to scavenge free radicals [63, 64].

TABLE 2
Amino acid profile (g/100g protein) of miracle berry seed flour samples

Samples	UMF	DMF	MSE	MPI
Non-essential amino acids (NEAAs)				
Glycine	4.15^{d}	4.40^{b}	4.24^{c}	5.40^{a}
Alanine	4.02^{c}	4.30^{b}	3.80^{d}	5.71^{a}
Serine	3.10^{d}	3.82^{a}	3.57^{c}	3.64^{b}
Proline	2.52^{d}	3.57^{b}	3.44^{c}	4.23^{a}
Aspartic	8.76^{b}	8.16^{d}	9.09^{a}	8.58^{c}
Cysteine	1.08^{c}	1.37^{a}	1.08^{c}	1.18^{b}
Glutamic	17.58^{a}	16.16^{b}	15.92^{c}	14.30^{d}
Tyrosine	3.34^{c}	4.27^{a}	4.03^{b}	3.04^{d}
Arginine	7.97^{c}	8.26^{b}	9.98^{a}	6.02^{d}
ΣNEAAs	52.52^{c}	54.31^{b}	55.15^{a}	52.10^{d}
Essential amino acids (EAAs)				
Phenylalanine	5.70^{c}	7.15^{a}	6.87^{b}	3.91^{d}
Histidine (His)	3.34^{b}	5.33^{a}	3.29^{c}	2.40^{d}
Metheonine	1.31^{b}	1.29^{c}	1.24^{d}	2.42^{a}
Valine	4.04^{b}	3.98^{c}	3.83^{d}	4.46^{a}
Tryptophan	0.16^{c}	0.22^{b}	0.18^{c}	1.02^{a}
Threonine	3.23^{c}	3.89^{a}	3.61^{b}	3.63^{b}
Isoleucine	2.43^{b}	2.15^{c}	2.05^{d}	4.08^{a}

Leucine	4.38[d]	4.85[c]	5.83[b]	7.68[a]
Lysine	6.08[b]	5.77[c]	5.70[d]	8.69[a]
ΣEAAs+His	30.67[c]	34.63[b]	32.60[c]	38.29[a]
Protein Quality				
ΣAA	83.19[d]	88.94[b]	87.75[c]	90.39[a]
TEAA/TNEAA	0.58[c]	0.64[b]	0.59[c]	0.73[a]
ΣSAA	2.39[c]	2.66[b]	2.32[d]	3.60[a]
ΣHAA	27.67[d]	31.86[b]	31.11[c]	35.31[a]
ΣPCAA	9.42[b]	11.10[a]	8.99[c]	11.09[a]
ΣNCAA	26.34[a]	24.32[c]	25.01[b]	22.88[d]
ΣAAA	9.20[d]	11.64[a]	11.08[b]	7.97[c]
PER (g/100g)	2.29[c]	2.56[b]	2.54[b]	2.63[a]
EAAI (%)	48.77[d]	54.42[b]	50.48[c]	76.66[a]
P-BV (%)	41.46[d]	47.61[b]	43.32[c]	71.86[a]
ΣBCAAs	12.89[c]	12.77[d]	13.58[b]	16.22[a]
Arginine/Lysine	1.31[c]	1.43[b]	1.75[a]	0.69[d]
BCAA/AAA	1.40[b]	1.10[d]	1.23[c]	2.04[a]
BCAA/Tyrosine	3.86[b]	2.99[d]	3.37[c]	5.34[a]

Total amino acids (ΣAA), Total essential amino acid (ΣEAA), Total non-essential amino acid (TNEAA), Sulphur containing amino acid (SAA= Cys, Met), Hydrophobic amino acid (HAA=Ala, Val, Iso, Leu, Try, Phe, Trp, Pro, Met, Cys), Positively charged amino acid (PCAA=His, Lys), Negatively charged amino acid (NCAA=Asp, Glu), Total Aromatic amino acids (ΣAAA=Phe, Try, Tyr), Branched chain amino acids (ΣBCAAs=Leu., Iso., Val), Protein efficiency ratio (PER), Essential amino acid index (EAAI), Predicted biological value (P-BV).

TABLE 3
Phytochemicals (mg/g) of miracle berry seed flour samples

Samples	UMF	DMF	MSE	MPI	Normal Range
Phenol	6.36±0.09[c]	14.64±0.21[b]	14.81±0.07[b]	19.41±0.20[a]	-
Flavonoid	0.55±0.02[d]	0.08±0.01[c]	1.31±0.05[b]	1.59±0.08[a]	-
Phytate	23.89±0.00[b]	25.13±0.23[a]	21.42±0.48[c]	23.48±0.23[b]	5-6g/100g
Tannin	1.86±0.03[a]	1.67±0.03[b]	1.58±0.03[c]	1.10±0.00[d]	3.0 mg/100g
Saponin	146.36±0.11[a]	95.82±0.11[c]	96.00±0.11[c]	108.91±0.11[b]	9-420 mg/day
Terpenoid	21.79±0.02[a]	20.01±0.02[b]	8.68±0.02[d]	18.87±0.02[c]	-
Oxalate	2.61±0.05[a]	2.21±0.03[b]	1.08±0.05[c]	1.17±0.00[c]	0.25g/100g
Glycoside	16.06±0.03[b]	13.84±0.03[c]	6.22±0.03[d]	21.88±0.03[a]	-
Alkaloid	38.21±0.02[a]	32.69±0.02[b]	21.31±0.02[c]	27.89±0.02[d]	1-75 mg/100g

Means (±SEM) with different alphabetical superscripts in the same column are significantly different at $P<0.05$ [UMF: un-defatted; DMF: defatted; MSE: extract; MPI: Protein isolate].

TABLE 3b
Phenolic profile (mg/100g) of miracle berry seed flour

Phenolic profile	Concentration
Catechin	3.975
Protocatechuic acid	0.001
Epicatechin	37.911
Vanillic acid	0.001
p-hydroxybenzoic acid	0.004
Gallic acid	0.001
Caffeic acid	5.36
Phloretin	16.367
Naringenic Chalcone	0.002
Avicularin	44.609
Luteolin	0.001
Phloridzin	2537.03
Epigallocatechin	0.005
Hyperoside	87.108
Quercetin	155.985
Chlorogenic acid	25.161
Isoquercitrin	206.052
Isoquercitrin	48.9
Rutin	11.07
Total	3179.539

Carbohydrate hydrolyzing enzymes inhibitory activity of miracle berry seed flour. The activity of miracle berry seed ethanol extract (MSE) and protein isolates on α-amylase and α-glucosidase enzymes is presented in Figure 2. The carbohydrate digestion involves two steps; it starts with the activity of α-amylase enzyme that hydrolyzes polysaccharides to oligosaccharides (sucrose), while α-glucosidase enzyme completes the final step by hydrolyzing oligosaccharides to absorbable monosaccharides (glucose). In this study, the inhibitory activities of the extract (MSE) and protein isolates (MPI) on α-amylase and α-glucosidase enzymes were concentration dependent, that is, as concentration of the MSE and MPI increased the percentage of enzymes inhibition also increased. In addition, it was observed in this study that the miracle berry seed bioactive compounds (MSE & MPI) exhibited weak inhibition on α-glucosidase enzyme compared with α-amylase enzymes. The 50% α-amylase inhibitory concentrations (IC50) of miracle berry seed protein isolates and extract ranged from 77.97 µg/mL in MSE to 82.02 µg/mLin MPI, while that of α-glucosidase ranged from 76.3 µg/mL in MSE to 79.3 µg/mL in MPI. This implies that the activity of MSE on α-amylase and α-glucosidase enzymes inhibition was higher than that of MPI. This observation could be attributed to the fact that the bioactive peptides are still inactive state in the parent protein, and unless the primary structure of the parent protein is further be broken down by the enzymes to release bioactive peptides that are more potent in therapeutic activities. This finding was in line with the reports of Arise et al. [65]. Recent evidences have established the use of plant seed bioactive compounds as possible alternatives to synthetic drugs in the treatment of diabetes mellitus [64, 66]. Scientific studies have revealed that ability to inhibit the activities of α-amylase and α-glucosidase enzymes on polysaccharides in the gastrointestinal tract (GIT) is an important aspect of diabetes therapy [67, 68].

Angiotensin converting enzyme (ACE) inhibitory activities of miracle seed flour. The effects of miracle seed protein isolates and extract on ACE is presented in Figure 3. The percentage ACE inhibitory activities of miracle seed protein isolate and extract were 72.3% and 87.8%, respectively. The percentage enzyme inhibitory activity of the miracle seed extract (MSE) was significantly ($p<0.05$) higher than that of protein isolate (MPI) (72.3%) and Captopril (a synthetic antihypertensive agent) (75.5%). The ACE inhibitory activities of the miracle seed extract and protein isolate could be attributed to the bioactive phytochemicals (flavonoid, saponin and phenols) [69, 70] and amino acids composition [71]. Previous studies have demonstrated that bioactive phytochemicals (polyphenols) and hydrolysed protein (peptides) possessed antioxidant and medicinal properties for the prevention of diseases in human like antiatherosclerotic, antihypertensive, etc. [72, 73]. The ACE inhibitory property of miracle seed protein isolate and extract agreed with other studies, which reported that regular consumption of plant-based seeds rich in antioxidant with ACE inhibitory activity is an alternative therapy for the treatment of hypertension [74] Scientific study has established that ability of plant bioactive compounds to inhibit angiotensin-1 converting enzyme from the conversion to angiotensin II (a vasoconstrictor) is the therapeutic approach to treat hypertension [75]. Hence, the ability of miracle seed bioactive compounds to inhibit ACE activity in this study indicates its potential as antihypertensive agent.

TABLE 4
In-vitro antioxidant activities of miracle berry seed extract, protein isolate and oil

	Concentration (µg/mL)				IC50 (µg/mL)	R2
	40	80	160	320		
DPPH (%)						
MSE	16.76±0.79b	41.59±0.50b	55.02±0.78b	80.27±1.10b	113.8a	0.94
MPI	8.12±0.38c	19.21±0.91c	32.48±1.54c	37.25±1.76c	94.61b	0.99
Glutathione	17.85±0.84a	44.28±0.62a	73.13±1.04a	85.46±1.17a	94.97b	0.99
ABTS (%)						
MSE	14.16±0.67a	35.11±0.49b	46.45±0.65b	67.77±0.93a	113.9a	0.94
MPI	6.86±0.32b	16.22±0.77c	27.43±1.29c	31.45±1.49c	94.60b	0.99
Trolox	15.07±0.71a	37.38±0.53a	53.56±0.76a	62.59±0.86b	88.51c	0.98
Fe^{2+} Chelation (%)						
MSE	12.59±0.59b	31.23±0.44a	41.31±0.58b	55.67±0.76b	103.2a	0.95
MPI	6.10±0.28c	14.43±0.68b	24.39±1.15c	27.97±1.32c	94.59c	0.99
Ascorbic acid	13.41±0.63a	33.25±0.47a	47.64±0.67a	60.27±0.83a	98.57b	0.97
OH- Free Radicals (%)						
MSE	15.75±0.75a	39.06±0.55b	51.67±0.72b	64.05±0.91b	91.99b	0.96
MPI	7.63±0.36b	18.05±0.85c	30.51±1.44c	34.98±1.66c	94.56a	0.99
Mannitol	16.77±0.79a	41.59±0.59a	59.58±0.85a	68.63±0.44a	86.74c	0.98

Means (±SEM) with different alphabetical superscripts in the same column are significantly different at P<0.05 [MSE: extract; MPI: Protein isolate]

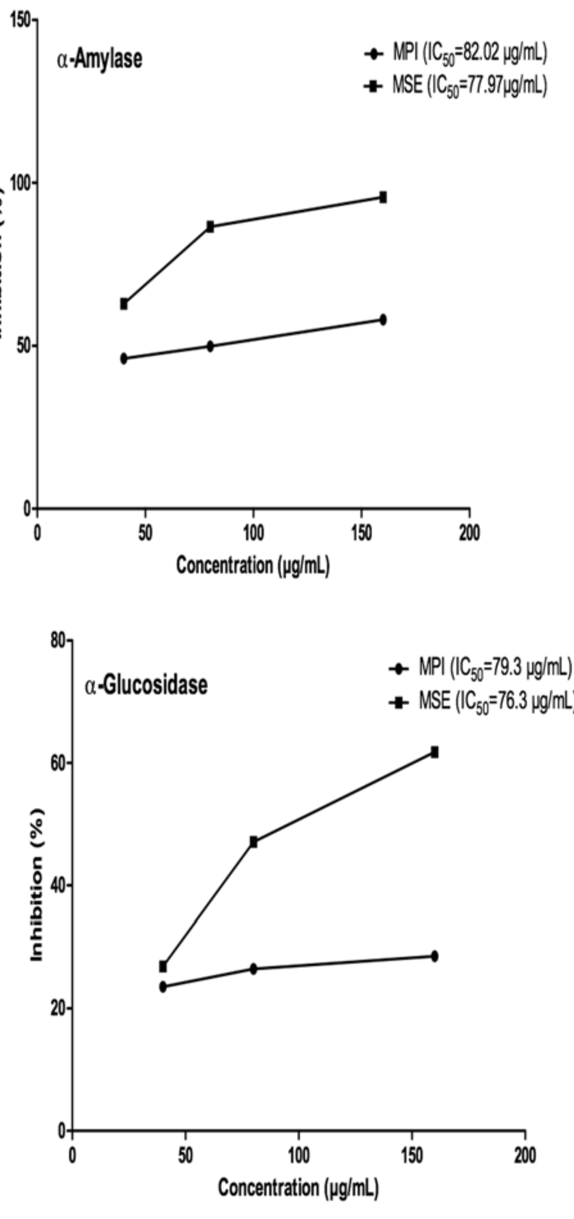
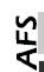


FIGURE 2
α-Amylase and α-Glucosidase inhibitory properties of miracle berry seed ethanolic extract and protein isolate [MSE: extract; MPI: Protein isolate]



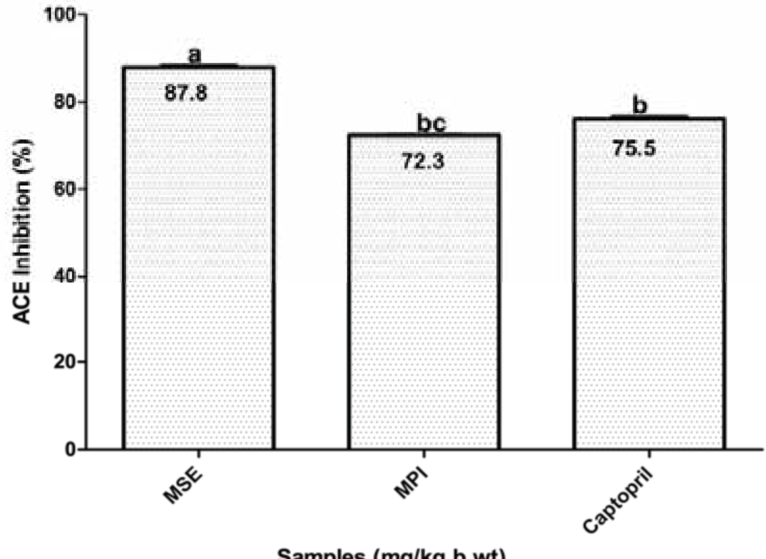


FIGURE 3
Angiotensin-1-Converting enzyme inhibitory properties (%) in rats administered with miracle berry seed extract and protein isolate (mg/kg b.wt) and Captopril for 28 days [Miracle berry seed extract (MSE) and Miracle berry seed protein isolate (MPI)]. Bars are expressed as means ± standard error of mean (SEM) of triplicate determinations (n=3). Bars with the same letters do not differ significantly while values with different letters are significantly different from one another at p<0.05.

CONCLUSIONS

The study reported on the amino acid profile, phytochemicals, carbohydrate hydrolyzing and angiotensin-converting enzymes inhibitory activities of miracle seed bioactive compounds (extract and protein isolate). The finding revealed that miracle berry seed contains essential amino acids, antioxidant activities, ability to inhibit angiotensin converting and carbohydrate hydrolyzing enzyme activities. However, the miracle berry seed extract was more potent in antioxidative activity, ability to inhibiting angiotensin-1 converting enzyme and carbohydrate hydrolyzing enzyme activities. Hence, encapsulating the seed extract may be suitable as antidiabetics and antihypertensive agent.

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THE EFFECT OF HERBAL THERAPY ON STRESS AND WELL-BEING MARKERS IN DEGENERATIVE JOINT DISEASE-RELATED HOSPITALIZED HORSES

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ABSTRACT

Transportation and living conditions are part of horse mode of life, but are itself a stressor often resulting in a reduction in feed intake, body weight gain, and nutrient digestibility. Something more stress factors induces physiological, behavioural, immunological and biochemical changes that directly affect health and well-being. For this reason the aim of this study was to evaluate some haematological, biochemical and hormonal changes, may able used successfully to identify the impact of mode of live on hospitalized horses suffering from degenerative joint disease (DJD) after consumation of herbal tea. Six animals were transported by road for approximate 1 h and accommodated in the Equine Veterinary Hospital of Trakia University, Stara Zagora, Bulgaria. The natural herbal tea (Herbacon GmbH, Berlin, Germany) was administered to three horses by water for drink in dose 10g/100 kg body weight together with standard therapy for osteoarthritis during 2 months. The results of this study confirmed that a linkage between stress and hormons and acute phase responce (APR) exists. In both groups horses Fib increased significantly after road transport and 24 and 48 h of accomadation compared to the previous data points. At the end of the period acute phase proteins, cortisol were insignificantly ($P > 0.05$) lower in treatmed group (Fb=3.69±0.15; Cortisol=0.93±0.16) with herbal tea in compare with control group (Fb=5.57±0.89; Cortisol=1.07±0.41). Based on the results presented above and considering changes in studied blood parameters which are useful indicators of animal health and welfare, we can recommend the daily use of natural herbal tea.

KEYWORDS:
Herbal, horses, hospitalization, stress, tea

INTRODUCTION

Hospital visits and hospitalization periods are generally stressful conditions among human [1] and companion animals [2; 3; 4; 5]. The unfamiliar conditions, transportation and people trigger to stress hence that the behavioral changes may ocur. The stress has multifactorial effects, resulted in alterations on physiologic and psychologic homeostasis [6]. Injury caused physical, and environmental circumstances induced stressors which are responsible for activation of stress-induced physiologic and psychologic phenomenons. The most common consequences are impairment of the general condition, suppression of the immunological response hence acute exacerbation of chronic diseases, hyperalgesia, or stress-induced analgesia, increased aggression, fight or flight behaviors, and emotional alterations [7; 8; 9].

Two major neuroendocrine pathways are activated through stressful conditions; the hypothalamic-pituitary-adrenocortical (HPA) and the sympathetic-adreno-medullar (SAM) axis which are led to increased cortisol and catecholamine levels physiologically and depression, anxiety psychologically [7;10] β-endorphin, adrenocorticotropic hormone (ACTH), cortisol and thyroid hormones are well established measurable physiological indicators of the stress level in companion animals [11].

According to analyses conducted in the past years, the horses are defined as the social animals able to demonstrate different feelings and emotions [9]. Hence, the housing (stall or paddock), hospitalization or transport conditions can directly effect horse behaviour such as resting quality, urination and, physiological stress parameters as heart and respiratory rates, blood pressure, body temprature, neutrophil:leucocyte ratio, cortisol level [12;13;14]. Herbal remedies have been using to alleviate the negative impacts of stressful conditions and, cure oxidative metabolism alterations, inflammation, anxiety, and depression for centuries [15]. Widespread alternative and complementary therapy options have been using among horse owners, whereas little data exist about herbal therapy up to date. Some medicinal herbs have adaptogenic effects which are metabolic regulators of the organisms for the maximum adaptation of the environmental factors [16; 17]. While adaptogens increase the strength of nonspecific resistance, also boost the tolerance of organism

against stress factors [18; 19]. Besides, some medicinal herbs have calming effects using serotonergic pathways neurophysiologically. The conservative methods of treatment through short-term medication with a mild sedative effect often do not give satisfactory results during long transportation and hospitalization periods [20].

Therefore, the purpose of this study was to clarify whether the intake of natural herbal tea developed by the Department of Animal nutrition, Trakia Uniersity and Innovation and Science Department of Herbacon GmbH would reducing stress in hospitalized horses suffering from degenerative joint disease (DJD). For the aim were trace the dynamics of the plasma levels of cortisol and thyroid hormones, as well as some acute-phase proteins (fibrinogen and ceruloplasmin) as markers of stress and inflammation in horses.

MATERIALS AND METHODS

Six horses 10-19 year old, weighing 400-450 kg affected by unilateral DJD of either tarsal or metacarpophalangeal joint were included in the examination over a half-year period from April 2019 to December 2019. Of these, three were selected at random to intake natural herbal tea (in dose 10g per 100kg body weight) dissoleved in 2 litres of drinking water. The patients were monitored and treatmented in the Equine Veterinary Hospital of Trakia University, Stara Zagora, Bulgaria for 2 month. Data obtained from the medical records included signalment (age and gender), duration of hospitalization and procedures performed. During the examination, the horses were housed in individual stalls, fed commercially available pellets and alfalfa hay with unrestricted access to water.

Standard views for each joint were applied during radiography of the diseased and contralateral joint. Arthocentesis of the same joints was done using aseptic technique and synovial fluid was analysed for some markers of chondral destruction and synthesis.

Morning (8 am) blood samples were taken from the *vena jugularis* of the patients after previous fixation. Disposable 18G needles and test tubes were used to obtain 10 mL from each animal. After being transported to the lab, the collected samples have been centrifuged at 3000 rpm for 5 min (Ohaus FC5515, Ohaus Corp., USA) at room temperature. After coagulation obtained serum was immediately separated and it was stored at -20°C until analysis. Serum cortisol, free triiodothyronine and free thyroxine analyses were conducted with ELISA analyzer, HumaReader HS (Human GmbH, Wiesbaden, Germany) and commercial equine ELISA kits (Monobind, Inc., USA). The concentration of fibrinogen was measured using the BN2 System (Siemens Healthcare Diagnostics GmbH). The obtained values were compared with reference ranges for horses.

The statistical analysis was performed using one way analysis of variance (ANOVA). The results were processed with software Statistica v.10 (StatSoft Inc., 2002). All results are presented as mean and standard error of the mean (Mean±SE). The statistical significance of parameters was determined in the LSD test at $P < 0.05$

RESULTS

The present work was designed to define the effect of stress caused by transportation and subsequent hospitalization on cortisol, thyroid hormones and acute phase proteins in horses. The acquired results of laboratory analysis on hospitalized patients are shown in table 1.

TABLE 1
Concentrations of cortisol, fibrinogen (Fib), free triiodothyronine (T3) and free thyroxine (T4) in hospitalized horses (n=6) suffering from degenerative joint disease (DJD) according to time intake of herbal tea

Time	Cortisol µg/dl		Fibrinogen g/L		Free T3 ng/mL		Free T4 mcg/dL	
	Control	Tea	Control	Tea	Control	Tea	Control	Tea
Day 0	5.56±0.83	6.36±1.00	9.23±0.89	10.81±0.54	1.10±0.19	0.90±0.01	1.48±0.10	1.29±0.15
Day 1	6.49±0.57	3.56±1.06	8.67±0.24	9.50±1.19	1.23±0.01	1.40±0.23	1.54±0.07	1.36±0.01
Day 2	2.87±0.59	2.45±0.51	8.40±0.11	8.48±1.12	1.10±0.02	1.18±0.15	1.36±0.08	0.98±0.12
Day 14	1.57±0.42	0.97±0.38	8.50±0.21	6.21±0.46	0.96±0.31	0.87±0.18	1.29±0.10	1.21±0.11
Day 30	1.74±0.66	1.36±0.79	5.75±063	5.91±0.58	1.03±0.04	1.17±0.11	1.20±0.06	1.45±0.02
Day 60	1.07±0.41	0.93±0.16	5.57±0.89	3.69±0.15	1.23±0.11	1.15±0.09	1.42±0.07	1.35±0.05

The maximal cortisol concentrations were recorded in control group on the 1st day (6.49±0.57 µg/dl) and were nearly 2 times higher than horses intake natural herbal tea (3.56±1.06 µg/dl). Between the 2nd day and 60th days, the cortisol concentrations decreased and ranged between 2.87 and 1.07 µg/dl for control group and 2.45 and 0.97 µg/dl for treatment horses respectively. Plasma fibrinogen concentrations were influenced by the DJD and were higher of norm for both group. The initial values averaged 9.23±0.89 mg/L for control group patients at 0 day and started to decrease. At the end of the survey remained unsignificantly elevated compared to reference value and treatment group. The thyroid hormones free T3 and free T4 levels slowly declined decreased but did not show any significant difference from basal values, as well as between the study groups ($P < 0.05$).

DISCUSSION

Nowadays, horses are often transported for different purpose including biomedical activities, medical examinations, clinical treatment etc., and their reactions can depend on duration of the stressor, physiological and physical factors and environmental conditions [21]. Cortisol is defined as the stress hormone and its concentration has also been reported to be a useful prognostic marker. Therefore, we examined the ability of serum cortisol concentration (SCC) as a stress marker in horses with osteoarthritis requiring admission at horses clinic.

In accordance with our results, higher levels of serum cortisol were detected at the beginning of the experiment. Although, this may be due to the fact that the animals are adults but also on the severity of the disease. Recently, it has been shown that the rate of cortisol production increases with age, which may partly explain the higher serum levels of cortisol in our patients (10-19 years old). In the current study, degenerative joint disease (DJD) was diagnosed, which also had an effect on the serum cortisol concentration. The main therapeutic agent often used to treat arthritis and related conditions is corticosteroid medications. These, along with acute stress (hospitalization and diagnostic manipulation), can also cause increased baseline levels of cortisol. In the present case, horse therapy included intra-articular administration of methylprednisolone acetate (Depo Medrol® 40 mg/weekly) and aseptic arthrocentesis. Of note, should not overlook the fact that in patients taking prednisone, the latter is converted to prednisolone after ingestion, which often leads to elevated levels of cortisol. All these facts taken together, indicate that arthritic diseases accompanied by stress create a greater stimulus for the hypothalamus-pituitary-adrenal (HPA) axis, which is manifested by higher levels of circulating cortisol. In animals consuming natural tea, there was clearly an adequate response to the severity of the disease and the stress of transport and accommodation, but serum cortisol levels were lower than the control group, which may be due to the effect of the medicinal plants contained in the tea.

Studies have shown that certain compounds and their derivatives in medical plants lead to stress relief through regulate the activities of certain receptors of stress hormones and mediators through targeting the HPA axis [22]. Prior to their administration in sport horses, it is recommended to pay serious attention to the FEI Equine Anti-Doping and Controlled Medication Programme. Measures that can be taken to prevent positive findings include using reputable suppliers of herbal and nutritional supplements. The created natural tea includes mainly anthodia et flores Chamomillae, folia Menthae and herba Verbenae, which not mentioned as a banned substances on the FEI Equine Prohibited Substances List. Chamomile is an effective traditional natural calmer and considered to have an positive effect on anxiety and overexcitement on horses [23]. Chamomile has active ingredients, containing α-bisabolol, chamazulene, and flavonoids. The flavonoid apigenin found in chamomile has been found to have an antianxient and mildly sedative effect. Researchers hypothesized that the mechanisms of effect of this medical plant involved the inhibition of cortisol production and the calming and anxiolytic effects. The Mentha (peppermint) still used effectively in anti-inflammatory, antioxidant and stress conditions. The extract include major active compounds such as hesperidin, diosmin, didymin, buddleoside, acacetin etc., and had a potent anti-stress effect that regulating the stress-related hormones (corticosterone, β-endorphin, and serotonin) and MAPK/COX-2 signaling pathways [24]. The traditional use of vervain includes depression, nervous breakdown, influenza and rheumatism due to a mild antidepressant, mild diaphoretic and astringent effects. Active constituents include iridoid glycosides, such as verbenalin, and caffeic acid derivatives [25]. According Preethi and Kuttan [26] some extracts increased level of antioxidants in the liver and lowers levels of acute phase proteins such as haptoglobin and orosomucoid in rats.

Acute phase proteins (APPs) are divided into positives whose plasma concentration increases during the APR and negative, the concentration of which decreases during the APR. At present, the measuring APPs concentrations is being used frequently in veterinary medicine. An acute phase response (APR) of the body is part of the nonspecific defense mechanisms, which initiate a very fast after the occurrence of disturbed homeostasis in a result of infection, inflammation, tissue damage or injury before they developed specific (cellular and humoral) immune reactions [27]. In the narrow sense, the term APR covers changes in the concentration of a large

number of plasma proteins. The APPs are reactants synthesized primarily by the liver during APR due to inflammation infection, trauma or stress [28].

However, in horses the major positive acute phase protein is serum amyloid A (SAA) which has very low concentration (or it is almost undetectable) in healthy animals but during APR can increase more over 100 times. Generally, the major APPs increase within a few hours after stimulus and reach maximal levels within 1–2 days, whereas the another APPs (moderate or minor) have a slower enhancement and persist in high concentrations for long periods of time. Actually, SAA is a sensitive indicator of the early phase of inflammation, and because of its rapid rise and short half-life, in most cases, fibrinogen is preferred to monitor the course of pathological processes requiring more time.

Furthermore, SAA is considered as the most precise marker of all the APPs which can elevate as a results of pathology conditions, along with an aseptic arthritis and laminitis, surgical trauma, enteritis etc., as well as in response to physical stress caused by transportation [29; 30]. According to Baghshani et al., [31] mean baseline pretransport concentrations of Fib and Cp did not change significantly during and after road transportation in dromedary camels. Whereas, Wessely-Szponder et al., [32] reported that fibrinogen increased in all studied groups of horses, especially in fillies after long distance transportation. Transportation stress can increase values rapidly and induce an elevanation of SAA concentration between 4 and 12 hours and significant peak 2 days later [30]. Thus, this enhancement is too soon to detect some differences agter longer studied priod. On the other hand, in our investigation SAA measurement cannot be useful stress indicator due to the fact that our groups included cases of DJD and also has a presence in a stressful situation, namely accommodation to a new location which must be taken into account as a horses are very sensitive animals. Therefore, we cannot be sure that an increase in SAA will be caused by joint disease, accommodation at a new location or transport stress.

Nevertheless, we focused on monitoring the dynamics of the changes in the other two APPs – fibrinogen and ceruloplasmin, which increase their values more slowly and persist for a longer time. In horses, fibrinogen (Fib) and ceruloplasmin (Cp) belong to the moderate APPs. Their levels can enhance up to 10 times during the APR and also may remain elevated for several weeks [33]. Ceruloplasmin is an APP which involved in copper metabolism responsible for the transport of approximately 95% of plasma copper. Biochemical studies have shown that copper helps to form the cross-link between cartilage and subchondral bone tissue, where its deficiency can predispose to osteochondral fragmentations [34]. Furthermore, Knight et al., [35] found that copper deficiency is responsible for problems of conformation, flexural and angular deformities, osteochondrosis, and physeal dysplasia in horses. The blood concentration of Cp indicates the highest peak of change between the seventh and the tenth of the onset of the stress process. So, because of the association of Cp with copper, we studied the levels of this protein, we found differences in concentrations, but because they may be due to the primary disease of the horses studied, we cannot say for certain that the increase is due to transport and accommodation and results were not included in this manuscript. The diagnostic potential of Cp in our study is not relevant, so we turned to the other moderate APP for horses – fibrinogen, which is a coagulant protein synthesized by liver. Normal blood level of Fib is between 2-4 g/L in agreement with that, noticeable increase in plasma tested fibrinogen was observed in current study with cases of DJD. Presumably that inflamed joints are develop a pro- or hypercoagulable state with a tendency to produce and coagulate more fibrinogen. If this applies to horses, the results indicate that the fibrinolytic pathways are intact in the initial inflammatory arthritis, and when fibrinogen and thrombin accumulate in the joint cavity, coagulation increases and fibrinolysis decreases, making the joint potentially vulnerable to fibrin deposition [36]. According to Shahabeddin Safi [37] fibrinogen react slowly, peak of change is 4-6 days after stimuli and also it can be considered relatively insensitive protein. Similarly, Fib exists in detectible levels in healthy horses, and amplitude of response of it is much lower than that of SAA. Further, it takes a few days for it values to rise after a stimulus, and its concentration remain increased for a longer period of time. Moreover, Fib and Cp concentrations reach maximal levels between 7 and 10 days after an inflammatory stimulus and may remain high for several weeks [33]. There are suggestions that the APPs can be used as markers of subclinical disease in cattle, but such data are not available for horses. Considering this, determination of APP can be used as useful tool to monitor the health of horses, thus avoiding the stress of transporting subclinically ill horses. The results obtained by us reveal a decrease in Fib levels after two months studied period, with significant increases above normal reference values initially. The presence of stress at the beginning of the study led to an increase in fibrinogen levels, but the use of tea and prednisolone resulted in a decrease in concentrations. In the literature available to us, there have been no reports concerning the relationship between the use of a medicinal plant and a decrease in fibrinogen levels as indicator of stress.

The assays used to measure thyroid hormones concentrations in horses are well established and accurate. However, laboratories reccomandate an evaluation of free T3 and T4 concentrations, which provides more useful information than total thyroid hormone concentrations. The main problem in measuring thyroid hormones in horses is that concentrations

vary over time and within the same individual and respond to many different environmental conditions. Common conditions such as different feeding regime, transport and training stress can directly affect as decrease thyroid hormone concentration. A persistently low free T3 and T4 for prolong period its not a normal and can be result from enviromental condition or medical problem. In fact, mechanisms by which thyroid hormones decrease during problem are due to decreased peripheral conversion of T4 to T3 by 5'-deiodinase and hypothalamic-pituitary dysregulation or suppression [38]. In the present study, exposure to transport stress did not lead in significant changes and serum free T3 and free T4 concentrations in animals of both groups remained within the reference values.

CONCLUSIONS

We acknowledge some important limitations must be stated. Our study was conducted with a small number of patients, so the information collected does not reflect the prevalence of the general horse population. Nevertheless, to our knowledge, this is the first study to investigate the association between herbal administration and decrese of stress in hospitalized horses. At the least, the results from our survey could alert the vet to pay close attention to hospitalized stress patients and to decide whether they need supplementary treatment to induce calm. The proposed natural herbal complex is easy to employ in real vet clinical practice. From other side tea provides the owners with a new perspectives to solving the psycho-emotional problem of their animal in the stables.

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ANTIMICROBIAL ACTIVITY OF TEN SEED OILS AGAINST GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA IN VITRO

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ABSTRACT

In recent years seed oils have become interesting by regulating authorities and were found to have nutritional quality, pharmaceutical and biomedical uses as therapy in medicine. *Staphylococcus aureus* represent Gram-positive and *Escherichia coli* represent Gram-negative are responsible of several diseases in humans and animals. The present investigation was carried out to determine the antimicrobial activity (AMA) and minimal inhibitory concentration (MIC) of ten seed oils against *Staphylococcus aureus* (*S.aureus*), *Escherichia coli* (*E.coli*), *Bacillus subtilis* (*B.subtilis*) and (*MRSA*) using agar diffusion method in vitro. Different values of inhibition zone diameters (4-16 mm) were estimated at 10% concentrations of seed oils against *S.aureus*, *E.coli*, *B.subtilis* and *MRSA*. The seed oils were more effective and exhibited the best AMA against *S.aureus* at 10% concentration but less effective and AMA against the other tested strains. Seed oils at 10% concentration, exhibited higher inhibition zone diameter of 16 mm against *S.aureus*. Decreases in inhibition zones diameters (4-12 mm) against *E.coli* and *MRSA* strains and (6-10 mm) against *B.subtilis* strain was observed as compared to those effects against *S.aureus* at 10% concentration. The inhibition zones of seed oils against *S. aureus* was decrease but no inhibition zone obtained against other bacterial strains at a low seed oils concentrations (10^{-1}, 10^{-2} and 10^{-3} respectively). Results indicated the increase of seed oil concentrations exhibited increase in the inhibition zone diameters. However, the inhibition zone diameters exhibited different levels of decreases with the seed oils concentrations decrease against *S.aureus, E.coli*, *B.subtilis and MRSA*. Moreover, the inhibition effects of seed oils against different microbial strains were found to be depending on the seed oils concentration. Minimal inhibitory concentrations (MIC) of seed oils against *S.aureus E.coli, B.subtilis* and *MRSA* were found in the range of 1-2.5mg/ml. Seed oils were also demonstrated the killing effect against *S.aureus E.coli*, *B.subtilis* and *MRSA* at 10% seed oils within 24 hours. However, the lowest concentration of seed oils produced lower inhibitory activity against *S.aureus B.subtilis E.coli* and *MRSA*. Results indicated the Gram-positive bacteria (*S.aureus*) were more sensitive than the Gram-negative bacteria (*E.coli*) at 10% concentration of seed oils used in the present study. Moreover, seed oils were found to be inhibited the growth of *S.aureus, E.coli, B.subtilis* and *MRSA* in vitro. This common seed oils consider an important sources of antimicrobial agents with minimal inhibitory concentrations of 1000-2500μg/ml may be used for inexpensive treatment of some infectious diseases.

KEYWORDS:
Seed oils, Microorganisms, Antimicrobial, In vitro

INTRODUCTION

Infectious diseases constitute a major public health problem, in developing countries, with higher rate of mortality, morbidity [1,2] and death in infants, children and adults affected with different bacteria [3, 4]. Microorganisms particularly bacteria (Gram-positive and Gram-negative bacteria) consider the major agents cause damage in several fields, including food industry, fish and farms [5, 6]. Bacteria, Salmonella sp., Pseudomonas sp., Escherichia coli and Staphylococcus aureus responsible for several diseases in humans and animals [7, 8, 9], showed pathogenesis of infections and constitutes a mechanical barrier against antibiotics [10,11]. Staphylococcus aureus, Clostridium perfringens and Staphylococcus epidermidis are producing toxin in food, followed by toxic symptoms in humans that able to cause diseases [12, 13,14] they reported many gram-negative bacteria are difficult food contaminants and pathogens. S. aureus and E. coli are of the major causes of hospital-acquired infections [6,15]. Moreover, bacteria species are responsible for upper respiratory, eye, ear, skin and urinary tract infections in general populations [11,16].Antibiotics agents are widely used for bacterial control in different regions of the world but their activities are failing to treat various infectious diseases [10,17] due to the increases of pathogenic bacteria resistant with side effects to traditional antibiotics [6,13,18] reported the control use of antibiotic need to develop the research studies for novel antibacterial compounds and efficient antimicrobial drugs [19,20]. Aguilar et al. [7], Sheikh et al. [10]

and Geetha et al. [11] reported the conventional chemotherapy drugs used for treatment and medicines are high cost and ineffective against bacteria. Several researches are being made in order to find new antimicrobial agents to overcome the problems of resistance and side effects of the available antimicrobial agents [21-23]. In last decades, chemotherapy stimulates many scientists to developed natural bioactive agents with antibacterial and anticancer properties without side effects for treatment of different diseases, including microbial infections, cancer and other diseases [24-26].Several research on going to identifying naturally occurring active compounds, capable of inhibiting and controlling some infectious bacterial diseases [10,27,28]. Antimicrobial drugs growing rapidly and strong demand to produce inexpensive antimicrobial agents from natural sources for treatment of infectious diseases without side effects [29,30]. Concentrated searches are still needed for production of new antimicrobial agents from natural sources [23,31,32] due to human pathogenic microorganisms resistant to antibiotics and failing in treatment of different types of infectious diseases [3,4,17]. Many studies suggested certain materials as natural product might be useful as anticancer and chemopreventive agents in a variety of bioassay systems and animal models due to phytochemical constituents [33-35]. Several researches are ongoing for production of new antimicrobial agents from natural sources due to a lower incidence of adverse reactions compared to synthetic pharmaceuticals and the reduced costs of preparations as natural therapeutics [11,22,31,33]. However, more than 60% of antitumor and antiinfectious drugs either under clinical trials or in the market are of natural origin. Plant seeds have a long history as a part of human culture by ancient people were contains nutritional and nutraceutical components used as food, feed or in medicine [36-38] reported plant seeds have medicinal therapeutic properties, used as therapeutic agent for protection against some diseases [39,40]. Plant seed extract and phytochemicals are showed several active therapeutic compounds represent a source of their chemical constituents, including polysaccharides, tannins, oils, phenolic, flavonoids, saponins, triterpenoids and alkaloids have active antibacterial properties [27,32,33] they reported these compounds were investigated as antimicrobial in vitro [9,21,28]. Different compoumds were used in medicine as anti-spasmodic and antidiuretic [14,41] reported some antibiotics were used for treated of bacterial pathogens responsible for respiratory, urinary tract, gastrointestinal and abdominal infection including gram negative and gram positive bacteria. Moreover, different plant extracts are widely used as antidiabetic [42], antimicrobial [32], antibacterial [11], antidiuretic [21,41], anticancer [25], antioxidants [43]and antiinflammatory agents [35] they found specific plant extracted compounds such as saponins, anthraquinones and dihhydroxyanthraquinones have direct antimicrobial activities [16,44,45]. Among plant extracts, oils were extracted in recent studies, showed pharmacological activities in vitro, in vivo and in medical trials [46-48]. Oils are most naturally occurring and widely diffused in plants, animals and microorganisms, represent largest products in the world and have different biological functions [25,30,39]. Oils extracted from plant seeds have become interesting in recent years and were found to have nutritional quality used as edible oil food ingredients in most diets [37,38,49] reported the majority of seed oils have health benefits, pharmaceutical and therapy in medicine [29,50]. Seed oils are acceptance by regulating authorities, nontoxic and biodegradable that consequently suitable for food, pharmaceutical and biomedical uses which play important roles in several physiological and pathological conditions [34,37,47] found oils component has indirect antimicrobial activity through stimulate phagocytic leukocytes. Oils used in treatment and protection against cancer [29,40,51]. Seed oils as natural compounds consider one of plant-derived bioactive molecules in medicine, have been demonstrated to have therapeutic [52,25], antitumor [24] anti-inflammatory [35], anticancer and cytotoxic activities [48]. Seed oils are consider biological macromolecules consisting of various types of fatty acids and phytochemicals necessary for biological activities and various mechanisms in human and animal [33,48,53]. Seed oils with their constituents of fatty acids and phytochemicals have various bioactivities including cytotoxicity [48,53], anticancer [24,26] and antidiabetic activities [54]. Oils extracted from plant seeds have antimicrobial [51], antifungal [55], antiviral [43] and antibacterial activities [56]. Several investigators [29,44,51] indicates the antibacterial activity against a variety of infectious agents has been attributed to oils containing different chemical compounds that recognized as active antimicrobial agents [32,56], reported the medicinal importance of plants come from the presence of bioactive oils in plants seeds. Zhang et al. [30] and Rawani et al. [32] reported the oils and their components are known to exhibit antimicrobial activities against clinical, food-borned pathogens and food spoilage bacteria. Many antibiotics used in medicine are derived from natural sources of fruits and vegetables involving phenolic, flavonoides and polyphenols [22, 23, 57]. Other investigators [9, 53, 56] reported the antibacterial activity derives from fatty acids, flavonoid and phenolic compounds of seed oils [11,30]. Antiinfectious and antitumor drugs either under clinical trials or in the market are of natural origin [16,17,24.47] Rapeseed (Brassica napus), watercress (Eruca sativa), pumpkin (Cucurbita pepo), radish (Raphanus sativus),purslane (Portulaca oleracea), safflower (Carthamus tinctorius), chickpeas (Cicer arietinum), dill (Anethum graveolens), lettuce (Lactuca sativa) and coriander (Coriandrum

sativum) seed oils are commonly used as food or in medicine in many region of the world [27,50]. In previous studies, we have extracted seed oils [48]. Fatty acid contents of seed oils were previous analyzed and identified using Gas Liquid Chromatography. Analysis of the prepared seed oils revealed the presence of different percentages of saturated and unsaturated fatty acids. Phenolic and flavonoid contents of these seed oils were also estimated. Interestingly, theses seed oils have anticancer and cytotoxic activities on various cancer cell lines in vitro [48]. However, the properties of many plants and plant derived compounds particularly it's cytotoxic, anticancer antioxidant and antimicrobial activities have not yet been fully investigated. The aim of this study was done to investigate the antimicrobial activities of ten seed oils on growth of four bacterial strains in vitro. The present study may be helpful to develop inexpensive antimicrobial drugs containing bioactive substances effectively used in treatment of different infectious diseases.

MATERIALS AND METHODS

Materials. 1-Ten seed oil samples of rapeseed (*Brassica napus*), watercress (*Eruca sativa*), pumpkin (*Cucurbita pepo*), radish (*Raphanus sativus*), purslane (*Portulaca oleracea*), safflower (*Carthamus tinctorius*), chickpeas (*Cicer arietinum*), dill (*Anethum graveolens*), lettuce (*Lactuca sativa*) and coriander (*Coriandrum sativum*) were prepared and used in the present study [48].

2- Four bacterial strains including *Staphylococcus aureus* (*S.aureus*), *Escherichia coli* (*E.coli*), *Bacillus subtilis* (*B.subtilis*) and Methicillin-Resistant *Staphylococcus aureus* (*MRSA*) as standard strain. All bacteria were obtained from Mercin faculty of agricultural, Ain shams University Cairo, Egypt. Stock cultures of all bacterial strain were grown on nutrient agar plates and maintained in the nutrient agar slants at 4°C. The bacterial strains were activated before the antibacterial test. After removal from the refrigerator, strains were incubated overnight in nutrient broth and then streaked on nutrient agar plate and kept for 24 hours at 37 ºC [58-60].

Preparation of oils stock solutions. Seed oils samples were dissolved individually in dimethylsulfoxide (DEMSO) using vortex mix. Solution of seed oils was freshly prepared from each seed oils sample separately to obtained a series of 5-fold dilutions (from 10^{-1} to 10^{-5}) of various concentrations of each seed oil sample in DEMSO before added to the agar and broth media used for antimicrobial tests [28].

In vitro antimicrobial activities (AMA). Bacterial strains of *S.aureus*, *E.coli*, *B subtilis* and *MRSA* cultures were incubated at 37˚C for 24-48h, each bacterial strain sub-cultured and strecked on agar medium and the antimicrobial activity (AMA) of each seed oils samples against each strain was detected. AMA was measured using agar-well diffusion method [60, 61]. 0.1 ml of each culture of each bacteria strain was introduced into a sterile Petri dish containing nutrient agar. Sterile nutrient agar has cooled and allowed to set. Three wells were made on the set medium at suitable space. The seed oil samples were dissolved in 1% DEMSO and prepared at concentration of 400µg/ml. The wells were respectively filled with different concentrations (50, 25 and 12.5 mg/ml) of each seed oil separately and they were incubated in an incubator at 37°C for 24 h. The seed oil solutions were diffused around the wells in Petri dishes and they were surrounded by circular clear zones of inhibition that could be analyzed. The results were recorded by measuring the diameters of growth inhibition zone around bacterial strains in millimeter (mm). These clear inhibition zones around the wells indicate the presence of antimicrobial activity. All data of antimicrobial activity are the average of triplicate analyses.

Determination of minimum inhibitory concentration (MIC). Agar diffusion test was used for determination of MIC [60, 61]. Muller hinton agar medium was used and a clear circular zone of growth inhibition (mm) was measured [62]. MIC of different seed oil samples against the four selected bacterial strains was determined.

RESULTS AND DISCUSSION

Seed oils. Seed oils associated with people from ancient time as food in many regions of the world and generally consumed for its nutritive values, health benefits, pharmaceutical and biomedical uses which play important roles in biological functions. Screening of seed oils previously prepared, revealed the presence of different percentages of fatty acids, phenolic and flavonoid compounds [48] they reported the seed oils have anticancer and cytotoxic activities against cancer cell lines. Seed oils used in the present study were analyses and the saturated fatty acid (5.6-15.6%), monounsaturated fatty acids (24.8-64.2%), polyunsaturated fatty acids (30.2-62.8), Phenolic (0.9-24.4 mg GAE/g) and flavonoid (0.2-32.6 CE/g) were estimated [48]. Previous results indicated the presence of 7 main fatty acids were detected (linolinic acid, linoleic acid, oleic acid, palmitic acid, stearic acid, arachidic acid and behinic acid) with different variations in their contents between seed oil samples under the present study [48]. Seed oils in the present study containing bioactive compounds which effective on growth inhibition of different bacterial strains and may be used in the treatment of infectious diseases. Many investigators [53, 55, 56]

were indicated the antibacterial activities were derives from fatty acids, flavonoid and phenolic compounds of seed oils. The main objective of this study was done to investigate the AMA of 10 seed oils against four bacterial strains in vitro [9, 27, 33] reported some seed oils have antibacterial properties and investigated as antimicrobial in vitro. Seed oils are effective in treating diseases as anticancer and antimicrobial [26, 51, 56]. Measurements of antimicrobial activity (AMA) of the 10 seed oils were determined against four strains of bacteria (S. aureus, E.coli, B.subtilis and MRSA) as shown in Table (1). The bacterial strain, S.aureus represents Gram-positive bacteria that can cause skin infection and E. coli strain represents Gram-negative bacteria which can be found in gastrointestinal tract. Moreover, S.aureus and E.coli are responsible of several diseases in humans and animals.

Determination of antimicrobial activity (AMA). The present results showed ten seed oil samples give AMA against S. aureus. The present results showed different percentages of growth inhibition of S. aureus by the all seed oil samples used in the present study (Table 1). Seven samples of seed oils used (rapeseed, watercress, pumpkin, radish, pursaline, safflower and lettuce) showed

TABLE 1
Activities of ten seed oils on growth of 4 bacterial strains.

Seed oil samples	Antimicrobial activity (AMA)			
	S. aureus	*E.coli*	*B subtilis*	*MRSA*
Rapeseed (*Brassica napus*)	+ve	+ve	-	-
Watercress (*Eruca sativa*)	+ve	+ve	+ve	+ve
Pumpkin (*Cucurbita pepo*)	+ve	+ve	-	+ve
Radish (*Raphanus sativus*)	+ve	+ve	+ve	+ve
Purslane (*Portulaca oleracea*)	+ve	+ve	-	+ve
Safflower(*Carthamu stinctorius*)	+ve	+ve	+ve	+ve
Chickpeas (*Cicer arietinum*)	+ve	-	-	-
Dill (*Anethum graveolens*)	+ve	-	-	-
Lettuce (*Lactuca sativa*)	+ve	+ve	+ve	+ve
Coriander (*Coriandrum sativum*)	+ve	-	-	-

+ve means the activity of oils against bacterial strains (Mean values of three samples)

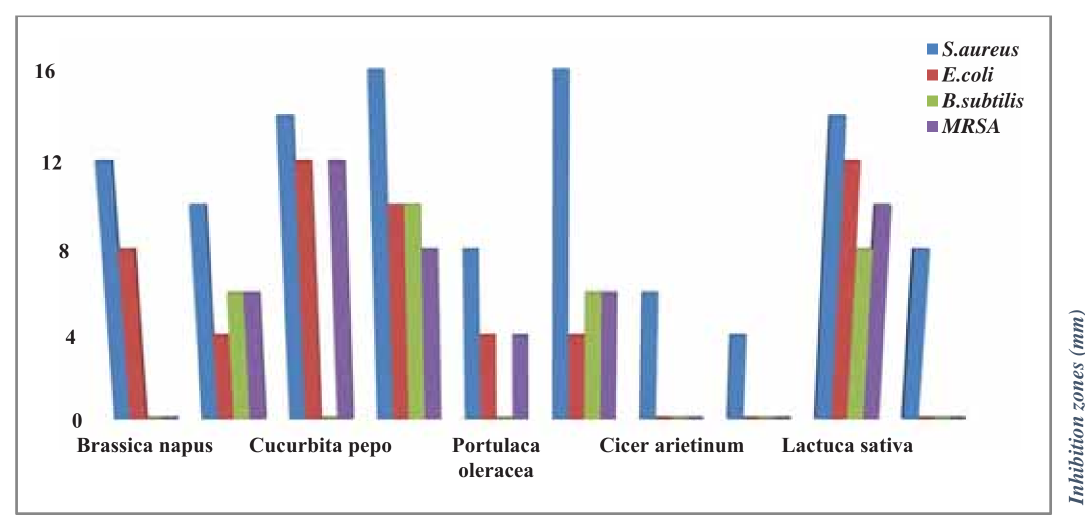

FIGURE 1
Antimicrobial activities of seed oils against four bacterial strains

Seed oils

AMA against E.coli (Table 1). Four samples of seed oils used (watercress, radish, safflower and lettuce) showed AMA against B subtilis (Table 1). Six samples of seed oils (watercress, pumpkin, pursaline, safflower, radish and lettuce) showed AMA against MRSA (Table 1). Chickpeas, dill and coriander seed oils showed no effect against E.coli (Figure 1). Rapeseed, pumpkin, purslane, chick peas, dill and coriander seed oils showed no effect against B subtilis (Figure 1). Rapeseed, chickpeas, dill and coriander seed oils showed no effect against MRSA (Figure 1). However, seed oils were inhibited the growth of S. aureus, E. coli, B subtilis and MRSA in vitro. The results obtained were found to be similar the results reported by several investigators [41, 46, 30, 56].

Antimicrobial activity (AMA) of seed oils at different concentrations was done using diffusion method test and inhibition zones were measured in mm diameter and recorded (Table 2). The results obtained with all seed oils showed best AMA against S. aureus than the other microbial strains used in the present study. The seed oil obtained from Pumpkin, radish and lettuce were active against S. aureus, E.coli, B subtilis and MRSA strains (Fig.1). The rapeseed, watercress, pumpkin, radish, safflower, pursaline and lettuce seed oils were active against E.coli strain. Watercress, radish, safflower and lettuce seed oil were active against B subtilis strain (Fig.1). Watercress, pumpkin, radish, safflower, pursaline and lettuce seed oils were active against MRSA strain (Fig.1). These results indicated that these seed oils have AMA against some bacterial strains. Similar results were obtained by other workers [41, 46, 56] found oils obtained from plant origin showing antibacterial effects against some common pathogens such as E. coli and S. aureus. Other investigators suggests some of plant seed oils are effective in treating diseases of microbial infections [32, 41, 63]. Seed oils were found to be used in treating different diseases as antidiabetic [42], antimicrobial [32], antibacterial [11], antidiuretic [21, 41], antifungal [55], antiviral [43] and anticancer [26]. However, different effects of seed oils were depend on their constituent compounds of fatty acids, phenolic and flavonoids [29,44,51] indicates the antibacterial activity against a variety of infectious agents has been attributed to oils containing different chemical compounds that recognized as active antimicrobial agents [32, 56]. Other investigators [9, 31, 56] reported the antibacterial activity derives from fatty acids, flavonoid and phenolic compounds of seed oils. Previous studies were reported the oils and their constituents are known to exhibit antimicrobial activities against clinical, food-borned pathogens and food spoilage bacteria [6, 30, 32]. Moreover, many antibiotics used in medicine are derived from natural sources of fruits and vegetables involving phenolic, flavonoids and polyphenols [22, 23, 57]. Different values of inhibition zone diameters (2-16 mm) were observed at 10% concentrations of all seed oil samples against microbial strains tested. The inhibition zones of rapeseed, watercress, pumpkin, radish, safflower, lettuce and coriander seed oils against S. aureus was decrease at a low seed oils concentrations (10-1, 10-2 and 10-3 respectively) as shown in Table (2). Results in Table (2) showed the seed oils of rapeseed, watercress, pumpkin, radish, safflower and lettuce at 10% concentration, exhibited higher inhibition zones (4-16 mm) against S. aureus. Decreases in inhibition zones (4-12 mm) against both of E.coli and MRSA strains and (6-10 mm) against B. subtilis strain as compared to the seed oils effect against S. aureus were observed at 10% concentration (Table 2) and (Figure 1). These results are similar to those reported by other investigators [6, 14, 41]. Inhibition zone ranged from 10 to 16 mm in diameter was observed with radish seed oil at a concentration of 10% against B. subtilis, E.coli and S.aureus respectively while the inhibition zone of 8 mm was observed against MRSA. Inhibition zone ranged from 4-16 mm in diameter was observed with safflower seed oil at a concentration of 10% against E.coli and S.aureus respectively while 6 mm inhibition were observed against B. subtilis and MRSA.Inhibition zone was 12-14 mm in diameter was observed with pumpkin seed oil at a concentration of 10% against E.coli, MRSA and S.aureus respectively while no inhibition effect was observed against B. subtilis (Figure 1) and Table 2. Inhibition zone ranged from 8-14 mm in diameter was observed with lettuce seed oil at a concentration of 10% against B.subtilis, MRSA, E.coli and S.aureus respectively. Inhibition zones, 8 and 12 mm in diameter were observed with rapeseed seed oil at a concentration of 10% against E.coli and S.aureus respectively while no inhibition effect was observed against B.subtilis and MRSA. Inhibition zone, 10 mm in diameter was observed with watercress seed oil at a concentration of 10% against S.aureus while inhibition zones ranges from 4-6 mm in diameters were observed against E.coli, B.subtilis and MRSA respectively (Figure 1) and Table 2. Inhibition zone was 8 mm in diameter was observed with purslane seed oil at a concentration of 10% against S.aureus while inhibition zone of 4 mm in diameter was observed against E.coli and MRSA. Inhibition zone was 8 mm in diameter was observed with coriander seed oil at a concentration of 10% against S.aureus while no inhibition effect was observed against E.coli, B.subtilis and MRSA (Figure 1). Inhibition zone was 6 mm in diameter was observed with chickpeas seed oil at a concentration of 10% against S.aureus while no inhibition zones were observed against E.coli, B.subtilis and MRSA. Inhibition zone was 4 mm in diameter was observed with dill seed oil at a concentration of 10% against S.aureus while no

inhibition effect was observed against E.coli, B.subtilis and MRSA. Inhibition effects of seed oils against different bacterial strains were found to be depending on the concentrations used. No inhibition zone against bacterial strains was obtained at low seed oil concentrations (10-3, 10-4 and 10-5). However, the present results indicated that the increase of seed oil concentrations exhibited increase in the inhibition zone diameter (Table 2). Moreover, the inhibition zone diameters exhibited different levels of decreases with the seed oils concentrations decrease against S.aureus, E.coli, B.subtilis and MRSA (Table 2). These results mainly attributed to the different percentages of fatty acids, phenolic and flavonoids compounds in seed oils [22, 27, 31, 64] reported the inhibition effect of seed oils associated with their phenolic and flavonoids contents. Other investigators reported the inhibitory effect of seed oils depend on structure, level, types and concentration of seed oil components as well as microorganism tested [6, 30, 41, 63]. A market effect was observed from these results is the E. coli, B.subtilis and MRSA strains were unaffected with chickpeas, dill and coriander seed oils used in the present study.

TABLE 1
Minimum inhibitory concentration (MIC) values of ten seed oils

Seed oil samples	Minimum inhibitory concentration (MIC) for *S.aureus*					
	10	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Rapeseed (*Brassica napus*)	12 mm	2 mm	1 mm	-	-	-
Watercress (*Eruca sativa*)	10 mm	4 mm	2 mm	-	-	-
Pumpkin (*Cucurbita pepo*)	14 mm	4 mm	2 mm	-	-	-
Radish (*Raphanus sativus*)	16 mm	4 mm	2 mm	-	-	-
Purslane (*Portulaca oleracea*)	8 mm	-	-	-	-	-
Safflower(*Carthamu stinctorius*)	16 mm	4 mm	2 mm	-	-	-
Chickpeas (*Cicer arietinum*)	6 mm	-	-	-	-	-
Dill (*Anethum graveolens*)	4 mm	-	-	-	-	-
Lettuce (*Lactuca sativa*)	14 mm	4 mm	1 mm	-	-	-
Coriander (*Coriandrum sativum*)	8 mm	-	-	-	-	-
Seed oil samples	Minimum inhibitory concentration (MIC) for *E.coli*					
	10	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Rapeseed (*Brassica napus*)	8 mm	2 mm	1 mm	-	-	-
Watercress (*Eruca sativa*)	4 mm	-	-	-	-	-
Pumpkin (*Cucurbita pepo*)	12 mm	4 mm	2 mm	-	-	-
Radish (*Raphanus sativus*)	10 mm	2 mm	1 mm	-	-	-
Purslane (*Portulaca oleracea*)	4mm	-	-	-	-	-
Safflower(*Carthamu stinctorius*)	4 mm	1 mm	-	-	-	-
Chickpeas (*Cicer arietinum*)	-	-	-	-	-	-

Dill (*Anethum graveolens*)	-	-	-	-	-	-
Lettuce (*Lactuca sativa*)	12 mm	2 mm	1 mm	-	-	-
Coriander (*Coriandrum sativum*)	-	-	-	-	-	-

Seed oil samples	Minimum inhibitory concentration (MIC) for *B.subtilis*					
	10	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Rapeseed (*Brassica napus*)	-	-	-	-	-	-
Watercress (*Eruca sativa*)	6 mm	4 mm	2 mm	-	-	-
Pumpkin (*Cucurbita pepo*)	-	-	-	-	-	-
Radish (*Raphanus sativus*)	10mm	2 mm	-	-	-	-
Purslane (*Portulaca oleracea*)	-	-	-	-	-	-
Safflower(*Carthamu stinctorius*)	6 mm	2 mm	-	-	-	-
Chickpeas (*Cicer arietinum*)	-	-	-	-	-	-
Dill (*Anethum graveolens*)	-	-	-	-	-	-
Lettuce (*Lactuca sativa*)	8 mm	1mm	-	-	-	-
Coriander (*Coriandrum sativum*)	-	-	-	-	-	-

Seed oil samples	Minimum inhibitory concentration (MIC) for *MRSA*					
	10	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Rapeseed (*Brassica napus*)	-	-	-	-	-	-
Watercress (*Eruca sativa*)	6 mm	-	-	-	-	-
Pumpkin (*Cucurbita pepo*)	12 mm	4 mm	1 mm	-	-	-
Radish (*Raphanus sativus*)	8 mm	2 mm	1 mm	-	-	-
Purslane (*Portulaca oleracea*)	4 mm	-	-	-	-	-
Safflower(*Carthamu stinctorius*)	6 mm	-	-	-	-	-
Chickpeas (*Cicer arietinum*)	-	-	-	-	-	-
Dill (*Anethum graveolens*)	-	-	-	-	-	-
Lettuce (*Lactuca sativa*)	10 mm	4 mm	1 mm	-	-	-
Coriander (*Coriandrum sativum*)	-	-	-	-	-	-

Mean values of three samples.

Determination of minimum inhibitory concentration (MIC). The minimum inhibitory concentration (MIC) was determined for the all seed oils used and the results are given in Table 2. Seed oils at 10% concentration exhibited best antibacterial activity against S. aureus. Seed oil of radish, pumpkin and lettuce exhibited high activity against E.coli and MRSA than the other seed oils. The seed oils

concentration at 10% were more effective against S.aureus with a zone of inhibition of 16 mm in diameter and was least effective against the other tested strains (Figure 1) and Table 2. E.coli, B.subtilis and MRSA strains showed a zone of inhibition was 4 mm in diameters (conc. 200µg). The MIC value of seed oils was found to have low MIC value of 1 mg/ml for S. aureus, while with the E.coli, B.subtilis and MRSA, showed a higher MIC value of 2.5 mg/ml. These results indicated higher activity of seed oils against S. aureus and less activity of the seed oils against E.coli, B.subtilis and MRSA. These results are in accordance with the previous reports [44, 65] and compared with the active concentrations to those reported by other investigators [66, 67]. Moreover, seed oils showed more effective than other extracts against E.coli, S.aureus and S.epidermidis [32, 68]. Results also indicated the Gram-positive bacteria were more sensitive than the Gram-negative bacteria at the higher concentrations of seed oils used in the present study. These effects are mainly attributed to the different constituents of seed oils [48] they identified different levels of saturated and unsaturated fatty acids as the major constituents of seed oils Saturated fatty acids were found to be active against Gram-negative but not Gram-positive bacteria [9, 30] they reported saturated fatty acids and unsaturated fatty acids are known to inhibit Gram-positive bacteria. Thus the antimicrobial activity of these seed oils is probably due to a complex action of the antimicrobial fatty acids with other bioactive antimicrobial compounds identified in these seed oils such as phenolic and flavonoids [48]. However, seed oils were inhibited the growth of S.aureus, E.coli, B.subtilis and MRSA in vitro. This common seed oils consider an important sources for antimicrobial agents with minimal inhibitory concentration (MIC) of 1000-2500µg/ml used as food, pharmaceutical and drug for treatment of different diseases.

CONCLUSIONS

Results of the present study demonstrate that bacterium *S. aureus*, *E. coli*, *B.subtilis* and *MRSA* were being inhibited by seed oils. Inhibition zone of *S. aureus* was found at 10% seed oils, whereas no inhibition zone was observed on lower concentrations of seed oils. Inhibition zone of *E. coli*, *B.subtilis* and *MRSA* were found at 10% seed oils and different percentages of decreases were observed on lower concentrations of seed oils. Seed oils used in the present study have different percentages of antimicrobial and inhibitory activities against *S. aureus*, *E. coli*, *B. subtilis* and *MRSA* in vitro.

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