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Effect of Fermentation Time on the Phenolic, Flavonoid and Vitamin C Contents and Antioxidant Activities of Okra (*Abelmoschus esculentus*) Seeds

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ABSTRACT

The objective of this study was to evaluate the effect of fermentation on the phenolic, vitamin C and total flavonoid contents and antioxidant properties of okra seed. The okra seeds were removed from the pod of matured and fibrous okra that cannot be easily cut with kitchen knife, and was allowed to undergo chance fermentation for 120 h. Samples were taken for antioxidant analysis at 24 h, 72 h and 120 h, respectively. The aqueous extracts of fermented and unfermented okra seeds were obtained and subsequently used for the analysis. The phenolic, vitamin C and total flavonoid contents and the antioxidant properties (ferric reducing antioxidant power (FRAP) and 1, 1-diphenyl-2 picrylhydrazyl (DPPH) free radical-scavenging ability) of the extracts were determined. The results revealed that fermented okra seeds had significantly ($p < 0.05$) higher phenolic content, vitamin C, total flavonoid and non-flavonoid contents and showed greater antioxidant activities than unfermented okra seed. Okra seeds fermented for 24 h exhibited the highest ferric reducing antioxidant power of 980 mgAAE/100 g and the least IC_{50} of 2.27 mg/ ml for DPPH free radical-scavenging ability than the others. Okra seeds fermented for 24 hours had significantly ($p < 0.05$) higher antioxidant activities. The product from fermented okra seeds could be used for the production of functional foods.

Keywords: Okra, phenolic, flavonoid, ferric reducing antioxidant power (FRAP) and DPPH Free radical-scavenging ability.

Introduction

Increased concern over the safety of synthetic antioxidants like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) has led to an increased interest in exploration of effective and economical natural antioxidants (Iqbal *et al*., 2005). Natural polyphenols exert their beneficial health effects by their antioxidant activity, these compounds are capable of removing free radicals, chelate metal catalysts, activate antioxidant enzymes, reduce α-tocopherol radicals and inhibit oxidases (Oboh, 2006).

Okra *(Abelmoschus esculentus L.)* is widely consumed as a fresh vegetable in both temperate and tropical countries. However, several studies have reported that okra is also useful for its seeds. In Turkey, the seeds of mature okra are roasted, ground and used as a coffee substitute (Çalisir *et al*., 2005). The seed powder has also been used as a substitute for aluminium salts in water purification (Camciuc *et al.,* 1998). In an earlier study, Karakoltsides and Constantinides (1975) found that the protein

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efficiency ratio (PER) of okra seed flour heated at 130°C for 3 h was not different from the non-heated flour, indicating the absence of anti-nutritional factors. According to these authors, the amino acid composition of okra seed protein is similar to that of soybean and the PER is higher than that of soybean. Moreover, okra seeds are a good source of protein and oil. The oil of okra seed appears to be as good as cottonseed oil (Aminigo and Akingbala, 2004). Savello *et al*. (1980) reported that okra seed oil is rich in unsaturated fatty acids such as linoleic acid which is essential for human nutrition. Okra seed is known to be rich in high quality protein especially with regards to its content of essential amino acids relative to other plant protein sources (Otunola *et al*., 2007). It is a good source of total phenol as reported by Adetuyi and Komolafe (2011). Its addition to predominantly carbohydrate foods therefore may potentially enrich such foods and considerably improve its nutritional status (Otunola *et al.*, 2007). Okra seed flour has been used to supplement 'ogi' and plantain flour (Aminigo and Akingbala, 2004; Otunola *et al.,* 2007; Adetuyi and Adelabu, 2011). The addition of okra seed flour to plantain flour caused a significant improvement in the nutritional and antioxidant properties of the plantain flour (Adetuyi and Adelabu, 2011; Adetuyi and Komolafe, 2011). Food processing method of defatting had caused a reduction in the antioxidant properties of okra seed flour (Adetuyi and Komolafe, 2011).

There is dearth of information on the effect of fermentation on okra seeds. This work was carried out to investigate the effect of fermentation on the antioxidant content and properties of okra seeds.

Materials and Methods

The okra used were freshly harvested from a farm in 'Ogbese' via Akure – Owo road, Ondo State; they were mature, fibrous okra that could not be easily cut with a kitchen knife. They were identified and authenticated in Agricultural Technology Department of Rufus Giwa Polytechnic, Owo, Ondo State, Nigeria. All the chemicals used were of analytical grade, while the water was glass distilled.

Reagents

Ascorbic acid was from Merck (Darmstadt, Germany), gallic acid and quercetin were from Aldrich (Steinheim, Germany), 1,1-diphenyl-2 picrylhydrazyl (DPPH) was obtained from Sigma Chemical Inc. (St Louis, MO, USA). All other chemicals and solvent used were of analytical grade.

Sample preparation

The okra seed (250 g) was removed from the pod of matured and fibrous okra that cannot be easily cut with kitchen knife, sun-dried and hot water was poured unto it and allowed to stand for 2 minutes; it was drained, covered with nylon and wrapped with jute sacks. It was kept in a dark and warm place (30 – 32^{o}C) and left to chance fermentation for 120 h. Samples were taken for antioxidant analysis at 24 h, 72 h and 120 h, respectively.

Aqueous extract preparation

The aqueous extracts of both dried fermented and unfermented okra seeds were prepared in distilled water as described by Oboh *et al.* (2010); briefly, one gramme (1 g) of each of the samples was homogenized in 100 ml distilled water for 5 min. Thereafter, the mixtures were centrifuged at 2,000 x g for 10 min. The supernatant was used for the determination of vitamin C, phenolic and total flavonoid contents and antioxidant activities (ferric reducing antioxidant power (FRAP) and 1, 1- diphenyl-2 picrylhydrazyl (DPPH) free radical-scavenging ability).

Vitamin C content determination

The vitamin C content of the aqueous extract was determined using the method of Benderitter *et al.* (1998). 75 µl DNPH solution (2 g dinitrophenyl hydrazine, 230 mg thiourea and 270 mg copper sulphate ($CuSO_4$. $5H_2O$) in 100 ml of 5 ml/L H_2SO_4) were added to 500 µl extract mixture (300 µl of an appropriate dilution of the extract with

100 µl 13.3% trichloroacetic acid (TCA) and water). The reaction mixture was subsequently incubated for 3 h at 37°C, then 0.5 ml of 65% H_2SO_4 (v/v) was added to the medium and the absorbance was measured at 520 nm in a UV spectrophotometer (JENWAY 6305, Barloworld Scientific Ltd., Dunmow, Essex, UK). The vitamin C content of the extracts was subsequently calculated using ascorbic acid as standard.

Phenolic content determination

The phenolic content was determined according to the method of Singleton *et al*. (1999).

Appropriate dilutions of the extracts were mixed with 2.5 ml of 10% Folin–Ciocalteau's reagent (v/v) and neutralised by 2.0 ml of 7.5% sodium carbonate. The reaction mixture was incubated for 40 min at 45°C and the absorbance was measured at 765 nm in a spectrophotometer (JENWAY 6305). The total phenolic content was subsequently calculated using gallic acid as standard.

Total flavonoid content determination

The total flavonoid content of both extracts was determined using a slightly modified method reported by Meda *et al.* (2005). Briefly, 0.5 ml of appropriately diluted sample was mixed with 0.5 ml methanol, 50 ml of 10% Aluminium chloride ($AlCl_3$), 50 ml of 1 mol/l potassium acetate and 1.4 ml water, and allowed to incubate at room temperature for 30 min. Thereafter, the absorbance of the reaction mixture was subsequently measured at 415 nm using a spectrophotometer (JENWAY 6305). The total flavonoid was calculated using quercetin as standard.

Ferric reducing antioxidant power (FRAP)

The reducing property of the extracts was determined by assessing the ability of the extract to reduce ferric chloride ($FeCl_3$) solution as described by Oyaizu (1986). A 2.5 ml aliquot was mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min; thereafter 2.5 ml of 10% trichloroacetic acid was added. This mixture was centrifuged at 2 000 x g for 10 min; 5 ml of the supernatant was mixed with an equal volume of water and 1 ml of 0.1% ferric chloride. The absorbance was measured at 700 nm in a spectrophotometer (JENWAY 6305) and ferric reducing antioxidant property was subsequently calculated using ascorbic acid as standard.

DPPH free radical scavenging ability

The free radical scavenging ability using 1, 1-diphenyl-2-picryl hydrazyl (DPPH) was determined as described by Singh *et al*. (2002). Different concentrations of the aqueous extract were taken in different test tubes and the volume was made up to 1 ml with distilled water. 4 ml of 0.1 mM methanolic solution of DPPH was added. The tubes were shaken vigorously and allowed to stand for 20 min at room temperature. A control was prepared as above without the sample and distilled water was used for base line correction. Changes in absorbance of samples were measured at 517 nm in a spectrophotometer (JENWAY 6305). Free radical scavenging ability was expressed as percentage inhibition and was calculated using the following formula:

Free radical scavenging ability (%) = [(absorbance of control – absorbance of sample) / absorbance of control] ×100

Statistical analysis

The results of the three replicates were pooled and expressed as mean ± standard deviation. Standard deviations were calculated using spread sheet software (Microsoft Excel©, version 2007). Analysis of variance (ANOVA) was performed in order to determine the significance of the fermentation (SAS, 2002). Mean separations were performed to indicate significant differences using the Duncan's multiple range test procedure as described in the SAS software. Significance was accepted at $p < 0.05$. IC_{50} (extract concentration causing 50% inhibition of antioxidant activity) was calculated using the linear regression analysis.

Results and Discussion

Total phenolic content

The total phenolic content of the okra seeds was expressed as mg gallic acid equivalent (GAE) per 100 g sample as shown in Table 1. Phenolic content for the okra seed samples analyzed in the study ranged from 185 mg GAE/100 g to 1460 mg GAE/100 g sample. Among the okra seed samples, okra seeds fermented for 24 h had the highest ($p < 0.05$) phenolic content of 1460 mg GAE/100 g, while unfermented okra seed sample had the lowest phenolic content of 185 mg GAE/100 g. The phenolic content of the fermented okra seeds in this work was higher than the phenolic content of fermented and unfermented bambara groundnut, African yam bean, pigeon pea and kidney bean (Oboh *et al.*, 2009; Guzmán-Uriarte *et al.,* 2013). The phenolic content of the fermented okra seeds was found to be significantly ($p < 0.05$) higher than the unfermented okra seeds. This is in agreement with reports on fermented seeds where fermentation caused increase in the phenolic content of the seeds (Moktan *et al*., 2008; Ademiluyi and Oboh, 2011; Dajanta *et al*., 2013; Guzmán-Uriarte *et al.*, 2013; Plaitho *et al*. 2013). In natural form, phenolic compounds are combined or bound with sugar which reduces their availability to organism. During fermentation, proteolytic enzymes from the starter organism hydrolyse complexes of phenolics into soluble-free phenols and other simpler and biologically more active ones that are readily absorbed (Shrestha *et al.,* 2010; Ademiluyi and Oboh, 2011). The results showed that as fermentation period increased, the phenolic content of the okra seeds decreased. At 24 h fermentation, the phenolic content of okra seed increased with 689.19% which reduced to 108.11% at 120 h fermentation. This was consistent with findings reported by Lasekan and Shabnam (2013).

This may be due to the diffusion of phenolics in cell liquids and oxidation of diffused phenolics by the action of an enzyme polyphenol oxidase.

Total flavonoid content

The total flavonoid content of the okra seeds was determined and reported as shown in Table 1. The fermented okra seeds have significantly ($p < 0.05$) higher total flavonoid content than the unfermented seeds. Okra seeds fermented for 24 h recorded 492.10% increase in total flavonoid, while fermentation for 120 h increased by 97.36%. This result is in accordance with the trend observed in phenolic content. The total non-flavonoid content of the okra seeds also followed the trend observed for both the phenolic and total flavonoid contents where the value for the fermented okra seeds was significantly ($p < 0.05$) higher than the unfermented okra seeds; with 24 h fermentation recording the highest percentage increase. This is in agreement with the observation of other investigators that fermentation caused an increase in the total flavonoid and total non-flavonoid content of legumes (Moktan *et al*., 2008; Ademiluyi and Oboh, 2011; Yao *et al*., 2010). The increase in flavonoid content of okra seeds as a result of fermentation may be due to the increase in acidic value during fermentation that is liberating bound flavonoid components and making it more bioavailable.

The total flavonoid content of okra seed decreased as the fermentation period increased, this could be attributed to either the sample concentration of flavonoid compound, or the duration of the fermentation process as observed by Ehsan *et al.* (2010) in the fermentation of pistachio hulls. However it is significant to note that there is a remarkable increase in non-flavonoid phenolic compounds over the total flavonoid compounds of the okra seed with fermentation. This has also been observed by Ademiluyi and Oboh (2011) in the fermentation of bambara groundnut. It shows that fermentation could enhance the release of several classes of phenolic phytochemicals. Flavonoids are polyphenolic compounds known for their high antioxidant properties and free radical scavenging ability (Scherer and Godoy, 2009).

Ascorbic acid

Ascorbic acid is an effective quencher of singlet oxygen and other radicals. It has a vitamin E sparing antioxidant action, coupling lipophilic and hydrophilic reactions. It reacts with superoxide and a proton to yield hydrogen peroxide or with the hydroxyl radical to yield water. It also enhances absorption of inorganic iron and inhibits the formation of nitrosamines in the stomach (Bender, 2009). The ascorbic acid content of the okra seeds is presented in Table 1. The ascorbic acid content of the okra seeds ranged from 70 mgAAE/100 g unfermented sample to 650 mgAAE/100 g 24 h fermented sample. The ascorbic acid reported in this work was higher than the value reported for underutilised legumes 0.5 – 0.9 mg/100 g (Oboh, 2006). Fermentation caused a significant ($p < 0.05$) increase in the ascorbic acid content of okra seeds. Fermentation of okra seeds for 24 h recorded 828.57% increase in ascorbic acid, while fermentation for 120 h recorded 435.71% increase over the unfermented okra seeds. That fermentation caused an increase in the ascorbic acid content is consistent with report of other investigators. It has been observed that vitamin C content of citrus peels and white cabbage increased with fermentation (Oboh *et al.*, 2011; Kusznierewicz *et al*., 2008). On the contrary, different authors have found that fermentation processes caused noticeable reductions in vitamin C content of *Vigna sinensis* and *Lupinus albus* (Doblado, *et al.*, 2005; Frias *et al.*, 2005). It is observed in this work that as the fermentation time increased, ascorbic acid content of okra seeds decreased. This was evident since okra seeds fermented for 24 h have the value of 650 mgAAE/100 g ascorbic acid and okra seeds fermented for 120 h have 375 mgAAE/100 g ascorbic acid. As the fermentation period increased the loss in ascorbic acid may be as a result of the increase in the activity of the enzyme ascorbate oxidase that might have been produced by the fermentation microorganism which strongly depends on the pH of the fermentation environment. The enzymes convert ascorbic acid to dehydroascorbic acid (Adetuyi *et al*., 2008).

Table 1: Phenolic, ascorbic acid, total flavonoid, total non-flavonoid and IC_{50} DPPH free radical-scavenging ability of okra seeds *(Abelmoschus esculentus)*

Okra seed fermentation period	Phenolic (mgGAE/100 g)	Ascorbic acid (mgAAE/100 g)	Total flavonoid (mgQE/100 g)	Total non-flavonoid (mgQE/100 g)	DPPH radical IC_{50} (mg/ml)
0 h	$185^{d} \pm 0.10$	$70^{d} \pm 0.01$	$38^{d} \pm 0.00$	$147^{d} \pm 0.10$	$6.38^{a} \pm 0.25$
24 h	$1460^{a} \pm 1.53$	$650^{a} \pm 0.43$	$225^{a} \pm 0.23$	$1235^{a} \pm 1.62$	$2.27^{d} \pm 0.01$
72 h	$615^{b} \pm 0.31$	$500^{b} \pm 0.21$	$150^{b} \pm 0.11$	$465^{b} \pm 0.32$	$2.91^{c} \pm 0.03$
120 h	$385^{c} \pm 0.20$	$375^{c} \pm 0.20$	$75^{c} \pm 0.01$	$310^{c} \pm 0.27$	$4.40^{b} \pm 0.11$

Values represent mean ± standard deviation of triplicate determination. Values with the same letter along the same column are not significantly different ($p < 0.05$). GAE – Gallic Acid Equivalent, AAE – Ascorbic Acid Equivalent, QE – Quercetin Equivalent.

Reducing power

There is an increasing interest in the measurement of total antioxidant activity rather than simply the contents of antioxidant (Ahmed and Beigh, 2009). The reducing power of antioxidants is an important indicator of potential antioxidant activity. The antioxidant effect exponentially increases as a function of the development of the reducing power, indicating that the antioxidant properties are concomitant with the development of reducing power (Oyaizu, 1986). The reducing powers of the extracts were assessed based on

their ability to reduce Fe^{3+} to Fe^{2+} and the results in ascorbic acid equivalent is presented in Fig. 1. The reducing power of the fermented okra seeds was significantly ($p < 0.05$) higher than that of the unfermented okra seeds. The unfermented okra seeds had a reducing power of 93 mgAAE/100 g, while fermentation for 24 h caused the reducing power to increase to 980 mgAAE/100 g. It has been previously reported that fermentation enhanced the reducing power of soy bean and bambara groundnut (Chang *et al.*, 2009; Ademiluyi and Oboh, 2011). The reducing power of the okra seeds reduced as the fermentation period increased.

The reducing power of okra seeds fermented for 24 h was found to be 980 mgAAE/100 g, while the reducing power of okra seeds fermented for 120 h was 134 mgAAE/100 g; it showed a reduction of 86.33%. This trend in the reducing power agreed with the total phenol, total flavonoid and total non-flavonoid contents of the okra seeds (fermented and unfermented). Ahmed and Beigh (2009) in the study of ascorbic acid, carotenoid and total phenolic contents, and antioxidant activity of various genotypes of *Brassica oleraceaencephala* reported a significant correlation between total phenolic content and antioxidant activity.

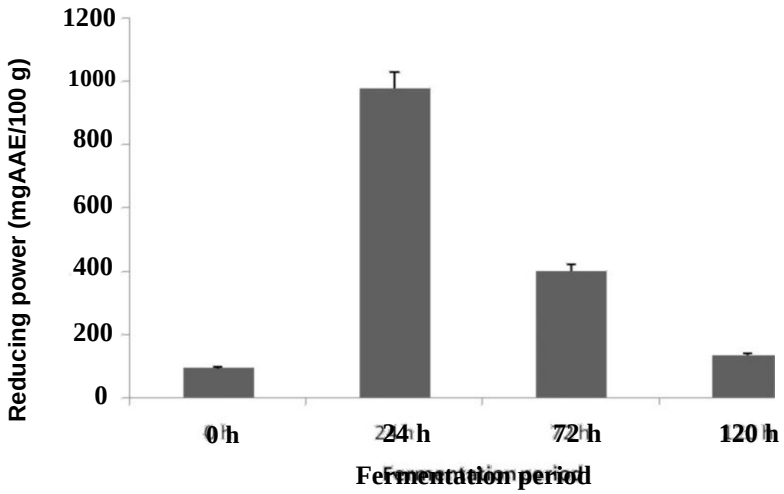

Fig. 1: Ferric reducing antioxidant power (FRAP) of aqueous extract of okra seed (*Abelmoschus esculentus*)
AAE - Ascorbic Acid Equivalent

DPPH free radical-scavenging ability

In the presence of hydrogen donors, DPPH is reduced and a free radical is formed from the scavenger. The reaction of DPPH is monitored by the decrease of the absorbance of its radical at 517 nm, but upon reduction by an antioxidant, the absorption disappears (Gupta and Prakash, 2009). DPPH radical-scavenging ability of the okra seeds (fermented and unfermented) at 0 – 3.33 mg/ml concentrations were measured and the results are presented in Fig. 2. A dose-response relationship was found in the DPPH radical-scavenging ability of the okra seed extracts; the ability increased with an increase in the concentration of the okra seed extracts. It shows from these results that fermentation caused significant ($p < 0.05$) increase in the DPPH radical-scavenging ability of okra seeds. Several researchers have found out that fermentation does increase the DPPH radical-scavenging ability of legumes, white cabbage and citrus peels (Ademiluyi and Oboh, 2011; Chang *et al.*, 2009; Dajanta *et al*., 2013; Kusznierewicz *et al.*,

2008; Oboh *et al.*, 2011). Okra seeds fermented for 24 h recorded the highest DPPH radical-scavenging ability which was significantly ($p < 0.05$) different from unfermented seeds and samples fermented for 72 h and 120 h. Fermentation time did significantly affect the DPPH radical-scavenging activities for all extracts, but it was not a matter of time dependant (Chang *et al.,* 2009). Solvent of extraction also plays an important role as observed by Chang *et al.* (2009) when studying the effect of fermentation time on the antioxidant activities of tempeh prepared from fermented soybean using *Rhizopus oligosporus* that except for 95% ethanol extract, the optimum fermentation time to obtain the extracts with the highest DPPH radical-scavenging activities was 10 days, while for the 95% ethanol extracts, the 5-day fermented tempeh showed the highest DPPH radical-scavenging activities. Table 1 shows IC_{50} value, which is the inhibition concentration of sample extract required to decrease initial DPPH activity by 50%. The lower IC_{50} value indicates stronger antioxidant activity in the sample. The fermented okra seeds exhibited the lower IC_{50} values than the unfermented okra seeds. It can also be seen from the table that okra seeds fermented for 24 h recorded the lowest IC_{50} value of 2.27 mg/ml. In addition, the DPPH radical-scavenging ability followed the same trend as the ascorbic acid, phenolic, flavonoid and non-flavonoid contents of the okra seeds and the FRAP of the okra seeds. It has also been observed by earlier researchers where correlation was established between phenolic content and antioxidant activities (Ademiluyi and Oboh, 2011; Dajanta *et al*., 2013; Ahmed and Beigh 2009; Ehsan *et al.,* 2010). Fermented okra seeds had higher antioxidant activities than the unfermented okra seeds at the concentrations tested. It has been observed that fermentation enhances the nutrient content of foods through the biosynthesis of vitamins, essential amino acids and protein, by improving protein quality and fibre digestibility.

It also enhances micro nutrient bioavailability and aids in degrading antinutritional factors (Oboh and Rocha, 2006). Fermentation also improves the phytochemical (phenolic) content and antioxidant properties of legume seeds (Ademiluyi and Oboh, 2011). The release of phenolic isoflavoneaglycones by the catalytic action of β-glucosidase during fermentation and the formation reductones during fermentation could contribute to such increase in antioxidant activities. Since okra seeds have been found to be of high protein content, the breakdown of protein to free amino acids and peptides by microbial protease activity could also account for the increase in the antioxidant activity. It has been observed by Murakami *et al*. (1984) that isoflavones were the major compounds responsible for the antioxidant activity in tempeh.

Two-thirds of the antioxidant capacities in tempeh were derived from the oligopeptides by proteolysis during fermentation (Sheih *et al.,* 2000). Watanabe *et al*. (2007) modified the fermentation conditions for preparing tempeh from soybean and observed that isoflavoneaglycones, free amino acids and peptides were responsible for the antioxidant activity of tempeh. Synergistic effect of the phenolic content of the fermented okra seeds with other components present in the aqueous extract could also cause an increase in the antioxidant activities. Moktan *et al*. (2008) reported that the total antioxidant activity of the aqueous extract cannot be predicted based on its total phenolic content alone but a synergism of soluble polyphenolic compounds, with one another, and/or other components present in the extracts, may contribute to the overall observed antioxidant activity.

The antioxidant activities of fermented okra seeds decreased with fermentation time. The antioxidant activities of okra seeds fermented for 24 h at all the concentrations tested were significantly ($p < 0.05$) higher than that fermented for 72 h and 120 h. These were also reflected in the IC_{50} values where the value for okra seeds fermented for 24 h (2.27mg/ml) was significantly ($p < 0.05$) lower to that of 72 h (2.91 mg/ml) and 120 h (4.40 mg/ml). The lower IC_{50} value is a representation of higher scavenging ability of the fermented okra seeds. Basically, the improvement in antioxidant activity

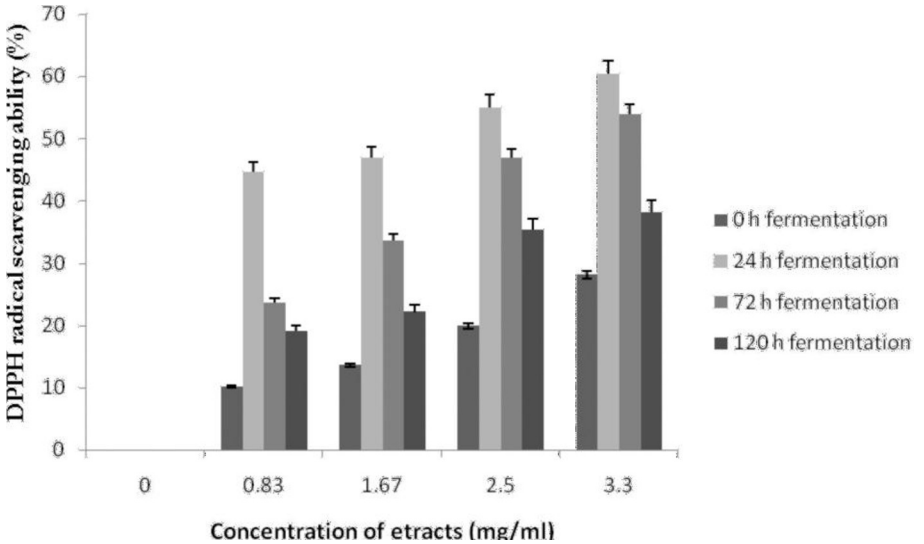

Fig. 2: 1,1-diphenyl-2 picrylhydrazyl (DPPH) free radical-scavenging ability of aqueous extract of okra seed (*Abelmoschus esculentus*)

occurs while the fermentation is taking place where microorganisms start breaking down the linkage of phenolic and flavonoid compounds, which free the compounds to actively play the role of antioxidants (Moktan *et al.*, 2008; Ademiluyi and Oboh, 2011). Increasing the fermentation time might allow the microorganisms to use those available compounds as substrates for their growth, thus reducing the sample concentration. Therefore, the fermentation period is critical to ensure optimal breaking down of the compound linkages but not to allow compounds to be substrates for microbial growth (Ehsan *et al.*, 2010).

Conclusion

This study demonstrated that fermentation caused a marked increase in phenolic, total flavonoid and vitamin C contents of okra seeds which then enhanced DPPH radical-scavenging ability and ferric reducing antioxidant power (FRAP) of the okra seeds. Fermentation time affected the antioxidant activities of okra seeds and fermentation for 24 h seemed to be applicable as exemplified by the least IC_{50} value of DPPH radical-scavenging ability and highest value of ferric reducing antioxidant power of the okra seeds. Considering phenolic, total flavonoid, vitamin C, DPPH radical-scavenging ability and ferric reducing antioxidant power of the okra seeds, 24 h fermented okra seeds showed greater qualities than unfermented, and 72 h and 120 h fermented okra seeds. Therefore, the product from fermented okra seeds, obtained in this work, could be used for the production of functional foods. This work was carried out within the purview of available methods of analysis. Further research can be done using HPLC characterisation method.

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