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PRODUCTION OF NATURAL DRINKS SWEETEN WITH STEVIA

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ABSTRACT

The present study was designed to evaluate the sweetness degree of stevia leaves compared to sucrose and used stevia leaves as a natural sweetener. Then, a sucrose replacement with stevia leaves at (25, 50, 75 and 100% replacements) to sweeten some hot - healthy herb drinks (anise, mint, ginger, cinnamon and hibiscus) individually. Drinks with different substitutions were evaluated organolyptically and content of phenols, flavonoids, tannins and antioxidant were measured. Results showed that 50% substitution of sucrose with stevia was the highest acceptable formula followed by 75% compared with control in most examined herb drinks. In addition to that, the highest antioxidant activity was observed in case of 100% replacement with stevia leaves for anise, mint, ginger, cinnamon and hibiscus, respectively compared with control of each herb drink. Stevia leaves could use in sweeten hot herb drinks up to 75% of used sucrose with high antioxidant activity.

KEYWORDS:
Antioxidant activity; herbs; natural drinks; phenols; sensory evaluation; stevia leaves; sweetening power

INTRODUCTION

Herbs with naturally healthy quality can be a valuable tool in the prevention and/or cure of several important diseases in developed countries plagued by dramatic and serious increases in the occurrence of obesity, cardiovascular disease, diabetes and cancer. Health is becoming a main concern for people around the world. However, many people are turning towards nature and or food ingredients that not only healthy, but also has a good taste[1]. *Stevia rebaudiana* is a natural plant derived sweetener which can be used to answer all of these health problems, namely. It is now widely obtainable and rapidly replacing artificial sweeteners in food products. Besides, its level of sweetness is 250 times sweeter than sucrose and it does not cause tooth decay. Stevia has many natural anti-oxidants that can help to lower blood pressure and cholesterol, and to control diabetes, hence it can prevent many chronic diseases . Also, Stevia has no bad effect or health risk so it is very safe and beneficial for everyone. It has become an alternative to calorie aware consumers who want to enjoy a sweet taste without added calories or glycemic response and is considered to be generally recognized as safe by FDA [2].

Sweet taste Foods have long been related with dietary energy As an example, in the current day, excessive consumption of sugar, especially in sugar-sweetened beverages, has been linked to the rising rates of obesity worldwide [3 and 4]. Refined sugars added to food and beverages have little to no nutritional value and contribute to increased energy intake [5 and 6].

Stevia rebaudiana Bertoni is a perennial herb/shrub-like plant belongs to the Asteraceae family. It is a natural sweetener plant and determined to be 300 times sweeter than cane sugar. The leaves of stevia are the source of diterpene glycosides, rebaudioside and viz. stevioside. Stevioside is regenerated as a valuable natural sweetening agent because of its relatively good taste and chemical stability. It is used as a sweetener for bitter medical preparations, teas and other beverages and sweetener is obtained from leaves extraction [7 and 8]. The crude stevia leaves and herbal green powder are 10-15 times sweeter than sucrose while refined stevia extracts are 200 to 300 times. Unlike some other high-intensity sweeteners, stevia is light, heat and acid stable, that makes it ideal for acidic juice drinks and pasteurized dairy products. *Stevia rebaudiana* leaves are available and used in several countries, including Japan and South American countries, as sweetener for a variety of foods and beverages [2 and 9].

Stevia leaves are good source for many micronutrients such as calcium, iron, sodium, potassium, magnesium, phosphorus, zinc, flavonoids and vitamins A and C. Stevia does not cause tooth decay and undesirable flavor tolerance. It prevents the formation of dental plaque, has a bactericidal effect, no calories and no harmful effects such as artificial sweeteners [10].

Stevia is stable at high temperatures and in aqueous solutions. It is without the caloric value and it is suitable for diabetics diet, people with obesity and phenyl ketonuria [11]. It is used as

sweetener for soft drinks, soy sauce, chewing gum, dairy products, tobacco products and many other foods. The main advantages of using stevia in the industry is its high stability in acidic and alkaline media, heat stability (up to 200°C), good light stability and good solubility. Stevia has a beneficial effect on human health [12 and13].

In addition to its natural, noncaloric sweetening characteristics, leaf extracts of stevia are reported to possess anti-hypertensive, ant hyperglycemic, antioxidant, anti-tumor, anti-inflammatory, anti-diarrheal, immunomodulatory and anti-viral effects [14]. Moreover, stevioside does not have mutagenic, teratogenic or carcinogenic effects. Likewise, allergic reactions have not been observed when it is used as a sweetener [15].

Stability of stevioside through different processing and storage conditions has been evaluated in tea and coffee drinks. Stevioside at elevated temperature for one h showed good stability up to 120 °C. In aqueous solution stevioside is stable in the pH ranged from 2 to 10. This knowledge seems to be essential for its effective application in hot coffee and tea beverages [16].

Sing and Rao [17] reported that a dried leaf is considerably sweeter than afresh one, and is the form of Stevia used in brewing herbal tea. Powdered stevia is 15-20 times sweeter than sugar. It has a greenish color and can be used in a wide variety of foods and drinks, including coffee, sweets, confectionaries and several edible dishes herbal tea blend. Its distinctive flavor is reminiscent of licorice, which will blend very well with different aromatic spices, such as cinnamon and ginger.

Stevia leaf extract exhibits a high degree of antioxidant activity and has been reported to inhibit hydroperoxide formation in sardine oil with potency greater than that of either DL-a-tocopherol or green tea extract. The antioxidant activity of Stevia leaf extract has been referred to the scavenging of free radical electrons and superoxides [9].

Stevia help's in weight loss because the glycosides cannot be degraded in the human body and they are not moved into the bloodstream and doesn't produce any calories. Since stevia sweetened products have lower caloric value there is a great possibility of its use in the food industry [18]. The usage of safe and effective low-calorie intense sweeteners is gaining popularity in processed foods with increased consumer awareness on dietary calorie intakes. The safety of steviol glycosides (steviol equivalents) and recommended an acceptable daily intake (ADI) limit of 4 $mgkg^{-1}$ body weight day^{-1} [19].

The objective of the current study was aimed to produce low-calorie hot drinks (anise, mint, ginger, cinnamon and hibiscus) sweetened with natural sweetener stevia leaves, as a source of antioxidants as well as healthy herbs, especially for diabetic's patients, overweight people and some categories that need such herbs and to maintain the weight.

MATERIALS AND METHODS

Materials. *Stevia* leaves *(S. rebaudiana* Bertoni) were obtained from Sugar Crop Research Institute, Agricultural Research Center, Giza, Egypt. Anise seeds (*Pimpinella anisum* L), dried mint leaves (*Mentha longifolia)*, ginger root (*Zingiber officinale*), cinnamon bark (*Cinnamomum verum*), and hibiscus (*Hibiscus sabdariffa* L) were purchased from local market in Giza, Egypt.

Chemical reagents. Catechin, Folin-Ciocalteu phenol reagent, Gallic acid, and DPPH (1,1-Diphenyl-2-picryl-hydrazyl) were obtained from Sigma-Aldrich Co. (Darmstadt, Germany). All chemicals were of analytical grade. All other chemicals were of analytical grade.

Preparation of stevia leaves. Fresh stevia leaves *(S. rebaudiana Bertoni*) were cleaned and washed from dust then dried indirectly in shadow at temperature ranged from 25 - 30°C for 24 - 48 h, packed in polyethylene bags after milled and stored at -18°C until used.

Sweetening power of stevia leaves evaluation. The sweetening power (The intensity of sweetness) of stevia leaves obtained by water extraction (around 15 times of sucrose at 5% concentrate proximately), determined by relative sweetness (equal sweetness matches) method, described by Isima and Kakayama [20].

Preparation of herbal hot drinks. One gram were weighed of each grinded herb (anise, mint, ginger, cinnamon and hibiscus) with (20-30 mesh) were mixed with or without the stevia leaves as sugar substitution which were early determined by relative sweetness intensity of stevia leaves obtained by water extraction (which were around 15 times of sucrose at 5% concentrate), then 200 ml hot water (100°C) were added to mixed herbs. Also, another extracts were repeated in this manner three times and filtered, reached to a known volume and kept at -18ºC until analysis.

Organoleptic evaluation of herbal hot drinks. Organoleptic evaluation of herbal hot drinks (anise, mint, ginger, cinnamon and hibiscus) substituted for sugar with stevia leaves as a sweetener. The sensory evaluations of herbs drink prepared by substituting 25, 50, 75 and 100% sugar

TABLE 1
Formulas of herbal hot drinks with different substitution of sugar with stevia†

Substitution	Sucrose (g)	Stevia leaves (g)	Herbs‡ (g)
Control (100% Suc)	15	----	1
75% Suc + 25% ST	11.25	0.25	1
50% Suc + 50% ST	7.5	0.50	1
25% Suc + 75% ST	3.75	0.75	1
100% ST	-----	1.00	1

†Stevia blends based on sweetening power of stevia leaves.
‡Herbs (anise or mint or ginger or cinnamon or hibiscus).
ST= Stevia leaves Suc= Sucrose

with their equivalent sweetness of stevia leaves (due to the sweetening power of stevia and based on preliminary trials). Samples were organolyptically evaluated for its sensory characteristics, *i.e.*, taste, flavor, sweetness, after test, color and overall acceptability. The evaluation was carried out by 10 trained panelists according to the method of Du-Bois and Stephenson [21].

Analysis of hot drinks. Determination of total phenol content. Total phenol content was determined according to Singleton and Rossi [22] using Folin-Ciocalteu method. Hot water extract of drinks was mixed with Folin-Ciocalteu reagent and sodium carbonate. A known volume (0.250 ml) was mixed with 0.250 ml Folin-Ciocalteau reagent and 0.50 ml of 10% sodium carbonate (Na_2CO_3) and the volume was completed to 5 ml with distilled water. After incubation in dark at room temperature for 30 min, the absorbance of the mixture was measured at 725 nm against blank. Gallic acid was used as a standard.

Determination of total flavonoids. Total flavonoids were determined according to the methods of Zhishen et al. [23]. Pervious extract (0.4 ml) was added to 4 ml of H_2O. Then 0.3 ml of 5% $NaNO_2$ was added. After five min, 0.3 ml of 10% $AlCl_3$ was added. After six min, 2 ml of 1 M NaOH was added and the total volume was made up to 10 ml with distilled water. The color was measured at 510 nm against a blank reagent. Catechin was used as standard compound.

Determination of tannins. Tannins were determined as described by Price et al.[24]. One ml extract was mixed with 5ml vanillin/HCl mixture (by mixing equal volumes of 2% vanillin in methanol and 8% methanol/HCl) in a test tube and kept for 20 min at room temperature. The color was determined at 500 nm. Catechin was used to prepare the standard curve.

Antioxidant activity. 2, 2 diphenyl-1-picrylhydrazyl (DPPH) radical assay. The stable 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the extracts according to. The antioxidant activity of plant water extracts was determined based on the radical scavenging ability in reacting with a stable DPPH free radical according to Brand-Williams et al. [25]. DPPH (2.4 mg) in 100 ml methanol was prepared and 3.9 ml of this solution was added to 0.1 ml of sample extract. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min in the dark. Then the absorbance was measured at 515 nm. The radical scavenging percentage was calculated by following equation:

Radical scavenging (%) = $[(A_0 - A_1/ A_0)] \times 100$

A_0= the absorbance of the control reaction (containing all reagents except the test sample).

A_1= the absorbance in the presence of the tested extracts after 30 min.

TABLE 2
Bioactive compound content, antioxidant activity and sweetening power of stevia leaves

Properties	Hot aqueous extract of stevia leaves
Total phenol ($mg100g^{-1}$)	2830.00±4.24
Flavonoid ($mg100g^{-1}$)	705.85±2.14
Tannins ($mg100g^{-1}$)	557.59±3.20
Antioxidant activity (%)	87.88±0.21
Sweetening power†	15

†Sweetening power was determined according to relative sweetness (equal sweetness matches) method.
Each value is expressed as mean ±standard deviation (n=3)

Statistic analysis. For the analytical data, mean values and standard deviation are reported. The data were subjected to one-way analysis of variance (ANOVA) at $P<0.05$.

RESULTS AND DISCUSSION

Bioactive compound content, antioxidant activity and sweetening power of stevia leaves. Bioactive compound content, antioxidant activity and sweetening power of stevia leaves aqueous extract are shown in Table (2). The results showed that aqueous extract of stevia leaves was high in content of total phenol, flavonoides and tannin. It could be noticed that stevia leaves water extract is considered natural source of antioxidants. These results are in the same line with Abou-Arab and Abu-Salem [26]. Also, Thomas and Glade [9] reported that, antioxidant activity of stevia leaf extract has been attributed to the scavenging of free radical electrons and superoxides . Gasmalla et al. [27] reported that, tannin content of stevia leaves it ranged from 5.20–6.0%.

Data indicated also, stevia water extract had the highest antioxidant activity in DPPH inhibition (87.88± 0.21). Stevia methanol and water extracts have the highest antioxidant activity in DPPH inhibition [28]. On the other hand, data show that, stevia leaves water extract around 15 times of sucrose at 5% concentrate. These results are in agreement with Vincent et al. [2].

TABLE 3
Sensory scores of (anise and stevia) hot drinks

Drinks \ Characters	Taste (20)	Flavor (20)	Sweetness (20)	After taste (20)	Color (20)	Overall acceptability (100)
Control (100% Suc+0% ST)	19.33±1.03[a]	19.33±0.81[a]	19.33±1.03[a]	19.00±1.09[a]	19.50±0.83[a]	96.50±4.46[a]
75% Suc + 25% ST	19.00±1.55[a]	18.83±0.98[a]	19.16±1.60[a]	19.00±1.55[a]	19.50±0.83[a]	96.00±5.54[a]
50% Suc+ 50% ST	18.16±1.47[ab]	18.33±1.21[ab]	18.83±1.60[a]	18.66±1.50[a]	18.66±1.21[a]	93.00±5.96[ab]
25% Suc +75% ST	17.00±1.41[bc]	17.16±1.32[bc]	17.66±2.06[ab]	17.50± 2.16[a]	17.16±1.47[b]	86.83±6.88[b]
0% Suc +100% ST	15.16±2.13[c]	16.5±1.51[c]	15.66±2.06[b]	15.33±1.96[b]	15.83±1.47[b]	78.50±8.06[c]

ST= Stevia Suc= Sucrose

Each value is expressed as mean ±standard deviation (n =10), number in the same column followed by the same letter are not significantly different at 0.05 level.

TABLE 4
Sensory scores of mint and stevia hot drinks

Drinks \ Characters	Taste (20)	Flavor (20)	Sweetness (20)	After taste (20)	Color (20)	Overall acceptability (100)
Control (100% Suc + 0% ST)	19.33±0.52[a]	19.33±0.51[a]	19.33±0.51[a]	17.67±3.82[a]	18.67±1.63[a]	94.33±5.08[a]
75% Suc + 25% ST	19.33±0.51[a]	18.84±0.75[ab]	18.83±1.60[a]	18.00±3.03[a]	18.34±1.63[a]	93.34±6.08[a]
50% Suc+ 50% ST	18.17±0.98[ab]	18.50±1.22[ab]	17.83±1.94[ab]	17.00±2.45[a]	18.50±1.87[a]	90.00±6.75[ab]
25% Suc +75% ST	17.16±1.47[b]	17.67±1.96[ab]	17.00±2.36[ab]	16.33±1.75[a]	18.00±2.00[a]	86.17±7.75[ab]
0% Suc +100% ST	15.00±2.00[c]	17.33±2.16[b]	16.17±2.92[b]	15.33±1.86[a]	17.68±2.06[a]	81.51±7.84[b]

ST= Stevia Suc= Sucrose

Each value is expressed as mean ±standard deviation (n =10), number in the same column followed by the same letter are not significantly different at 0.05 level.

TABLE 5
Sensory scores of ginger and stevia hot drinks

Drinks \ Characters	Taste (20)	Flavor (20)	Sweetness (20)	After taste (20)	Color (20)	Overall acceptability (100)
Control(100% Suc + 0% ST)	18.20±1.55[a]	17.40±1.78[a]	17.80±1.55[a]	16.90±2.99[a]	18.40±1.43[a]	88.70±7.21[a]
75% Suc + 25% ST	17.90±2.08[a]	17.60±1.90[a]	17.90±1.73[a]	17.00±2.26[a]	18.30±1.64[a]	88.70±8.01[a]
50% Suc+ 50% ST	17.00±1.70[a]	16.30±2.11[a]	16.90±1.91[ab]	16.20±1.55[ab]	17.80±1.81[ab]	84.20±6.66[ab]
25% Suc +75% ST	15.50±1.51[b]	15.80±2.30[a]	15.60±1.43b[c]	15.90±1.45[ab]	16.90±2.07[ab]	79.30±7.42[bc]
0% Suc +100% ST	14.80±1.03[b]	16.10±2.60[a]	14.70±1.77[c]	14.70±1.77[b]	16.10±3.14[b]	76.40±8.74[c]

ST= Stevia Suc= Sucrose

Each value is expressed as mean ±standard deviation (n=10), number in the same column followed by the same letter are not significantly different at 0.05 level.

TABLE 6
Sensory scores of cinnamon and stevia hot drinks

Characters / Drinks	Taste (20)	Flavor (20)	Sweetness (20)	After taste (20)	Color (20)	Overall acceptability (100)
Control(100% Suc + 0% ST)	19.33± 0.82[a]	19.50± 0.71[a]	19.35±1.33[a]	19.50±0.85[a]	9.00±0.94[a]	96.65±3.57[a]
75% Suc + 25% ST	18.05±2.41[ab]	18.30± 2.41[ab]	17.60±3.06[ab]	17.20±2.86[ab]	17.80±2.89[ab]	88.95±3.32[ab]
50% Suc+ 50% ST	16.60± 2.84[bc]	17.60± 2.41[ab]	16.40±2.99[b]	16.60±2.88[b]	17.30±3.37[ab]	84.50±3.82[b]
25% Suc +75% ST	14..90±2.75[c]	16.35±3.10[bc]	15.10±2.64[b]	14.10±2.96[c]	16.85±2.58[ab]	77.30±1.46[bc]
0% Suc +100% ST	12.50± 3.37[d]	14.80±4.39[c]	12.50±2.95[c]	11.40±2.95[d]	15.80±4.02[b]	67.10±5.78[c]

ST= Stevia Suc= Sucrose
Each value is expressed as mean ±standard deviation (n =10), number in the same column followed by the same letter are not significantly different at 0.05 level.

TABLE 7
Sensory scores of hibiscus and stevia hot drinks

Characters / Drinks	Taste (20)	Flavor (20)	Sweetness (20)	After taste (20)	Color (20)	Overall acceptability (100)
Control(100% Suc +0% ST)	18.70±0.82[a]	18.20±1.75[a]	18.70±0.67[a]	17.80±1.69[a]	19.50±0.71[a]	92.90±2.56[a]
75% Suc + 25% ST	17.75±1.23[ab]	17.80±0.79[a]	17.80±0.63[a]	17.20±1.23[a]	19.60±0.52[a]	90.15±2.71[a]
50% Suc+ 50% ST	17.05±2.06[ab]	18.05±0.83[a]	17.30±1.49[ab]	16.40±1.43[ab]	18.90±1.66[ab]	87.70±5.25[ab]
25% Suc +75% ST	16.20±1.32[b]	17.20±1.39[ab]	16.00±2.11[bc]	15.15±1.33[b]	18.10±2.64[ab]	82.65±6.92[b]
0% Suc +100% ST	13.10±3.07[c]	16.40±1.84[b]	14.60±2.46[c]	13.40±2.72[c]	17.60±2.72[b]	74.50±1.90[c]

ST= Stevia Suc= Sucrose
Each value is expressed as mean ±standard deviation (n =10), number in the same column followed by the same letter are not significantly different at 0.05 level.

Sensory evaluation. The sensory evaluations of hot herbs and stevia drinks (anise, mint or ginger or cinnamon or hibiscus) prepared by substituting 25, 50, 75 and 100% sugar with their equivalent sweetness of stevia are shown in Tables (3-7). Results indicated that there were no significant differences on taste, flavor, sweetness, after test, color and overall acceptability between all herbs and stevia hot drinks and control (100% sugar as sweetener) at 50% substitution with stevia except for taste, sweetens and overall acceptability in cinnamon drinks. It could attribute to their strong test as Pundir et al. [29] suggested that cinnamon and ginger have been defined as plant substances from indigenous or exotic origin, aromatic or with strong taste, used to enhance the taste of foods.

However, significant differences were found in sensory attributes at 75 and 100% substitution with stevia in herbal drinks.

It was observed that different addition of stevia caused variation in drinks color, while the sugar has no effect on herbal drinks color. The leaves of Stevia has functional and sensory properties superior to those of many other high-potency sweeteners, and is likely to become a major source of high-potency sweetener for the growing natural food market in the future [30].

As the results showed, 50% substitution with stevia was the highest acceptable formula followed by 75% compared with control in most examined herb drinks due to the bitter after taste of stevia.

Phenols, flavonoids, tannins and antioxidant activity. Total amount of phenolic, flavonoids, tannin content and antioxidant activity aqueous extracts of herbs is shown in Fig. 1. It could be noticed that the content of total phenolic compounds and flavonoids of herb drinks (hot water extracts) was increased by increasing the substitution level of stevia in herb drinks (water extracts) due to the higher phenolic and flavonoids content of stevia (2830 and 1900, respectively). Abou-Arab and Abu-Salem [26] reported *Stevia rebaudiana* is considered as natural source of antioxidants, which contained phenolic compounds and flavonoids at levels 2401 and 1893 mg100g^{-1} dry weight basis of leaves .

The same figure illustrated that tannin content of herb drinks was increased with increasing stevia replacement level (hot water extract of herbs). The 100% substitution with stevia had highest tannin content compared with control level of all herb drinks.

Regarding to antioxidant activity, the DPPH assay provides basic information on the antioxidant activity of the extracts. The present study was carried out to evaluate the DPPH free radical scavenging activity of herbal water extracts, the highest antioxidant activity was observed in case of 100% replacement with stevia leaves were 86.77, 85.33, 85.98, 87.44 and 88.63% for Anise, mint, ginger, cinnamon and hibiscus, respectively compare with control of each herb drink, that's mean substitution with stevia increased the antioxidant activity of herbal drink and this due to

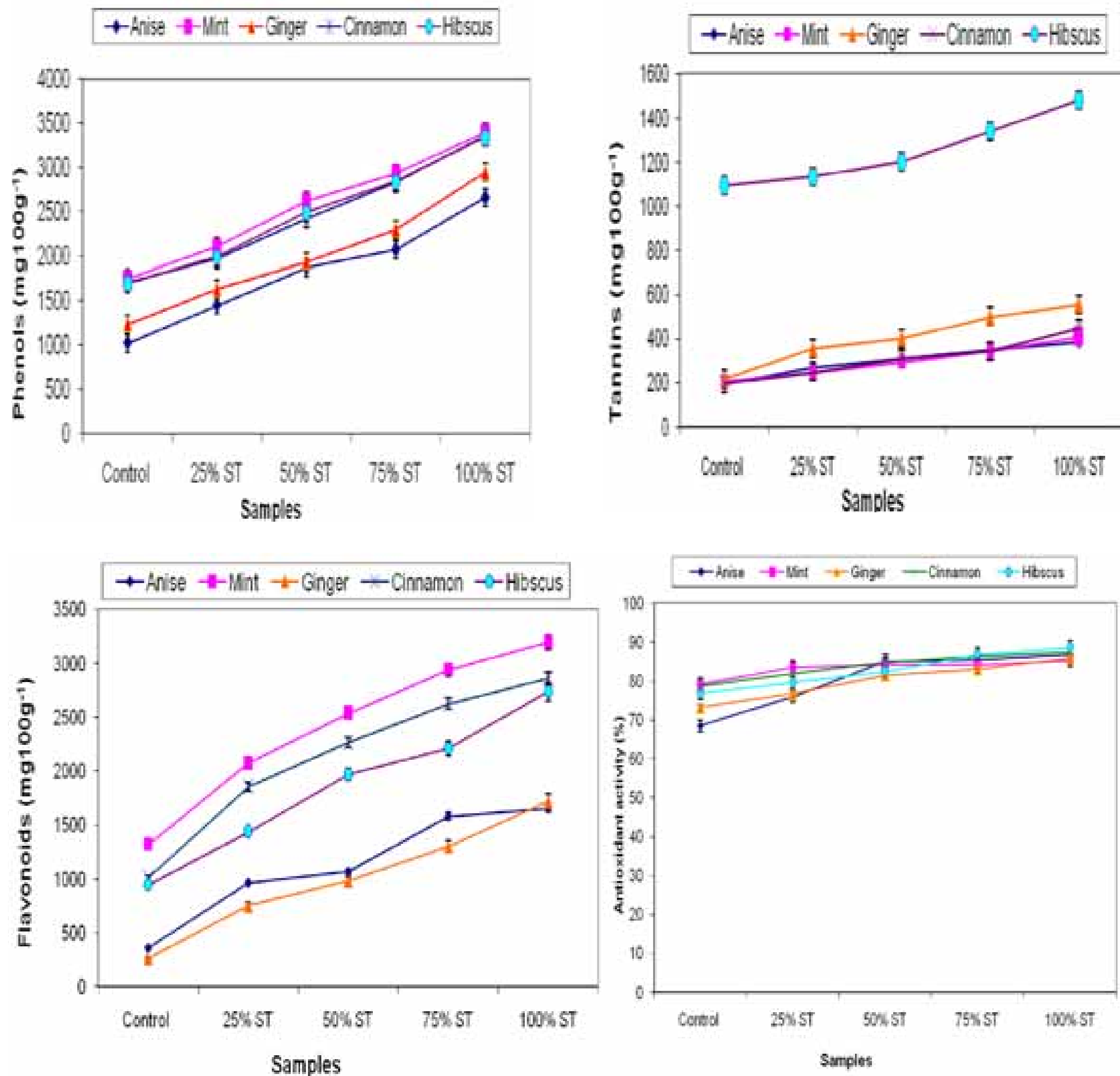

FIGURE 1
Phenols, tannin, flavonoid content and antioxidant activity of different herb drinks.

higher antioxidant activity of stevia leaves (88.70%). Results indicated that stevia has a significant potential to be used as a natural antioxidant and contribute to various food formulations using this property as well apart from sweetening purposes.

CONCLUSION

From the data of the present study, it could be concluded that stevia leaves could be used in sweeten hot - healthy herb drinks up to 75% of the sucrose percent with high phenols, flavonoids, tannin content and antioxidant activity. Moreover, substitution of 50% sucrose with 50% stevia was the highest acceptable formula in most examined herbal drinks (anise, mint or ginger or cinnamon or hibiscus).

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EFFECT OF PRESERVATIVES POTASSIUM METABISULPHITE($K_2O_5S_2$)& SODIUM BENZOATE ($NaC_6H_5CO_2$)ON SEABUCKTHORN PULP

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ABSTRACT

Seabuckthorn (*Hippopheae rhamnoides*) is one of the most important and underutilized fruit crops of the high altitude especially Gilgit-Baltistanregion of Pakistan. It has immense industrial importance because of its medicinal, cosmetic and nutraceutical value.The present study pertains the effect of different concentration of potassium metabisulphite ($K_2O_5S_2$) and sodium benzoate ($NaC_6H_5CO_2$) at overall quality parameters of Seabuckthorn (*Hippopheae rhamnoides*) pulp. Preservative effect of potassium metabisulphite ($K_2O_5S_2$) and sodium benzoate ($NaC_6H_5CO_2$) on proximate/chemical and organoleptic characteristics were tested on Seabuckthorn pulp stored at roomtemperature ranging from -14 to 25ºC from November2014 to April2015 at PCSIR Processing unitSkardu for a period of 6months. Protein and fats decreased, while ash content and Total soluble solid (TSS) increased during the storage period. The pH of pulp decreased while total titrate able acidity increased.Both the preservatives showed controlled effect on total bacterial count up to four months after increasing temperature above 20ºC, a nominal increase of total plate count was observed at pulp preserved with sodium benzoate. Storage time significantly ($p<0.05$) increased the colony forming unit (CFU/g) of the pulp samples as the maximum growth was observed after 5 months of storage.Organoleptic characteristics of thejuice prepared from Seabuckthorn pulpwith preservative have a little negative effect as compared to Seabuckthorn pulp without preservatives however; the samples with preservative were accepted by the judges even after sixmonthsstorage.

KEYWORDS:
Seabuckthorn, pulp,chemical preservatives,storage effect

INTRODUCTION

Seabuckthorn (*Hippopheae rhamnoides* L.) is a one of the important natural resources of the mountainous region of Pakistan.It is widely distributed throughout the temperate zone of Pakistan especially in Gilgit-Baltistan region at an altitude of 1200-4500m.It is deciduous shrub 2-4m high and can survive up to -27°C to 45°C.Total area in Pakistan is 7000 hectares, total production is 5000ton berries/annum. Nutritionally, Seabuckthorn fruit (berries) contain 190 bioactive compounds in seed and pulp. The pulp composition consist of Vitamin C (ascorbic acid) 200-500mg/100g, Vitamin E-180mg/100mg,carotenoids content1167mg/100g, Vitamin K 189mg/100g anthocyanin content 9.69 mg/100g etc.Oil contains phtosterol 4.47 mg/100g and 68-88% unsaturated fatty acids, linolic &linolenic acid are chief components. Seabuckthorn can be suitable forcardiovascular system, hypertension, diabetes, high blood fat contents, cancer therapy, gastric ulcer, liver cirrhosis, skin diseases etc. Seabuckthorn totally wild thesenutritional, medicinal and economical values of this plant attracted a special attention and become an important resource for domestication. The aim of this research study is to exploitation of this important natural resource and to aware the industrialists about chemically preserved seabuckthorn pulp usage. (Zhang et al 1989, Tong et al 1989)

Preservation of pulp with chemical preservatives like potassium metabisulphite ($K_2O_5S_2$) and sodium benzoate ($NaC_6H_5CO_2$) isthe most common and cheapest wayworldwide. These preservatives are used to control thepulp spoilage with microbial growth. Both the chemicals can be usedeffectively in combinations for better preservation. Potassium metabisulphite (PM) and sodium benzoate (SB) alone are not completely effectiveagainst all microorganisms (Chipley.(1983).Due to their better antimicrobial activity both the chemicals are commonly used as preservatives for long term storage of fruit pulp (Sofos. &Busta.(1981) Manganelli.&Casolari, (1983) LuÈcK, 1990). The effect of SB on the growth and survival of some yeast strains, food poisoning and spoilage organisms has been widely reported (Warth. (1985) Sofos *et al*.(1986).

A combined dose of 1500ppm of both chemicals i.e. 1000ppm PM and 500ppm SB showed no sign of micro flora up to 90 days. Use of these preservatives mayhave an adverseeffect on its chemical and sensory profile. It is also reported beforethat pulp stored with chemical preservatives have influence on chemical characteristics of mango pulp, asstorage caused an increase in acidity, brix, reducing sugars and decrease in sucrose (Hussain *et al*., 2003).Dosage of SB has a direct impact on the inhibition of microorganism (Ogiehor& Ikenebomehclearly, 2004) as higher dose of SB manifested the greaterantimicrobial effect, exercised on various species of *Aspergillus* (Gould, 1989;Ogunrinola *et al*., 1996).

According the Codex Standards adopted in 2001 and 2006the maximum levels of preservatives in fruitpulp, purees and fruit is described as 1000 mg/kg $NaC_6H_5CO_2$ as benzoic acid and 500 mg/kg $K_2O_5S_2$ as residual SO2(Codex Standard, 1995). Indiscriminate and non-judicious use of these preservatives is agreat threat to the health and well-being of the consumers and has been the cause of theappearance of resistant microorganisms, leading to the occurrence ofemerging food borne diseases (Gibbons, 1992; Kaur & Arora, 1999; Akinpelu, 2001).The setting of optimum dose of chemical preservatives as well as their individual and synergistic effect on its variousquality attributes under the local conditions in all foods as well as in fruitpulps is a challenge for concern organization.

This research work was conducted in PCSIR Labs Skardu in order to investigate the inhibitory effect of potassium metabisulphite ($K_2O_5S_2$) and sodium benzoate ($NaC_6H_5CO_2$) when usedin various concentrations (0.0ppm, 500ppm, and 1000ppm) for controlling thegrowth and survival of microorganisms in Seabuckthorn pulp stored under ambient conditions for seven months. This research work further explored the adverse effect of these preservatives on chemical, organoleptic characters of Seabuckthorn pulp**.**

MATERIALS AND METHODS

Procurement of Seabuckthorn Berries. Fully ripened and clean Seabuckthorn berries were purchased from District Ghagchae (village Siling) Baltistan on 15th November 2014. The berries were thoroughly washed with berry washer to remove dirt, dust and other material. Commercial grade preservative Potassium metabisulphite ($K_2O_5S_2$) and Sodium benzoate ($NaC_6H_5CO_2$) were purchased from Skardu local market.

Pulp extraction, packaging and storage. The washed berrieswere passed through pulpierto extract pulp and separate the seed, stem and outer cover. The pulp was obtained in stainless steel pans, weighed, preservatives were addedand transferred toa 30litter plastic cane. Before filling the plastic cans werewashed with hot water and rinsed with potassium metabisulphite solution for sterilization this is common practiceof PCSIR Demonstration Cum Training Center (PCSIR DCTC) Skardu as they are working in Seabuckthorn since 1999. Seabuckthorn pulp is being used as basic finished raw material for other value added products development (jam, juice, squash, fruit candies and tonic) the seeds are used for extraction of Seabuckthorn seed oil and outer cover used for the development of Seabuckthorn powder.

The pulp samples were then transferred to pre washed and rinsed with potassium metabisulphite solution plastic cans andstored at room temperature (-12 to +25ºC) in plastic drums for a period of 6 months andthe samples were analyzed for their chemical/proximate composition, organoleptic and microbiological evaluation in duplicate as per standard proceduresafter every two months.

Proximate/Chemical/ analysis of the Seabuckthorn pulp. For the estimation of protein, fat and ash content in stored Seabuckthorn pulp samples following methods were used: microKjeldahl for protein [(N x 5.7) (method 960.52) (Glass Model Pyrex-1)]; incineration

TABLE 1
Proximate/Chemical composition of Seabuckthorn fresh pulp

Brix	11.8%
Moisture content	80.0%
Dry matter	20%
Total sugar	4.79%
Reducing sugar	2.12%
Oil content	1.73%
Protein	1.14%
Ash	0.83%
Titrate able acidity (as malic acid)	1.97%
Fiber	0.075%
PH	3.13
Color	Yellow

TABLE 2
Mineral composition vitamin C and Anthocyanin content of Seabuckthorn fresh pulp

Vitamin C	294mg/100g
Anthocyanin content	9.67mg/100g
K	259mg/litter
Na	47mg/litter
Ca	99.8mg/litter
Mg	198.7mg/litter
Fe	133.2mg/litter
P	123mg/litter

at550°C for ash [(method 923.03)(PCSIR-Lhr)]; defatting in a Soxhlet apparatus(J.P.Selecta–Spain) with 2:1 (v/v) chloroform/methanol for lipids (method 920.39C).Total soluble solids (TSS) were determined directly in each sample by usingrefractometer (Atago 3810-Japan) and expressed as Brix. Acidity and pH determination (Jenway 3510-UK) was carried out by the same methods (Anon., 2000). The experiment was repeated twice and the values are presented as means

Determination of Total Bacterial Count. The total microbial contamination determination of the Seabuckthorn pulp was performed after 60 days until six months by the recommended methodsoutlined in compendium of methods for the microbiological examination of foods (Anon., 1992). The total bacterial count (TBC) determined using Nutrient agar, in the stored Seabuckthorn pulp samples. Nutrient medium was suspended per liter of the distilled water, mixed thoroughly, heated and mixed regularly until nutrient agar became clearly dissolved and autoclaved at 121°C for 15 minutes. One-gram sample was taken from each treatment using aseptic techniques, placed inlabeled sterile dilution bottles and made into a volume of 100 ml by distilled water toachieve 10-1 suspension under sterile conditions. The contents were mixed thoroughlyand aliquots were serially diluted and .

enumerated onto Nutrient agar. Plates wereincubated (Memmert 100-Germany) for 48h at 37°C and TBC wascalculated using colony counter. The experiment was repeated twice and reported datarepresent mean values (CFU/g) of these measurements.

Organoleptic evaluation of Seabuckthorn pulp. Seabuckthorn ready to serve drinks (20ml Seabuckthorn pulp,5 gram sugar and volume made up to 250ml) were prepared from each sample of Seabuckthorn pulp and were presented toa panel of judges for organoleptic/sensory evaluation for color, taste, flavour and overall acceptabilityusing a hedonic scale in accordance with the method described by Larmond (1977). Thepanel members were selected on the basis of their ability to discriminate and scale abroad range of different attributes of Seabuckthorn andSeabuckthorn products. An orientation programwas organized for the panel members to brief them the objective of the study. The drinksamples were served to the panelists for organoleptic/sensory analysis. Thejudges were provided with prescribed questionnaires to record their observation. Theinformation contained on the performa was 9 = Liked extremely; 8 = Liked very much; 7 =Liked moderately; 6 = Liked slightly; 5 = Neither liked nor disliked; 4 = Disliked slightly; 3 =Disliked moderately; 2 = Disliked very much; 1 = Disliked extremely. The panelistsexpectorated the drinks and rinsed mouth using distilled water between samples. Organoleptic/Sensorytesting was made in the office of director completely free of food/chemical odor, unnecessarysound and mixing of daylight. The experiment was repeated twice and the values arepresented as means (SD±).

Statistical analysis. Data were statistically analyzed, using analysis of variance technique (Steel *etal*., 1997). Duncan's Multiple Range Test was applied to assess the difference betweenmeans (Duncan, 1955). Significance was defined at $p \leq 0.05$. Values are means of twoexperiments (SD±).

TABLE 3
Treatment combinations (ppm) of Sodium benzoate and Potassium metabisulphite inSeabuckthorn pulp

Treatment	Sodium benzoate ($NaC_6H_5CO_2$)	Potassium metabisulphite ($K_2O_5S_2$)
T_0 (control)	--	--
T_1	**1000**	--
T_2	--	**500**
T_3	**500**	**500**
T_4	**500**	--
T_5	--	**1000**

TABLE 4
Treatments effect on chemical composition of chemically preserved Seabuckthorn pulp

Treatment	Protein (%)	Fat (%)	Ash (%)	TSS (Brix)	Acidity	pH
T_0	0.56±0.013**z**	0.62±0.013**z**	0.35±0.006**y**	18.50±0.30	0.68±0.031**z**	3.80±0.086
T_1	0.50±0.023**x**	0.60±0.014**zy**	0.39±0.009**z**	18.19±0.47	0.63±0.024**zy**	3.83±0.062
T_2	0.52±0. 020**yx**	0.56±0.020**y**	0.37±0.007**zy**	18.02±0.40	0.61±0.021**yx**	3.84±0.081
T_3	0.50±0.024**x**	0.58±0.017**y**	0.35±0.006**y**	18.13±0.39	0.61±0.022**yx**	3.84±0.070
T_4	0.55±0.015**zy**	0.59±0.016**zy**	0.37±0.006**zy**	17.88±0.36	0.58±0.018**x**	3.89±0.057
T_5	0.49±0.024**x**	0.58±0.018**y**	0.37±0.008**y**	18.23±0.34	0.60±0.021**yx**	3.83±0.091

Means (± SD) sharing similar subscripts in a column are statistically non-significant ($p<0.05$)

RESULTS AND DISCUSSION

Proximate/chemical analysis of the Seabuckthorn pulp. Incorporation of chemical preservatives potassium metabisulphite ($K_2O_5S_2$) and sodium benzoate (NaC6H5CO2)exhibited a significant ($p<0.05$) effect on proximate/chemical profile of Seabuckthorn pulp (Table 4).Addition of 500ppm of ($NaC_6H_5CO_2$) alone did not show any effect on protein content of thesamples while rest of the combinations (Table 5) decreased protein content. Benzoic acid atvarious concentrations exerted no effect on lipid content; however SO_2 was able todecrease the fat content in Seabuckthorn pulp samples. ($NaC_6H_5CO_2$) at the concentration of 1000ppmit increased ash content while Total Soluble Solids (TSS) content of all the treated samples remained unchanged.The results revealed that benzoic acid only increased the acidity of Seabuckthorn pulp with acorresponding decrease in pH value of the samples (Table 4).

Storage time had shown a pronounced effect on proximate/chemical attributes ofchemically preserved Seabuckthorn pulp. Progressive decrease in protein and fat content of pulpsample was observed over the entire storage period of 180 days (Table 5). Similarly, asignificant ($p<0.05$) decreasing pattern in ash content was noticed when the sampleswere tested after every 60 days. Total Soluble Solids (TSS) was not affected up to a period of 120 days storage,however, the increase in TSS was apparent in the last month of the storage period. Abouthalf of the soluble sugars of mango pulps are mainly composed of fructose, with about30% sucrose and 20% glucose. The high sugar content of pulps from ripe fruits might beattributed to the transformation of starch into soluble sugars under the action ofphosphorylase enzyme during ripening (Germain & Linden, 1981; Favier *et al.*, 1993).Amin*et al.*, (2008) reported the effect of the time of fruit harvest on most of the fruitquality attributes. The authors showed a significantly higher TSS value, measured in thefruit harvested at noon as compared to other times of the day.

Addition of preservatives $K_2O_5S_2$ and $NaC_6H_5CO_2$ increased the acidity of the Seabuckthorn pulp samples until 120days and no further change in acidity could be observed in the last month. Interestingly,the pH of the stored pulp samples decreased concomitantly for 180 days but thedifferences remained non-significant (Table 5). Germain *et al.*, (2003) howevercorrelated the changes in lipid profile of Seabuckthorn pulp samples with the state of ripening ofthe Seabuckthorn berries. Abbassi *et al.*, (2009) attributed the increase in pH and the decrease intitratable acidity with increased storage time of the berries.

The results pertaining to the increase in acidity and decrease in pH during storage ofSeabuckthorn pulp were in complete agreement with other researchers (Fulya *et al.*, 1999; Doreyyapa*et al.*, 2001). The increase in acidity might be ascribed to rise in theconcentration of weakly ionized acid and their salts during storage. This increase inacidity might also be due to formation of acid by degradation of polysaccharides andoxidation of reducing sugars or by breakdown of pectic substances and uronic acid (Hummel & Okay 1950; Iqbal *et al.*, 2001; Hussain *et al.*, 2008).ThepH

TABLE 5
Storage effect on chemical composition of chemically preserved Seabuckthorn pulp

(Days)	Protein (%)	Fat (%)	Ash (%)	TSS(Brix)	Acidity	pH
0	0.61±0.009**z**	0.67±0.007**z**	0.35±0.004**y**	17.38±0.30**y**	0.51±0.009**x**	3.93±0.064
60	0.54±0.008**y**	0.57±0.009**y**	0.38±0.006**z**	18.01±0.30**zy**	0.62±0.012**y**	3.85±0.054
120	0.49±0.008**x**	0.58±0.009**y**	0.37±0.005**z**	18.40±0.23**zy**	0.66±0.013**z**	3.81±0.058
180	0.45±0.014**w**	0.55±0.008**y**	0.38±0.008**z**	18.90±0.32**z**	0.68±0.016**z**	3.77±0.059

Means (± SD) sharing similar subscripts in a column are statistically non-significant ($p<0.05$)

plays dual role in the fruit juices by acting as a flavour promotion andpreservative factor. Decrease in pH of the fruit pulp samples proportional to increase inacidity has been
confirmed by several researchers and might be attributed to the presenceof SB in the pulp samples (Bajwa *et al.*, 2002; Hussain *et al.*, 2008).

Microbiological evaluation of the Seabuckthorn pulp. The data inTable 8 revealed inhibitory effects of$K_2O_5S_2$ and $NaC_6H_5CO_2$ on the microbial growth of the Seabuckthorn pulp at concentrations commonlyused in the food industry. The highest inhibitory effects on bacterial growth in Seabuckthornpulp samples were exerted by $K_2O_5S_2$ alone at a concentration of 1000ppm followed bycombination of $K_2O_5S_2$ and $NaC_6H_5CO_2$ at 500ppm each. Increasing $NaC_6H_5CO_2$ concentration from 500ppm to 1000ppm reduced the growth velocity to two times and 500ppm of $K_2O_5S_2$was shown to be equally effective as compared to 1000ppm of the $NaC_6H_5CO_2$ (Table 8). Theresults of the present study also demonstrated an inhibitory effect of $K_2O_5S_2$ and $NaC_6H_5CO_2$ inSeabuckthorn pulp stored for a period of six months.

Table 8 indicated the bacterial growth pattern at various time intervals and thesystematic increase in CFU/g of the Seabuckthorn samples, corresponding to the storage time. The growth was found to be highest at the termination of the storage period which mightbe attributed to the variability in the temperature and the chemical changes, specificallyalteration in pH of the system that would take place resulting from the presence of the chemical preservatives ($K_2O_5S_2$ and $NaC_6H_5CO_2$) in the samples.

Inhibitory effect of $K_2O_5S_2$ and $NaC_6H_5CO_2$ in specifiedcombinations and concentration levelsin relation to storage periods have been presented in Table 8. The highest level ofcontamination in Seabuckthorn pulp samples was observed in control (no preservative added) after 180 days of storage while the minimum growth was shown in the presence of 1000ppm$K_2O_5S_2$. Periodical analysis of the Seabuckthorn pulp samples for the total bacterial count (TBC) showed aprogressive increase in the growth all though the rate of growth varied with differenttreatment combinations
with exception of 1000ppm of $K_2O_5S_2$ where the growthremained stagnant for 120 days, suggesting the $K_2O_5S_2$ to be relative inhibitor in Seabuckthorn pulp. The results of the present study substantiated that none of the two preservatives ($K_2O_5S_2$ and $NaC_6H_5CO_2$) were able to completely inhibit the bacterial growth in the specified concentrationsfor a period of 180 days storage; however, the preservative had been synergistically activein inhibition of the microbial growth in the Seabuckthorn pulp samples.

A previous study on inhibitory effects of 1000ppm $K_2O_5S_2$ confirmed the results of the present study suggesting that $K_2O_5S_2$ is more effective against the microbial growth of the stored Seabuckthorn pulp samples at 1000 ppm(Hussain *et al.*, 2003).

Organoleptic/Sensory evaluation of Seabuckthorn pulp. Organoleptic/sensory evaluation of the ready-to-serveSeabuckthorn drinks prepared from the treatedSeabuckthorn pulp samples was carried out for color, flavour,taste and overall acceptability. It was evident that addition of chemical preservatives ($K_2O_5S_2$ and $NaC_6H_5CO_2$) greatlyinfluenced these attributes with a little loss in pulp quality (Table 6). The resultspertaining to the effect of addition of chemical preservatives ($K_2O_5S_2$ and $NaC_6H_5CO_2$) to Seabuckthorn pulp were presentedin Table 6. Concentration and synergistic addition of $K_2O_5S_2$ and $NaC_6H_5CO_2$seem to have no effecton their ability to act differently regarding deteriorating the color, flavour and overallacceptability of stored Seabuckthorn pulp. However, the exception was noticed in taste scoresshowing greater variability in relation to treatment combination and the concentration ofthese chemical compounds (Table 6).

Periodical analysis for colour scores manifested a drastic decline ($p<0.05$) in the firstmonth of storage which did not alter in the last five months. The panelist's clearlyidentified the changes in flavour profile of the samples rating the stored sample inferioras compared to the freshly prepared drinks. Storage time perpetually

TABLE 6
Treatments effect on organoleptic/sensory characteristics of preserved Seabuckthorn pulp

Treatment	Colour	Flavour	Taste	Acceptability
T_0	7.24±0.174**z**	7.24±0.141**z**	7.00±0.170**z**	6.74±0.121**z**
T_1	6.74±0.182**y**	6.24±0.150**y**	5.74±0.174**w**	6.00±0.170**y**
T_2	6.24±0.167**y**	6.24±0.141**y**	6.24±0.141**yx**	6.00±0.153**y**
T_3	6.74±0.202**y**	6.24±0.279**y**	6.53±0.220**y**	6.24±0.259**y**
T_4	7.24±0.174**z**	6.24±0.150**y**	6.00±0.177**xw**	6.00±0.135**y**
T_5	6.49±0.201**y**	6.49±0.220**y**	5.74±0.167**w**	6.00±0.170**y**

Means (± SD) sharing similar subscripts in a column are statistically non-significant ($p<0.05$)

TABLE 7
Storage effect on organoleptic/sensory characteristics of preserved Seabuckthorn pulp

(Days)	Colour	Flavour	Taste	Acceptability
0	7.66±0.138**z**	7.49±0.128**z**	7.32±0.115**z**	7.16±0.095**z**
60	6.66±0.142**y**	6.32±0.120**y**	6.32±0.100**y**	6.32±0.105**y**
120	6.49±0.145**y**	6.00±0.135**x**	5.82±0.135**x**	6.00±0.117**x**
180	6.32±0.125**y**	6.00±0.140**x**	5.35±0.088**w**	5.16±0.057**w**

Means (± SD) sharing similar subscripts in a column are statistically non-significant ($p<0.05$)

TABLE 8
Inhibitory effect of different preservatives (CFU/g) during six months storage at room temperature of Seabuckthorn pulp

Treatment	Storage time (days)			
	0	30	60	**90**
T_0	24.00±0.50**mn**	101.00±5.32**ef**	354.00±8.19**b**	515.00±14.7**a**
T_1	20.00±0.79**mn**	41.00±0.52**kl**	83.00±2.87**gh**	96.00±6.04**efg**
T_2	23.00±0.53**mn**	50.00±2.39**jk**	91.00±5.30**fg**	108.00±2.20**e**
T_3	17.00±0.59**mn**	21.00±1.08**mn**	52.00±0.82**jk**	60.00±2.30**ij**
T_4	23.00±0.49**mn**	71.00±1.08**hi**	187.00±9.66**d**	216.00±4.90**c**
T_5	10.00±0.21**n**	15.00±0.22**mn**	10.00±0.30**n**	28.00±0.44**lm**

Means (± SD) sharing similar subscripts in a column are statistically non-significant ($p<0.05$)

decreasedflavor score until 180 days storage, nevertheless, the drink samples were still liked by the judgesfor colour and flavour (Table 7). The maximum deterioration was noticed in theSeabuckthorn pulpsample for taste and overall acceptability as a function of storage for five months. Auniform pattern of decline in these sensory attributes of the Seabuckthornpulp samples wasevident in relation to storage time, albeit the samples were not rejected instantaneously fortaste and overall acceptability at any time period (Table 7).

Numerous studies have confirmed the effect of adding chemical preservatives andsubsequent storage at room temperature of the fruit pulp samples on the organolepticcharacteristics. One study revealed that pulp could be preserved up to one year at ambienttemperature by adding $K_2O_5S_2$ alone (1200ppm) or in combination with $NaC_6H_5CO_2$(600ppmeach) without a significant loss of sensory properties of the fruit pulp (Sonia *et al.*, 2003).Other studies (Kapse *et al.*, 1995 ; Mercadante & Rodriguez, 1998) verified theresults of the current research reporting notable changes in sensory characteristics(appearance, smell and flavour) of the pulp fruit during ripening which altered itsphysico-chemical profile by inter-conversion of the starch to soluble sugar, decrease inacidity, carotene content, modification in texture and conversion of protopectin to pectin.

In another study, pulp of *chaunsa* mango tested for various sensory attributes wasrated highly acceptable to the judges (Akhtar *el al.*, 2009). A similar study conducted on the sensory evaluation of chemically preserved mango pulpsamples confirmed that all the samples tried for organoleptic evaluation were satisfactoryup to 270 days of storage at ambient temperature (Hussain *etal.*, 2003).

CONCLUSIONS

This study suggested that potassium metabisulphite ($K_2O_5S_2$) and sodium benzoate ($NaC_6H_5CO_2$) applied synergistically at concentrations of 500ppm each had been equally effective as1000ppm $K_2O_5S_2$ used individually in inhibiting bacterial growth. Further the chemical preservativeshad significantlyincreasedtheacidity and corresponding decrease in pH during storage for six months. Addition ofpotassium metabisulphite ($K_2O_5S_2$) and sodium benzoate ($NaC_6H_5CO_2$) adversely influenced the sensory attributes of the stored pulp however; theproduct remained acceptable after six months of storage.

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IMPROVING THE ROASTING PROCESS AND EFFICIENCY OF HAZELNUT

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ABSTRACT

The total amount of world production of hazelnut is mainly around 800.000 tons/year and 75% produced by Turkey. Hazelnut is a crucial and non-equivalent material for praline production, which is semi-finished product of chocolate industry. Roasting of hazelnut is an energy intensive process which commonly involved drying, sterilization, and enhancing of total palatability. Product quality depends on the methods of process. Therefore, it is important to determine the suitable methods. The purpose of this study are the presentation of various possible types of dryer/roaster, tips for creating better competition conditions for industrial practitioners and investors, and making some influences on product quality of roasted hazelnut.

KEYWORDS:
Hazelnut, roasting, process, dryer, efficiency

HIGHLIGHTS

Suitable dryers for hazelnut roasting; Tray cabinet dryer, Belt conveyor dryer, Rotary dryer, and Fluidized bed dryer were introduced. Effects of roasting and expected properties were discussed. Desirable features of the roasted hazelnut were listed. Some outstanding features in fluidized bed dryer over other conventional dryer were also listed.

INTRODUCTION

Hazelnut, which is one of the main materials for praline, is undispensible for chocolate industry, Figure 1. Roasting is an essential process for hazelnut and roasted product is preferred by manufacturer because of extension of shelf-life, disposal of aflatoxin and enhancing of total palatability. Flavour can be improved due to Maillard reaction which occurs during roasting. Maillard reaction is possible in two different conditions. One is at room temperature, which occurs in long time (few months). Second is at high temperature in short time. Main effect of roasting is dehydration of the kernel. Additionally, sterilization, which occurs above 120 ºC, is very imported for food industry. The temperature and duration of the process is a vital issue. Blanching, which is commonly made by rotating brushes immediately after roasting, is a closely related and combined process to the roasting. Blanching means skin removal for hazelnut. Both process are energy-intensive and directly influence product quality. The selection of dryers and improving of process efficiency which must be solved carefully and attentively appears to be matter because of depleted fossil resources, greenhouse effect and global warming, modifying and improving consumer behaviour.

Generally, the roasting process is performed by dryers which are not specially built for hazelnut roasting but other purposes. Fundamentally, one of the main and most important issues is drying during roasting of hazelnut. The other issues are change of colour, flavour, texture, and nutritional value. Thus, these type of dryers do not suitable to arrange special conditions such air velocity, heat intensity etc for improving product quality. Drying is an essential operation in wide range of industries such as the chemical, agricultural, biotechnology, food, polymer, ceramics, pharmaceutical, pulp and paper, mineral processing, and wood processing industries. Over 400 types of dryers have been reported whereas over 100 distinct types are commercially available [1]. The basic objective of food dehydration is to remove water to a level at which microbial spoilage is minimized in order to extend the shelf-life of the product. The second objective of drying is to produce dried foods of good quality from a nutritional and organoleptic standpoint [2].

a) in husk b) shelled d) unshelled

e) roasted f) roasted and blanched

FIGURE 1
Hazelnut in different stages

Today, the dehydration sector of the food industry is huge and extends to all aroun the world. Drying facilities range from simple sun or hot air dryers to high capacity, sophisticated spray drying or freeze drying installations. A very large range of dehydrated foods is available and makes a significant contribution to the convenience food market, commercially [3].

Conventional dryers have well-established records of performance for drying most materials. However, they may not be optimal in terms of energy consumption, quality of dried product, safety in operation, ability to control the dryer in the event of process upsets, ability to perform optimally even with large changes in throughput, ease of control, and minimal environmental impact due to emissions or combustion of fossil fuels used to provide energy for drying [4]. Classification of the dryer, structure and properties of hazelnut, and the parameters of roasting process have been given in the following sections. Dryers used for roasting process in industrial scale, and some comparative informations about them has been mentioned as well.

Drying. Food dehydration is a combined heat and mass transfer process, involving handling of solids and particles. The design of industrial dryers in food processing is based mainly on practical experience, since handling and processing of solid and semi-solid food materials cannot be described adequately by mathematical models and computer simulations[5]. Besides, in order to select proper dryer, one must have detailed information about them and detailed investigation of heat and mass transfer phenomenon occurs during drying process.

Dryer classification. Dryer are classified by following different methods;

-Type of operation: batch, continuous

-Type of feed:: liquid, suspension, paste, granules, fibrous solids, porous solids, dense solids, and sheets

-Heating method: convection, contact, radiation, dielectric

-Product sensitivity (vacuum, low temperature) [5].

Dryer can be named as follow; Indirect Dryers, Rotary Dryers , Fluidized Bed Dryers, Drum Dryers, Industrial Spray Dryer, Freeze Drying, Microwave and Dielectric Dryer, Solar Dryer, Spouted Bed Dryer, Impingement Drying, Pneumatic and Flash Drying, Conveyor Dryers, Infrared Drying, Superheated Steam Drying, and Special Drying Techniques and Novel Dryers [1].

Due to the wide variety of material characteristics and the diversity of dried foodstuffs, variety of types of dryers are used in the food industry. Typical dryers and representative applications in food processing are summarized in Table 1 [2]. Batch dryers are used for solids as well as hazelnut throughputs (flows) below 50 kg/h, while continuous operation is preferred above 1000 kg/h. [5].

TABLE 1
Typical dryers and representative applications in food processing.

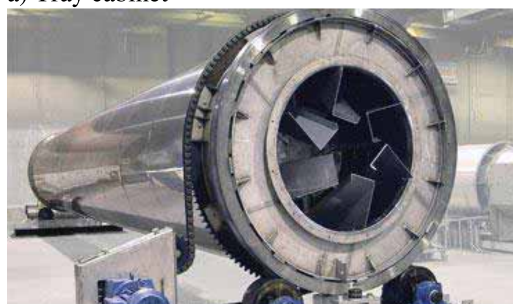
Dryer Type	Product Application
Tray or cabinet	Fruit vegetable, meats and confectionary
Tunnel	Fruits, vegetables
Belt conveyor	Grains, vegetables, fruits, cereals, nuts
Rotary	Seeds, grains, starch, sugar crystals
Pneumatic or flash	Starch, pulps, crops, granules, powders
Fluid bed	Vegetables, granules, grains, peas
Spray	Milk, cream, coffee, tea, juices, eggs, extracts, syrups
Drum	Milk, soups, flakes, baby cereals, juices, purees
Foam mat	Fruit juices and purees
Puffing	Fruits and vegetables
Freeze	Flakes, juices, meat, shrimp, coffee, vegetables, extracts

Drying periods. In general, drying process is involved four stages such as heating, constant drying rate, first decreasing drying rate, and second decreasing drying rate periods. The drying process of kernel which come at low moisture content into the dryer, however, consist of three stages; heating, first decreasing drying rate, second decreasing drying rate stages. The occurring of second decreasing drying stage depends on the final moisture content of the kernels [6].

Varieties of dryer for hazelnut roasting. Hazelnut, a non-hygroscobic porous material with high oil content, is accepted as grains. Therefore, it is very sensitive for lipid oxidation which can cause toxic compounds such as aflatoxin, and acrylamide. Traditionally, tray-cabinet, belt conveyor, and rotary dryer have been used for hazelnut roasting fluidized bed dryer is practised recently (Figure 2). Metal body structure of the dryers, and increasing touching of the product with the metal can cause lipid accumulation on the kernel surface which reduces shelf-life, possibly increase to aflatoxin formation. On the other hand, increasing the solid wood structure on the touching surfaces of dryers may solve this problem and produces better flavour on the final product.

a) Tray cabinet

b) Belt conveyor



c) Rotary

d) Fluidized bed

FIGURE 2
Suitable dryers for hazelnut roasting [7].

Tray-cabinet dryer. Cabinet dryers are the most popular equipment for fruit drying. One of the drawback of this dryer can be non-uniformity of moisture content on end product. These dryers are simple in structure, low in installation cost, and can be employed in almost all environmental conditions [8]. In this basic type of indirect dryers, the material to be dried is placed in pans or trays on the hollow shelves, which are heated by the heating medium. Tray dryers are relatively small batch units for drying small quantities of product [5]. The trays are generally metal to ensure good heat transfer between the trays and the shelves [1]. It can vary in size from a bench-scale unit holding one or two small trays of food to a large unit taking stacks of large trays. The air may be directed by baffles to flow the across surface of the trays of food or through perforated trays and the layers of food, or both ways [3].

In conventional cabinet dryers, hot air is usually introduced from under first tray (bottom tray) and passes through the other trays sequentially. Therefore, the kernels located on the bottom trays would be exposed the highest energy and could be over dried, while the upper trays may not receive enough energy to be dried due to increase in drying air relative humidity and decrease in air velocity and temperature [6]. For heat-sensitive materials drying can take place at low temperature under vacuum application to reduce the boiling point of the liquied to be removed. Vacuum is applied to the drying chamber and vapor is removed through an exhaust pipe connected to the chamber [1].

Belt conveyor dryer. The conveyor dryer is conceptually very simple. Product is carried through the dryer on conveyors and hot air is forced through the bed of product [1]. Belt (conveyor) dryers are used widely in food processing for continuous drying. In belt dryers, the feedstock is spread on a moving perforated conveyor in order to dry the products in a continuous process [9].

In this type of drier, the food products are conveyed through the drying tunnel on a perforated conveyor, made of hinged, perforated metal plates, wire or plastic mesh. The heated air usually flows through the belt and the layer of food, upward in the early stages of drying and downward in the later stages, and vice versa [3].

Industrial conveyor-belt dryers are the most popular type for removing moisture from agricultural products [9]. Since the air temperature and velocity may be set to different levels in each stage, the use of multistage conveyor drying is more preferable. Minimizing heat demage on the product may be possible by exercising exact control over the drying process. On the other hand, drying in this type of drier is relatively expensive because of using two or more stages [3].

In general, the conveyor dryer is the best suited for drying of particulate materials in the 1- to 50-mm diameter range [1]. Belt dryers are more convenient to take advantage of low-grade and waste heat because they operate at lower temperatures than rotary dryers [9].

Rotary dryer. Comparing the other types of dryers, conventional rotary dryers are used across a wide range of industries because of their simplicity and their flexibility to handle a wider range of solid materials [10], [11]. They consist basically of a cylindrical shell inclined at a small angle downward to the horizontal [11]. The process uses a continuous rotary drum in which the wet material is tumbled, or mechanically turned over, usually in the same direction, while hot air with regulated temperature is blowing continuously [12]. Internal set of flights lift the particles through the air stream, while the drum rotates slowly at 4–5 rpm [2, 3]. The direction of gas flow through the cylinder relative to the solids is decided mainly by the properties of the processed material [1]. Rotary dryers are less expensive than belt dryers, but they cannot handle large food pieces, which may be damaged by mechanical abrasion during tumbling [5].

Fluidized bed dryer. Fluidized bed drying is an efficient and most successful drying technique among the different drying systems in the food industry [13], [14]. Fluidized bed dryers are used commonly for fast drying of food products and particles that can be suspended in a stream of hot air. Increased drying rates are obtained due to high heat and mass transfer [5]. Two different types of fluidized bed are used in food industry as a batch and continuous fluidized bed dryers. A batch fluidized bed dryer is used when production capacity required is small (normally 50 to 1000 kg/h) [1], and continuous fluidized bed dryer is used for larger capacities.

The known advantages of fluidized bed drying are as follow;

-High rate of moisture removal, high thermal efficiency,

-Easy material transport inside dryer,

-Ease of control,

-Low maintenance cost.

Limitations of fluidized bed dryer include;

-High pressure drop,

-High electrical power consumption,

-Poor fluidization quality of some particulate products

The type of distributor has to be chosen carefully to ensure uniform and stable fluidization [1]. The charecteristic of different selected dryer is given in Table 2 [2].

TABLE 2
Charecteristic of suitable dryer for hazelnut roasting

Dryer type	Evaporation capacity kg/m^2h	Energy comsumption kJ/kg	Thermal efficiency %
Tray or cabinet	0,1-1	3000-4500	50-80
Tunnel and conveyor	5-18	4000-6000	35-60
Rotary	30-120	3500-6000	40-70
Fluidized bed	30-90	3100-6000	40-80

Effects and expected properties of roasting. Roasting at high temperatures may change the moisture level, color, texture, and flavor of the nuts. Exposure to high temperatures for elongated time can cause some toxic compound such as acrylamide; especially drying starchy food products. Moisture level can be reduced until equilibrium moisture related to the process temperature. Required moisture level in dry basis for roasting effect is below 3 %. However, to develope crunchiness and crispiness of the product, and to enhance total palatability, the moisture content should be reduced below 1%. Desirable features of the roasted hazelnut can be listed as follows;

-low moisture content,
-long shelf-life,
-crispiness,
-crunchiness,
-improvement of total palatability,
-not-causing of undesired colour changes,
-insignificant reduction of nutritional value of the product,
-destroying of allergenic substances,
-destroying mold bacteria such as aflatoxin.
-prevent acrylamide formation

Dehydration develops a crispy and crunchy texture [15]. At the same time, the native microstructure of the nuts is damaged and the cell compartments, as well as the oleosomes, are destroyed causing intracellular oil diffusion and increasing porosity[16], [17].

Free amino acids, peptides, fatty acids, vitamin E, phytosterols and lignans are found to be changed during roasting process. By roasting, enzymes that cause nutrient loss can be inactivated and undesirable microorganisms, toxins or allergens and food contaminants can be destroyed, and the pellicules of hazelnut kernels can be removed [18]–[21].

The effects of roasting process on the hazelnut have been studied by various researchers given below.

Amaral et al, (2006) have been searched the effects of different temperatures and (125-200 °C) and times of exposure (5, 15, and 30 min)[22]. They noticed minor changes in the fatty acid and triacylglycerol compositions. As temperatures and roasting periods increased, generally, a modest increase of oleic and saturated fatty acids and a decrease of linoleic acid, expressed as relative percentages, occurred. Similarly, an increase of triacylglycerols containing oleic acid moieties and a decrease of those containing linoleic acid moieties were found in the roasted samples. Roasting caused a modest decrease of the beneficial phytosterols (maximum 14.4%) and vitamin E homologues (maximum 10.0%) and a negligible increase of the trans fatty acids [20].

Alamprese et al (2009) reported that thermal processing and roasting conditions used had a lesser effect on the individual phenolic composition of the kernel and thus roasted and unroasted hazelnuts without skin contain comparable amounts of health promoting compounds[16]. It should be emphasized that hazelnut is a rich source of tocopherols, which largely survive the roasting thermal treatments [16], [22].

The effect of roasting on taste-active components of 18 native hazelnut varieties, grown in the Giresun province of Turkey, was assessed by Alasalvar et al (2010)[23]. In their work, the samples were examined for their sugars, organic acids, condensed tannins, and free phenolic acids. Six sugars (fructose, glucose, sucrose, myo-inositol, raffinose, and stachyose), seven organic acids (oxalic, maleic, citric, malic, lactic, succinic, and acetic), and one phenolic acid (gallic acid) were positively identified in natural and roasted hazelnut varieties; among these, sucrose, malic acid, and gallic acid predominated, respectively. Total sugars among hazelnut varieties ranged from 1.99 to 4.94 g/100 g, organic acids from 0.96 to 2.72 g/100 g, condensed tannins from 3.99 to 40.56 mg of catechin equivalents/g, and gallic acid from 0.159 to 0.871 mg/100 g. Differences existed in the sugar and organic acid contents between natural and roasted hazelnut varieties, but they did not follow any particular trend. Significant losses ($p < 0.05$) in condensed tannins (approximately 97.3%) and gallic acid (approximately 66.7%) were noted when the hazelnuts were roasted. The present work suggests that roasting resulted in significant loss in condensed tannins and gallic acid due to the removal of the brown skin. The effect of roasting on sugars and organic acids was not noteworthy [23].

Alasalvar et al. (2003) presented that the natural (raw) and roasted hazelnuts were compared for their differences in volatile components and

sensory responses [24]. A total of 79 compounds were detected in both hazelnuts, of which 39 (27 positive, 5 tentative, and 7 unknown) were detected in natural hazelnut and 71 (40 positive, 14 tentative, and 17 unknown) were detected in roasted hazelnut. These included ketones, aldehydes, pyrazines, alcohols, aromatic hydrocarbons, furans, pyrroles, terpenes, and acids. Pyrazines, pyrroles, terpenes, and acids were detected in roasted hazelnut only. Concentrations of several compounds increased as a result of roasting and these may play significant roles in the flavor of roasted hazelnut. Pyrazines together with ketones, aldehydes, furans, and pyrroles may contribute to the characteristic roasted aroma of hazelnut. Descriptive sensory analysis (DSA) showed that some flavor attributes such as "aftertaste", "burnt", "coffee/chocolate-like", "roasty", and "sweet" were rated significantly higher in roasted hazelnut compared to its natural counterpart. Natural and roasted hazelnuts can be distinguished using these attributes [24].

Burdack-Freitag and Schieberle (2010) detect 46 aroma compounds in roasted and 37 odor-active compounds in the raw nuts from Italian hazelnut varieties [25].

Colour and colour uniformity are vital components of visual quality of fresh foods and play a major role in consumer choice. For low temperature processes such as chilling, freezing or freeze-drying, the colour changes little during processing. For more severe processing, the colour may change markedly during the process. The texture of raw materials is frequently changed during processing. Textural changes are caused by a wide variety of effects, including water loss, protein denaturation which may result in loss of water-holding capacity or coagulation, hydrolysis and solubilisation of proteins. Flavour is a rather subjective property which is difficult to quantify, and may be altered during processing [3].

Thermal changes in the hazelnut microstructure developed gradually with increasing air temperature, air velocity and roasting time in the roasting proces. Roasting causes some degrees of cell wall separation and intercellular spaces, partial disruption of cytoplasmic network, swollen and aggregated protein bodies. These changes were observed in the microstructure of extremely liked quality of hazelnuts (roasted at 165 C, 1 m/s, 25 min). Hazelnut microstructure was related with hazelnut texture such that the increase in the volume of intercellular spaces caused an increase in crispiness and crunchiness [26].

DISCUSSION

Commonly, roasting of hazelnut has been carried by tray cabinet dryer among small size enterprises or by conveyor band dryer among big sizes enterprises for enormous capacities until today. Final product quality was of secondary importance, because ideal mixing of kernels, monitoring and control of process parameters are very complicated in tray cabinet dryers. Although it is possible to get better conditions in conveyor band dryer, the negative effects of directly touching of the kernel with burned gas should not be ignored. Additionally, providing of free competition seems very difficult in conveyor band dryers.

Mainly, roasting process is a non-enzimatic browning because of low moisture content (5-6%) at the entry of dryer [26]. The highness and the duration of roasting temperature influence the color change of hazelnut extremely. Therefore, process duration at temperatures higher than 150 ºC should be strictly limited during roasting. Commonly, applied roasting temperature at first stage is slightly above 150 ºC, and slightly below 145 ºC at the second stage of the process. First roasting stage is mostly performed for skin fracturing. In order to establish sterilization, the temperature above 120 ºC even in most critical inner parts of the product (around the kernel cavity) should be provided for a sufficient long duration. On the other hand, acrylamide is formed in the Maillard reaction by the reaction of the free asparagines and reactive carbonyls (e.g. reducing sugars) at temperatures above 120 ºC [7], [17]. Acrylamide has neurotoxic and carcinogenic properties and classified as probably carcinogenic to humans [17], [27]. All these mentioned points should be considered during process design, and establishment of the automation systems. Moreover, fullfillment of cooling and skin removal, which must be followed adjacent to roasting, should be targeted together simultaneously.

Heat and mass transfer occurs simultaneously during the drying of porous materials. While the heat is transferred from the kernel surface to the inner cavity, mass (moisture) is tranferred in opposite direction from the inner parts to the surface. Using of continuously internal circulated hot air may cause accumulation of high moisture content in air. Thus, the dragging of the moisture from the surface of hazelnut by hot air may be negatively effected, which also caused a worsening effect on the final product quality. Therefore, it should be expected to use the systems running at high rate of fresh air. Blanching is a relative combined process of the roasting; therefore this should be performed within the process. Another important parameter of system selection is being suitable for all sizes enterprises. Thus, small and medium-sized enterprises may have chance of starting and surviving in the sector. Hence, more creative ideas may arise by the improved competition condition. On the other hand, consumption of hazelnut, which is a rich food

source of folic acid, zinc, and vitamin E may be enlarged and expanded.

interaction influence the final product quality. Therefore, these are issues for further studies.

CONCLUSION

The effects of different temperature and duration of roasting process on variety of hazelnut have been given by above researchs. However, the effects of different types of dryer on roasting process have not been investigated sufficiently. Optimization of process parameters, however, is depend on the detailed analyses of dryer for roasting obviously.

It is essential and crucial to promote the production of healthy, nutritious, and good-quality product. In the meantime, compliance to food regulations should be ensured. When using new methods, rehabilitation of existing methods should not be neglected. Preferring of energy-efficient processes, which can help to provide sustainability, should be among the first priorities. In this point, fluidized bed dryer presents strong future perspectives for hazelnut roasting. Some outstanding features in fluidized bed can be counted as follows;

- Applying of both batch and continue system for roasting process
- Performing of roasting and blanching at a single process,
- Shorter process time,
- Facility of batch system (decreasing lost during breakdown)
- Construction possibility from very small to big size capacity
- To possible to work with 100% fresh air by taking of measures for efficient heat recovery

As mentioned earlier drying process is commonly involved four categories such as heating, constant dying rate, first decreasing drying rate, and second decreasing drying periods. The constant drying rate period is not occurring during hazelnut roasting because of low moisture content of sun-dried hazelnut by farmers. An ideal roasting process should be taking into account all the drying periods and regulation possibility of them. Roasting and skin removal (blanching) are very close and mutual-influencing unit operations for hazelnut. Therefore, the proper dryer should respond most of the expectations mentioned above. Actually, a proper dryer may be a combination from tray cabinet (optimum fracturing performance of skin optimal), fluidized bed (self-implementing skin removal) , and fixed bed (better regulation of process parameters such as air velocity and duration). Consequently, a compact dryer design for hazelnut roasting must fulfill all the expectation for the final product and in meantime, it shoul be energy efficient. The material and kind of the dryers, particle-particle interaction, and particle-gas

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BIOCHEMICAL CHANGES ASSOCIATED WITH POLY-Γ-GLUTAMIC ACID SYNTHESIS DURING SPONTANEOUS AND *BACILLUS SUBTILIS* FERMENTATATION OF *PARKIA BIGLOBOSA* SEED INTO *IRU*

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ABSTRACT

This work investigated biochemical changes associated with poly-γ-glutamic acid (γ-PGA) production during fermentatation of Parkia biglobosa seed. The pH, microbial count, γ-PGA concentrations and amino acid profiles of spontaneous fermented (SF) and Bacillus subtilis fermented (BSF) Parkia biglobosa seed were determined. The pH, microbial load, and γ-PGA concentrations were monitored daily until day 4, while the amino acid profiles and calculated amino acid quality were assessed on day 0 and 3. The pH ranged from 6.63 to 9.37 for SF and 6.47 to 9.73 for BSF, while the microbial loads of SF and BSF were in the range 1.03 x10^7 - 2.2x10^9 cfu/g and 1.10x10^7 - 5.97x10^9 cfu/g respectively. The γ-PGA concentrations increased from 0 to16.38 gkg^{-1}for SF and 0 to14.61 gkg^{-1} for BSF. The most abundant essential amino acid (EAA) was leucine (6.23 g/100gcp) while the least was tryptophan (0.65 g/100gcp). The limiting amino acids for BSF_3 are methionine and tryptophan, while those for SF_3 are methionine, tryptophan and leucine. The percentage range of different group of amino acids are, essential with histidine, 33.04 - 36.17 %; essential without histidine, 30.65 - 32.99 % ; non-essential, 44.18 - 47.82 %; neutral, 36.01 - 36.66 %; acidic, 34.2 - 37.21 %; basic 14.01 - 14.73 %; sulphur, 1.97 - 2.31 %; and aromatic , 7.62 - 8.32 %. The results showed that the fermentation of Parkia biglobosa seeds was accompanied with increase in pH, poly-γ- glutamic acid concentrations and improved protein quality.

KEYWORDS*:*
Parkia biglobosa seed, fermentation, biochemical changes, poly-γ- glutamic acid, iru.

INTRODUCTION

Fermentation is one of the oldest methods of food preservation known to man. In Africa, the art of fermentation is widespread including the processing of fruits and other carbohydrate sources to yield alcoholic and non-alcoholic beverages, and sour-tasting *ogi* - the fermented cereal product, which provides instant energy in breakfast and convalescent diets [1]. Oil seeds and legumes such as African locust bean, melon seed, castor oil seed, and soybean are also fermented to give condiments which are known to contribute to calories and protein intake. The condiments are generously added to soups as low-cost meat substitute by low-income families in parts of Nigeria [2].

The fermentation of oil seeds and legumes is usually carried out in a moist solid state, involving contact with appropriate inocula of assorted microorganisms and is accomplished by the natural temperatures of the tropics. One of the microorganisms whose inoculum could be used for solid state fermentation is *Bacillus subtilis*.

Bacillus subtilis is a Gram-positive, member of the genius *Bacillus,* rod-shaped, catalase-positive bacterium [3] and has the ability to form a tough, protective endospore, allowing the organism to tolerate extreme environmental conditions. There have been reports on the use of *Bacillus subtilis* as one of the components of starter culture for fermentation of *Parkia biglobosa* seed [4] to a tasty food condiment called *iru* which is used as a flavour enhancer for soups and stews in Nigeria [2, 5] and other developing and under developed countries of the world where there is high cost of animal protein. The desired state of fermentation of the condiments is indicated by the overtones of ammonia produced as a result of the breakdown of amino acids and formation of whitish mucilage during the fermentation [6]. The major component of the mucilage secreted during such solid state fermentations as soybean and African locust bean has been identified to be poly-γ-glutamic acid (γ-PGA) [7]. Several strains of *Bacillus subtilis* that are responsible for biosynthesis of γ-PGA have been isolated and characterized from different fermented vegetable products. These include *Bacillus subtilis* (*natto*) from *natto* and *Bacillus subtilis (chungkookjang)* from *chungkookjang* [8].

γ-PGA is a polymer produced outside the cells by the producing microorganisms [9]. γ -PGA consists of D-and /or L-glutamate connected by amide bonds

between γ -carboxyl and α-amino acid groups. Its molecular weight ranges from 10 to 2000 kDa [10]. γ -PGA is water-soluble and anionic, and confers osteoporosis-preventing and mineral (calcium) absorption properties on the food containing it [8].

This study focuses on the production of γ-PGA during the fermentation of *Parkia biglobosa* seeds, considering its health significance in such fermented legume products as *iru* and dearth of this information in the literature.

MATERIALS AND METHODS

The isolation and identification of Bacillus subtilis from the fermented product

Commercially available *iru* prepared using traditional method was purchased from a local market at Akure in western Nigeria. About 10 g of the sample was homogenized with 90 ml of sterile distilled water to give the 10^{-1} dilution level. From this, dilution levels of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} were made and cultured on Nutrient Agar (NA) (Biomark laboratories, India) using pour plate technique. Subsequent streaking on plates and slants was done to obtain pure culture of the isolated *Bacillus subtilis*. After identification using appropriate morphological and biochemical tests as described by [11], *Bacillus subtilis* was used in the subsequent fermentation of prepared seed of *Parkia biglobosa* for 96 h at 37 ^{0}C.

Inoculum production. A loop-full of *Bacillus subtilis* from the slant was used to inoculate 5-ml sterile Nutrient Broth (NB) (Biomark laboratories, India) in McCartney bottle and incubated at 37 ºC for 18 h, then the culture obtained was used to inoculate 50 ml NB in 250 ml flasks, and incubated for 18 h. Culture was centrifuged using Stuart microcentrifuge, at 6,000 x g for 3 mins., and cells' pellets were washed in sterile distilled water and centrifuged again and re-suspended in 5ml sterile distilled water to obtain *Bacillus subtilis* inoculum. Cell density of the inoculum was determined by mixing 1 ml of the inoculum with 9 ml of sterile distilled water to give the 10^{-1} dilution level. From this, dilution levels of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} were made and cultured on NA using pour plate technique in such that the inoculum contained 8.4 x 10^8 cells/ml [12].

Pure culture fermentation of Parkia biglobosa seeds. *Parkia biglobosa* seeds were fermented as previously described [12]. Dried seeds were soaked in water (ratio 1: 5 w/v) for 2 h, autoclaved at 121 ^{o}C for 15 min. and dehulled manually. Clean 300 g cooked cotyledons were portioned into each of two aluminium containers with paper laminated aluminium foil lid and sterilized in autoclave at 121 ^{o}C for 15 min. and allowed to cool to room temperature. One was opened and exposed to the atmosphere for 3 h to be inoculated with natural microflora-wild fermentation (SF), while the other was inoculated with 2.5 ml of pure culture of *Bacillus subtilis* under sterile conditions using sterile syringe and thoroughly mixed to give final concentration of 7.0 x 10^6 cells/g-*Bacillus subtilis* fermentation (BSF). Fermentation was carried out at 37 ºC for 96 h, with 24-hourly monitoring of pH, total viable count and concentration of poly-γ-glutamic acid. Amino acid profiles of the samples with highest concentrations of poly-γ-glutamic acid were also determined.

Determination of pH. Exactly 10 g portion of the sample was homogenized with 90 ml of distilled water. The pH of the homogenate was measured using Eco Testr pH_1 digital pH meter [11].

Determination of total microbial counts. Exactly 10 g sample was taken at 24-hourly intervals and homogenized in 90 ml 0.8 per cent saline sterile distilled water. From the 10^{-1} dilution, other decimal dilutions (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}) were prepared. The total viable count was determined by pour plate method, using NA as the medium. Plates were incubated at 37 ^{0}C for 18 h, and examined for growth [11].

Determination of poly-γ-glutamic acid γ-PGA) concentration. The sample was treated by the method reported by. Exactly 5 g sample was taken for poly-γ-glutamic acid water extraction. Cells and solid particles were separated by decantation and centrifugation using a micro-centrifuge (Stuart, UK) at 10,000 x g for 25 min. and the supernatant was mixed gently with four volumes of absolute cold ethanol. After centrifuging again at 10,000 x g for 15 min., the sediment was collected and dissolved in deionized water and made up to 1 ml, and then any insoluble contaminants were removed by centrifugation at 10,000 x g for 25 min. 0.1 ml of the solution was diluted with 1.9 ml of deionized water to obtain 2 ml of γ-PGA solution which was used for γ-PGA concentration determination using UV visible spectrophotometer (Schimadzu 1800, USA). Standard solution (500 µg/ml) of γ-PGA (Kem Light, India) was prepared from which a series of working standards (50–300 µg/ml) were prepared by diluting standard solution with deionized water and subsequently used for constructing calibration curve [13].

Determination of amino acid composition. The amino acid composition of samples was determined using AOAC methods [14]. Dried and pulverized sample was made to be free of water by ensuring constant weight for a period of time. 10 g sample was weighed into 250 ml conical flask capacity. The sample was defatted by extracting the

$$Amino\ acid\ score = \frac{\text{amount of amino acid per test protein } \left(\frac{g}{100gcp}\right)}{\text{amount of amino acid per protein in reference pattern } \left(\frac{g}{100gcp}\right)}$$

fat content of the sample with 30 ml of petroleum spirit three times with soxhlet extractor that was equipped with thimble. The sample was hydrolyzed three times for complete hydrolysis to be achieved for the totality of amino acids recovery. The pulverized and defatted sample was soaked with 30 ml of the 1M potassium hydroxide solution and was incubated for 48 hours at 110 ^{0}C in hermetically closed borosilicate glass container. After alkaline neutralization of the hydrolysate to a pH in the range of 2.5-5.0. The solution was purified by cation-exchange solid-phase extraction. The amino acids in the purified solution were derivatised with ethlchloroformate. The derivatising reagent removed by scavenged with nitrogen gas for proper mop up of the excess reagent. The derivatised amino acids that are free of derivatizing reagent was made up to 1 ml in a vial for free amino acid analysis using gas chromatography-mass spectrophotometer (GC-MS).

The essential amino acid scores were also calculated using the formula above:

[15]. It was also calculated based on whole hen's egg [16].

Statistical analysis. The data obtained were subjected to statistical analysis using the SPSS 20 software (IBM, New York, USA).

RESULTS AND DISCUSSION

The colony of the isolate selected as *Bacillus subtilis* was large and spreading which differentiated it from a very closely related *Bacillus* species, *Bacillus licheniformis* whose colony is small and distinct. The isolate whose growth in Hugh-Leifson's medium was aerobic, was Gram positive, rod shaped, spore forming, acid producing on sugar fermentation, catalase and methyl red positive. Citrate utilization test further differentiated the isolate (negative) from *Bacillus licheniformis* (positive). *Bacillus subtilis* has been identified as one of the major desirable fermentative organisms in some legumes [17, 4] including *Parkia biglobosa* seed.

Table I shows the changes in pH during the fermentation period. The pH of the fermenting seeds increased till third day after which the pH either decreased for SF or remained constant for BSF. The pH values for SF on days 0 and 1 were higher ($p \leq 0.05$) than those of BSF on the same days. The pH ranged from 6.47 to 9.67 which indicated strong alkaline fermentation. This agrees with findings of Aderibigbe *et al.* [12] for *Parkia biglobosa* seed, Enujiugha *et al.* [11] for *Pentaclethra Macrophylla* Benth seed and Ogueke *et al.* [18] for *ogiri egusi*. It has been established that during fermentation there is breaking down of proteins to amino acids by proteases and deaminase activity by the fermenting bacteria [19, 20]. These activities resulted in ammonia production and consequently increase in pH.

The total viable counts in the fermenting *Parkia biglobosa* seeds during the fermentation period are shown in Table II. The total viable counts for SF increased from 1.03×10^7 to 2.20×10^8 cfug^{-1} while those for BSF increased from 1.10×10^7 to 5.97×10^9 cfug^{-1} within 3 days only and later decreased. The microbial counts for SF were only significantly higher ($p \leq 0.05$) than BSF in days 2 and 3.The increase in the counts implies that the seed cotyledons have the required substrates for the growth and multiplication of the fermentative organisms.

Other workers have observed increases in microbial counts during fermentation of plant protein sources [20, 21].

The γ-PGA concentrations of SF and BSF (Table III) increased till day 3 with maximum concentrations of 16.38 and 14.61 gkg^{-1} of sample, respectively after which they decreased. However, there was no significant difference ($p \geq 0.05$)

TABLE 1
The changes in pH during the fermentation of *Parkia biglobosa* seed

Fermentation Period /days	SF	BSF
0	6.63 ± 0.06^{a1}	6.47 ± 0.59^{a2}
1	7.97 ± 0.15^{b1}	7.07 ± 0.15^{b2}
2	8.57 ± 0.06^{c1}	8.67 ± 0.06^{c1}
3	9.67 ± 0.06^{e1}	9.73 ± 0.06^{d1}
4	9.37 ± 0.12^{d1}	9.73 ± 0.06^{d1}

Values are means of triplicate determinations (mean ± standard deviation)
Means on the same column with different superscript letter are significantly different ($p \leq 0.05$), and means on the same row with different superscript number are significantly different ($p \leq 0.05$)

TABLE 2
Total viable counts of fermenting organisms (cfu/g) in the samples

Fermentation Period /days	SF	BSF
0	$1.10x10^{7a1}$	$1.03x10^{7a\,1}$
1	$3.20x10^{7a1}$	$2.06x10^{7b1}$
2	$5.03x10^{8c2}$	$1.07x10^{8c1}$
3	$5.97x10^{9d2}$	$3.44x10^{7d1}$
4	$3.00x10^{8b1}$	$2.20x10^{8e1}$

Values are means of triplicate determinations (mean ± standard deviation)
Means on the same column with different superscript letter are significantly different ($p \leq 0.05$), and means on the same row with different superscript number are significantly different ($p \leq 0.05$)

between the two values of concentration. A similar trend was reported by Xiaoyu *et al.* [21] for *Bacillus amyloliquefaciens* C1 fermentation of substrate mixture of dairy manure compost and monosodium glutamate production residues for γ-PGA production. The decreases in concentrations after day 3 might be caused by γ-PGA degradation by γ-PGA depolymerise [22] and [23]. This suggests that fermentation of *Parkia biglobosa* seed should be allowed for 3 days for maximum γ-PGA production.

The amino acid profiles of SF_0, SF_3, BSF_0 and BSF_3 are presented in Table IV. SF_0 and SF_3 are spontaneous fermented samples taken on day 0 and 3 respectively, while BSF_0 and BSF_3 are *Bacillus subtilis* fermented samples taken on day 0 and 3 respectively. The result showed that the non-essential amino acids content of the samples ranged between 4.19±0.01 mg/100g for serine and 15.74±0.11 mg/100g for aspartic acid in SF_0; and 4.03±0.02 mg/100g for serine and 16.39 ±0.10 mg/100g for aspartic acid in SF_3; 4.21±0.03 mg/100g for serine and 16.37±0.01 mg/100g for aspartic acid in BSF_0; and 4.43±0.03 mg/100g for serine and 17.00±0.11 mg/100g for aspartic acid in BSF_3. For the conditionally essential amino acids (TCEA), the values ranged between 1.08±0.02 mg/100g for cystein and 4.67±0.01 mg/100g for glycine in SF_0; 1.26±0.06 mg/100g for cystein and 5.03±0.02 mg/100g for glycine in SF_3; 0.86±0.01 mg/100g for cysteine and 4.6±0.05 mg/100g for arginine in BF_0; and 1.22±0.01 mg/100g for cysteine and 5.30±0.06 mg/100g for arginine in BSF_3. The essential amino acid values ranged between 0.60±0.01 mg/100g for tryptophan and 5.81±0.01 mg/100g for leucine in SF_0; 0.71±0.00 mg/100g for tryptophan and 6.48±0.02 mg/100g for leucine in SF_3; 0.58±0.11 mg/100g for tryptophan and 5.58±0.11 mg/100g for leucine in BSF_0; and 0.72±0.00 mg/100g for tryptophan and 7.04±0.14 mg/100g for leucine in BSF_3. Nutritionally, the recommended daily allowances (RDA) of all the essential amino acids except methionine and tryptophan were adequately met by BSF_3, and SF_3 met RDA of all the essential amino acids except methionine, tryptophan and leucine; while BSF_0 and SF_0 met RDA of valine, isoleucine, phenylalanine and histidine only.

Table V presents calculated essential, non - essential, basic, acidic, neutral, aromatic and total sulphur amino acids, leucine/isoleucine ratio, leucine and isoleucine difference (g/100gcp). The essential amino acids were further analyzed with and without histidine. Histidine is important for the synthesis of red and white blood cells. It is a precursor for histamine which is good for sexual arousal and improved blood flow. High dosage of histidine however increases stress and anxiety [24]. BSF_3 had the highest value for the total essential amino acid (TEAA) with histidine (349.2 mg/gcp) while BSF_0 had the least value (274.3 mg/gcp). BSF_3 had the highest total non –

TABLE 3
The changes in γ-PGA concentration (gkg^{-1} sample) during the fermentation of *Parkia biglobosa* seed

Fermentation Period /days	SF	BSF
0	0.00 ± 0.00^{a1}	0.00 ± 0.00^{a1}
1	6.12 ± 0.10^{b1}	3.48 ± 0.34^{b1}
2	10.69 ± 1.46^{c1}	5.57 ± 0.00^{c2}
3	16.38 ± 2.88^{d1}	14.61 ± 0.19^{d1}
4	16.24 ± 1.19^{d1}	14.24 ± 0.04^{d1}

Values are means of triplicate determinations (mean ± standard deviation)
Means on the same column with different superscript letter are significantly different ($p \leq 0.05$), and means on the same row with different superscript number are significantly different ($p \leq 0.05$)

TABLE 4
The Amino Acid Composition (g/100 gcp) of Spontaneous (SF_0 and SF_3) and *Bacillus subtilis* (BSF_0 and BSF_3) fermented *Parkia biglobosa* Seed

Amino acids	SF_0	SF_3	BSF_0	BSF_3	*RDA
Non essential amino acids (TNEAA)					
Ala	4.59 ± 0.04^{c}	4.98 ± 0.05^{b}	4.59 ± 0.04^{c}	5.21 ± 0.04^{a}	-
Asp	15.74 ± 0.11^{b}	16.39 ± 0.10^{b}	16.37 ± 0.01^{b}	17.00 ± 0.11^{a}	-
Ser	4.19 ± 0.01^{bc}	4.03 ± 0.02^{c}	4.21 ± 0.03^{ab}	4.43 ± 0.03^{a}	-
Glu	15.50 ± 0.14^{a}	15.85 ± 0.03^{a}	14.48 ± 0.22^{b}	16.02 ± 0.01^{a}	-
Conditionally essential amino acids (TCEA)					
Pro	4.54 ± 0.04^{ab}	4.78 ± 0.12^{ab}	4.42 ± 0.07^{b}	4.92 ± 0.04^{a}	-
Gly	4.67 ± 0.01^{b}	5.03 ± 0.02^{a}	3.58 ± 0.01^{c}	5.30 ± 0.06^{a}	-
Arg	4.67 ± 0.04^{a}	4.65 ± 0.03^{a}	4.60 ± 0.05^{a}	4.73 ± 0.01^{a}	2
Cys	1.08 ± 0.02^{ab}	1.26 ± 0.06^{a}	0.86 ± 0.01^{b}	1.22 ± 0.01^{a}	-
Tyr	2.40 ± 0.09^{ab}	2.75 ± 0.06^{ab}	2.37 ± 0.00^{b}	2.80 ± 0.02^{a}	-
Essential amino acids (TEAA)					
Lys	5.51 ± 0.02^{b}	5.94 ± 0.01^{a}	5.00 ± 0.05^{c}	6.42 ± 0.12^{a}	5.8
Thr	3.27 ± 0.03^{b}	3.74 ± 0.03^{ab}	2.88 ± 0.01^{c}	3.81 ± 0.04^{a}	3.4
Val	3.93 ± 0.02^{b}	4.12 ± 0.06^{ab}	4.00 ± 0.07^{b}	4.36 ± 0.07^{a}	3.5
Met	0.77 ± 0.01^{b}	0.88 ± 0.01^{a}	0.77 ± 0.01^{b}	0.93 ± 0.00^{a}	2.2
Ile	3.86 ± 0.08^{b}	3.99 ± 0.07^{ab}	3.23 ± 0.07^{c}	4.27 ± 0.03^{a}	2.8
Leu	5.81 ± 0.01^{b}	6.48 ± 0.02^{a}	5.58 ± 0.11^{b}	7.04 ± 0.14^{a}	6.6
Phe	4.21 ± 0.00^{ab}	4.25 ± 0.03^{a}	3.38 ± 0.01^{b}	4.30 ± 0.04^{a}	2.8
His	2.43 ± 0.10^{b}	2.87 ± 0.02^{ab}	2.02 ± 0.05^{c}	3.07 ± 0.10^{a}	1.9
Try	0.60 ± 0.01^{b}	0.71 ± 0.00^{a}	0.57 ± 0.00^{b}	0.72 ± 0.00^{a}	1.1

Values are means of duplicate determinations (mean ± standard deviation)
Means on the same column with different superscript letter are significantly different ($p \leq 0.05$)
SF_0-spontanenous fermentation for 0 day, SF_3-spontanenous fermentation for 3 days, BSF_0- Bacillus subtilis fermentation for 0 day and BSF_3 – Bacillus subtilis fermentation for 3 days, RDA-Recommended Daily Allowance.
*source of RDA: [15]

TABLE 5
Calculated amino acid quality of spontaneous (SF0 and SF3) and *Bacillus subtilis* (BSF0 and BSF3) fermented *Parkia biglobosa* Seed

Amino Acids	SF_0	SF_3	BSF_0	BSF_3
Total amino acid [TAA]	87.77	92.70	82.91	96.55
Total non-essential amino acid [TNEAA]	40.02	41.25	39.65	42.66
Total essential amino acid [TEAA] + His	30.39	32.98	27.43	34.92
Total essential amino acid [TEAA] - His	27.96	30.11	25.41	31.85
% TNEAA	45.60	44.50	47.82	44.18
% [TEAA + His]	34.62	35.57	33.04	36.17
% [TEAA – His]	31.86	32.48	30.65	32.99
Total neutral amino acid [TNAA]	31.61	33.63	28.78	35.40
% TNAA	36.01	36.29	34.71	36.66
Total acidic amino acid [TAAA]	31.24	32.24	30.85	33.02
% TAAA	35.60	34.78	37.21	34.20
Total basic amino acid [TBAA]	12.61	13.46	11.62	14.22
% TBAA	14.37	14.52	14.01	14.73
Total sulphur amino acid [TSAA]	1.85	2.14	1.63	2.15
% TSAA	2.11	2.31	1.97	2.23
% Cys in TSAA	58.38	58.88	52.76	56.74
Total aromatic amino acid [TArAA]	7.21	7.71	6.32	7.82
% TArAA	8.21	8.32	7.62	8.10
Leu/Ile ratio	1.51	1.62	1.73	1.65
Leu – Ile	1.95	2.49	2.35	2.77

Values are means of duplicate determinations (n=2)
TAA-Total Amino Acids, TNEAA -Total Non-Essential Amino Acids, TEAA- Total Essential Amino acids, HIS – Histidine, TNAA-Total neutral amino Acids, TNAA-Total neutral amino Acids, TAAA-Total Acidic Amino Acids, TBAA -Total Basic Amino Acids, TSAA-Total Sulphur Amino Acids, TArAA-Total Acidic Amino Acids, Cys-Cystine, Leu- Leucine, Ile-Isoleucine.

TABLE 6
Amino acid whole hen's egg scoring pattern for spontaneous (SF_0 and SF_3) and *Bacillus subtilis* (BSF_0 and BSF_3) fermented *Parkia biglobosa* Seed (EAA g/100gcp).

Sample	Lys	Thr	Val	Met	Ile	Leu	Phe	His
[a] Standard(mg/gcp)	6.20	5.10	7.50	3.20	5.60	8.30	5.10	2.40
SF_0	0.89	0.64	0.52	0.24	0.69	0.70	0.83	1.01
SF_3	0.96	0.73	0.55	0.28	0.71	0.78	0.83	1.20
BSF_0	0.81	0.56	0.53	0.24	0.58	0.67	0.66	0.84
BSF_3	1.04	0.75	0.58	0.29	0.76	0.85	0.84	1.28

[a] Source: [27]

TABLE 7
Amino acid chemical scores for spontaneous (SF_0 and SF_3) and *Bacillus subtilis* (BSF_0 and BSF_3) fermented *Parkia biglobosa* Seed (EAA g/100gcp)
(EAA g/100gcp)

Sample	Lys	Thr	Val	Met	Ile	Leu	Phe	Try	Met+Cys	Phe+Try
[b]Standard (mg/gcp)	55	40	50	35	40	70	60	10	35	60
SF_0	1.00	0.82	0.79	0.22	0.97	0.83	0.70	0.60	0.53	0.80
SF_3	1.08	0.94	0.82	0.25	1.00	0.93	0.71	0.71	0.61	0.83
BSF_0	0.91	0.72	0.80	0.22	0.81	0.80	0.56	0.57	0.47	0.66
BSF_3	1.17	0.95	0.87	0.27	1.07	1.01	0.72	0.72	0.61	0.84

[b] Source: [14]

essential amino acid value of 426.6 mg/gcp which was comparable to 407 mg/gcp for Kidney bean seed [25], and to 341 mg/gcp for fermented cocoa nibs [26], while BSF_0 had the lowest value of 396.5 mg/gcp. The content of TSAA was lower than the recommended values for children (5.8 g/100gcp) [27]. The TArAA ranged from 6.32 g/100gcp for BF_0 to 7.82 g/100gcp for BSF_3. This is within the range suggested for an ideal protein (6.8-11.8 g/100 gcp). The % CYS in TSAA ranged from 56.74 % for BSF_3 to 58.88 % for SF_3, this is higher than previously reported value of 44.4% for *Parkia biglobosa* [28]. Cysteine has a positive effect on mineral absorption, especially zinc [29]. In overall, BSF_3 had the highest essential amino acid profile, followed by SF_3, SF_0, and BSF_0, suggesting that fermentation has the potential of improving the amino acid content of *Parkia biglobosa* seeds.

Table VI presents the essential amino acid scoring pattern for SF_0, SF_3, BSF_0 and BSF_3 (EAA g/100gcp). The limiting amino acid was methionine, while the most abundant was lysine. The values of other amino acids were also considerable implying that the vegetables are good sources of protein and amino acids. Methionine has been reported to be the limiting amino acid in *P. mildbraedi* and fermented/non-fermented cocoa nibs [30].

Table VII shows the chemical score for the essential amino acid for SF_0, SF_3, BSF_0 and BSF_3 (EAA g/100gcp). Methionine was further confirmed to be the limiting amino acid while the most abundant was lysine. Generally, all the samples had high values of amino acids and this indicates that they possess high biological protein value. The results from this study greatly reveal the need to encourage the consumption of iru in rural households towards solving the pervading protein-energy malnutrition that currently give many third world governments serious concern. This is a great step towards exploiting an underutilized forest seed for improved food and nutrition security [31].

CONCLUSION

Bacillus subtilis and spontaneous fermentation of *Parkia biglobosa* seed were accompanied by increase in pH, titratable acidity and microbial counts. γ-PGA production was at maximum on the third day of fermentation. Fermentation improved amino acid composition of *Parkia biglobosa* seed with BSF_3 having the highest amount of essential amino acids.

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