Antioxidant potential of *Fraxinus floribunda* Bark Extracted through Various Aqueous Processing

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ABSTRACT

Introduction: *Fraxinus floribunda* Wallich (Oleaceae) is an ethno-medicinal plant found in Sikkim, India. The leaves of the plant are traditionally used in fracture, dislocation and other inflammatory conditions. From our survey, it was found that the successive aqueous decoction of the bark of this plant is prescribed to diabetic patients by herbal practitioners. In the present study the bark of *Fraxinus floribunda* was extracted through various boiling procedures and cold percolation method to assess the effect of process variation on the antioxidant activity. **Methods:** The dried bark sample of *Fraxinus floribunda* was subjected to three hydrothermal procedures and cold percolation method. The obtained extracts were investigated spectrophotometrically against DPPH, $ABTS^+$, nitric oxide, superoxide and ferric reducing power. Metal chelating activity and anti-lipid peroxidation assay were also performed along with quantitative estimation of total phenolics, flavonols and ortho-dihydric phenol content. **Results:** The lowest IC_{50} values for DPPH (0.241 mg/ml), $ABTS^+$ (0.0284 mg/ml), nitric oxide (5.3151 mg/ml), anti-lipid peroxidation assay (2.29 mg/ml) were exhibited by the extract obtained through pressure boiling. The same sample also showed highest ferric reducing power and total phenol content. Maximum total flavonol and ortho-dihydric phenol content were exhibited by the extracts obtained through normal boiling and Soxhletion respectively. **Conclusion:** The bark of *Fraxinus floribunda* contains potentially active antioxidants. Extracts obtained through pressure boiling showed significant antioxidant activity therefore it seems to be the most effective method of extraction for acquiring antioxidants. The providing data can enrich the existing comprehensive data of antioxidant activity of plant materials.

Key words: Hydrothermal processing, Lipid peroxidation, Metal chelation, Percolation, Phenolics, superoxide,

INTRODUCTION

Fraxinus floribunda Wallich is a tree occurring in the Eastern Himalayas, Sikkim and Khasi hills. The leaves of the plant have been traditionally used for the treatment of fracture and dislocation.[1] To our knowledge, no studies are provided on the pharmacological properties of the bark of this plant. Hence the present study reveals free-radical scavenging activity and quantitative phytochemical screening on the bark of *F.floribunda* **a**nd their dynamic alteration during various thermal processing (boiling, pressure boiling and soxhletion) and cold percolation was also done to assess the effect of extraction process variation on antioxidant availability.

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MATERIALS AND METHODS

Chemicals

Methanol, 2,2-diphenyl-1-picryl hydrazyl (DPPH), 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), nitro blue tetrazolium (NBT), phenazine methosulphate (PMS), reduced nicotinamide adenine dinucleotide sodium salt monohydrate (NADH), sulfanilamide, glacial acetic acid, naphthyl ethylenediamine dihydrochloride (NED), potassium ferricyanide [$K_3Fe(CN)_6$], (TCA), thiobarbituric acid (TBA), trichloroacetic acid,$FeSO_4.7H_2O$, potassium hydroxide

(KOH),potassium dihydrogen orthophosphate (KH_2PO_4), ethylene-diamine tetra acetic acid (EDTA), 2-deoxyribose, ferric chloride ($FeCl_3$), ferrous chloride ($FeCl_2$), ferrozine, sodium nitroprusside, gallic acid, catechol,quercetin, Folin-Ciocalteau reagent, sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$), aluminium chloride (AlCl3), sodium hydroxide (NaOH), hydrochloric acid and potassium chloride. All these chemicals were either purchased from Himedia, India or of Merck, Germany made.

Plant material

The bark of *Fraxinus floribunda* Wallich was collected from Sankhu, Dentam, West Sikkim, India in the month of April, 2012. The specimen was identified and authenticated by Dr. A.P. Das, Professor, Taxonomy & Environmental Biology Laboratory, Department of Botany, University of North Bengal. A voucher specimen was deposited at laboratory herbarium, Taxonomy and Environmental Biology Laboratory, University of North Bengal and identified against the accession number 9632/ Tag no E.S.03 for the further references.

Animal material

For anti-lipid peroxidation assay, goat liver used was collected from slaughter house immediately after slay and the experiment was conducted within an hour after collection.

Sample preparation

The bark of *F. floribunda* was sun-dried & reduced to coarse powder using laboratory grinder. Powdered bark (10 g) was extracted with 100 ml distilled water separately through boiling at atmospheric pressure, soxhlet apparatus, autoclave and cold percolation at low temperature. The extracts were collected & filtered through Whatman No. 1 filter paper and finally subjected to evaporation in a heating mantle. Final volume make up of each of the sample were done by adding distilled water. This process was repeated three successive times to obtain the extracts at three different stages. The samples were then kept in bottles and stored in refrigerator for further analysis.

Extraction procedures

- ***Soxhletion***

The dried bark powder (10 g) was extracted successively with 100 ml distilled water in a soxhlet extractor (Merck, Germany) for 8 hours.

- ***Boiling at atmospheric pressure***

The conventional extraction was done through boiling / refluxing the sample for 2 hours in a conical flask.

- ***High pressure boiling***

The powered sample was put into an autoclave for 15 mins under high pressure (1.5 kg cm^{-2}) and high temperature (121°C).

- ***Cold percolation***

The powdered sample was placed in a glass beaker with water at below 4^{0}C in a refrigerator for 48 hours. The sample was then filtered with Whatman no 1 filter paper.

Evaluation of antioxidant activity

Determination of the free radical scavenging activity of each of the crude extract was carried using the following methods.

- ***DPPH radical scavenging activity***

DPPH based radical scavenging activity was measured.[2] Aqueous extract (0.2 ml) was added to 1.8 ml of DPPH (0.1 mM). The mixture was shaken well and allowed to stand in dark at room temperature for 10 minutes. The absorbance was measured at 517 nm with UV-Visible Spectrophotometer (Systronics, 2201). A reaction mixture without test sample was considered as control.DPPH scavenging activity (%) was measured using the following formula:

$$DPPH\ scavenging\ activity\ (\%) = \left[\frac{Absorbance_{control} - Absorbance_{sample}}{Absorbance_{control}}\right] \times 100$$

Where, absorbance of the control and absorbance of the extract or standard.

Then IC_{50} was calculated from the graph of percentage inhibitions plotted against concentration.

- ***ABTS^{+} radical scavenging assay***

ABTS^{+} assay was done by the method[3] with few modifications. The reaction mixture contained 7mM ABTS^{+} radical cation (s) in methanol with 2.4 mM potassium per sulfate ($K_2S_2O_8$) in water which was stored in the dark at room temperature for 12 hrs. Before usage, the ABTS^{+} solution was diluted 8 times. Then, 2 ml of ABTS^{+} solution was added to 1 ml of the aqueous extract. After 10 mins, absorbance at 734 nm was measured.

$$ABTS^{+}\ scavenging\ effect\ (\%) = \left[\frac{Absorbance_{control} - Absorbance_{sample}}{Absorbance_{control}}\right] \times 100$$

- ***Superoxide anion radical scavenging activity***

For superoxide radical scavenging activity method[4] was followed with slight modifications. The solutions were prepared in 0.1 M phosphate buffer (pH-7.4). The reaction mixture contained 1 ml of NBT solution (312 μM prepared

in phosphate buffer, pH-7.4), 1 ml of NADH solution (936 μM prepared in phosphate buffer, pH-7.4), and 1 ml of aqueous extract of different concentrations. After 5 minutes of incubation, 20 μl of PMS (120 μM) was added to the reaction mixture. The reactant was illuminated by fluorescent lamp for 30 minutes. The absorbance was measured at 560 nm against control. The percentage inhibition of generation of superoxide anion was calculated by using the following formula:

$$\text{Superoxide scavenging effect (\%)} = \left[\frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}}\right] \times 100$$

- ***Metal chelating activity***

The method[5] was used for the estimation of chelating activity of the extracts for ferrous ions Fe2^{+}.To 0.4 ml of aqueous extract, 1.6 ml of water was diluted and mixed with 0.04 ml of $FeCl_2$ (2 mM). After 30 minutes, 0.08 ml ferrozine (5 mM) was added. The mixture was shaken vigorously and left standing at room temperature for 10 minutes. The absorbance of the solution was measured at 562 nm. The chelating activity was calculated as:

$$\text{Chelating effect (\%)} = \left[\frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}}\right] \times 100$$

- ***Ferric reducing antioxidant power assay***

The ferric reducing antioxidant power (FRAP) can be determined.[6] The reaction mixture was prepared by mixing 1ml extract, 2.5 ml of phosphate buffer (200 mM, pH 6.6), 2.5 ml of potassium ferricyanide (30 mM) and the mixture was incubated at 50°C for 20 minutes. To the reaction mixture, 2.5 ml of trichloroacetic acid (600 mM was added and centrifuged for 10 minutes at 3000 rpm. The upper layer of solution (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (6 mM) and absorbance was measured at 700 nm in UV-VIS spectrophotometer. Ascorbic acid was used as control.

- ***Nitric oxide radical scavenging assay***

Nitric oxide was generated according to the method of Marcocci *et al*. (1994) from sodium nitroprusside and measured by the Greiss reaction.[7] The reaction mixture was prepared with 2 ml (15 mM) sodium nitroprusside, 0.5 ml PBS solution and 0.5 ml aqueous extract. After incubation at room temperature for 160 minutes, 3 ml Greiss reagent was mixed and again the mixture was left for incubation at 25°C for 30 minutes. Lastly absorbance was taken at 546 nm. Radical scavenging activity was expressed as percent inhibition from the given formula:

$$\text{Nitric oxide scavenging effect (\%)} = \left[\frac{\text{Absorbance}_{\text{cont}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{cont}}}\right] \times 100$$

- ***Anti-lipid peroxidation assay***

A modified thiobarbituric acid reactive species (TBARS) assay was used to measure the lipid peroxide formed using liver homogenates as lipid-rich media.[8] $FeSO_4$induces lipid peroxidation. Malondialdehyde (MDA), produced by oxidation of polyunsaturated fatty acids, reacted with two molecules of thiobarbituric acid (TBA) yielding a pinkish red chromogen with an absorbance maximum at 532 nm was measured. The inhibition percentage of lipid peroxidation by the extract at different concentration was calculated with the formula:

$$\text{Lipid peroxidation effect (\%)} = \left[\frac{A_0 - A_1}{A_0}\right] \times 100$$

Where, A_0 = absorbance before reaction and A_1 = absorbance after reaction has taken place.

Quantitative estimation of bioactive phytochemicals

- ***Total phenol content***

The total phenolic content of the aqueous extract of *F. floribunda* was carried out with Folin-Ciocalteau reagent.[9] One ml of aqueous bark extract, 1 ml of 95% ethanol, 5 ml of distilled water, 0.5 ml 50% Folin-Ciocalteu reagent was mixed and after 5 mins 5% of sodium carbonate (Na_2CO_3) was added and after 1 hr the absorbance value was taken at 725 nm.

- ***Total flavonol content***

Total flavonol content in the aqueous extract of *F. floribunda* was done by the method[10] Sodium nitrite (0.5 ml) was mixed to form the reaction mixture. After 5 mins 0.3 ml 10% aluminium chloride (AlCl3), was added and diluted with 2.5 ml of distilled water and mixed well. The absorbance was taken at 510 nm.

- ***Total ortho-dihydric phenol content***

The determination of ortho-dihydric phenolics was based on the method.[11] Firstly; 0.5 ml of aqueous extract was added to a mixture containing 0.5 ml of 0.05 (N) HCl and 0.5 ml of Arnow's reagent. After proper shaking, 5 ml of water was added, followed by the addition of 2 ml of 1 (N) NaOH. Finally, after 5 min the absorbance was recorded at 515 nm. The total ortho-dihydric phenolics content in different extracts was calculated as catechol equivalent (CE)/gm fresh weight tissue (FWT).

- ***Statistical analysis***

The standard software SPSS (ver. 15.0) was used for all statistical analysis. One-way analysis of variance (ANOVA) was used to compare the differences and the means which were considered significant at $p \leq 0.05$. Correlation and regression analysis was done by using Excel Software of MS Office 2009.

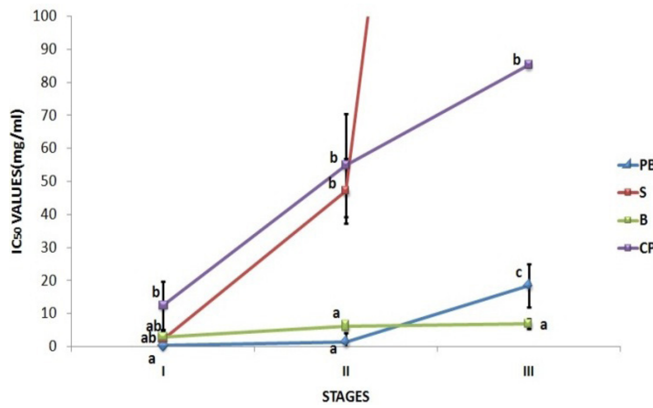

Figure 1: DPPH Scavenging Activity

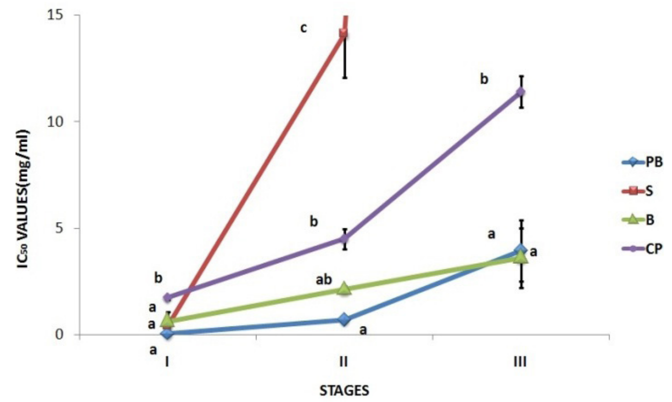

Figure 2: $ABTS^{+}$ Scavenging Activity

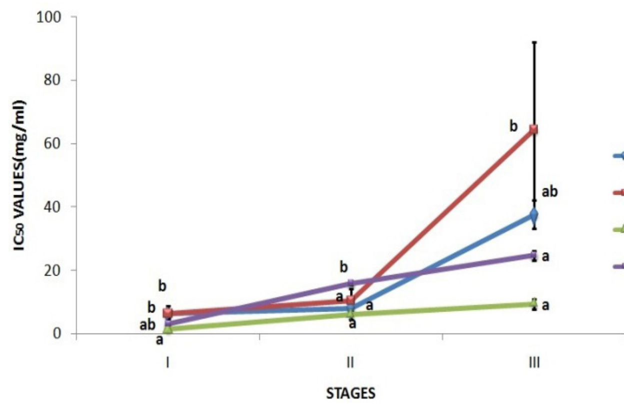

Figure 3: Superoxide Scavenging Activity

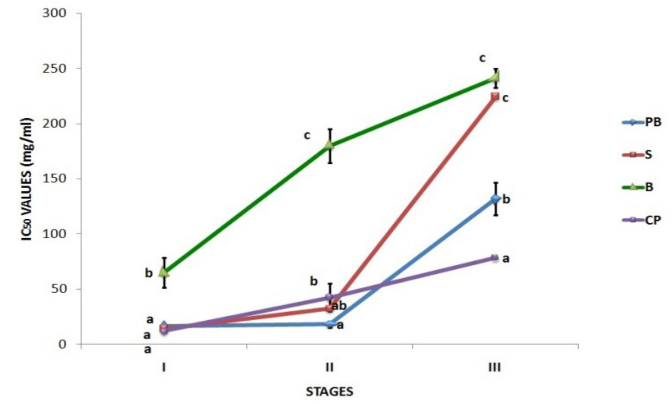

Figure 4: Metal Chelating Activity

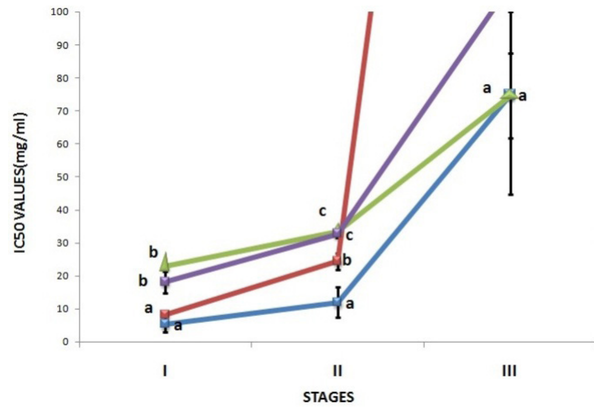

Figure 5: Nitric oxide Scavenging Activity

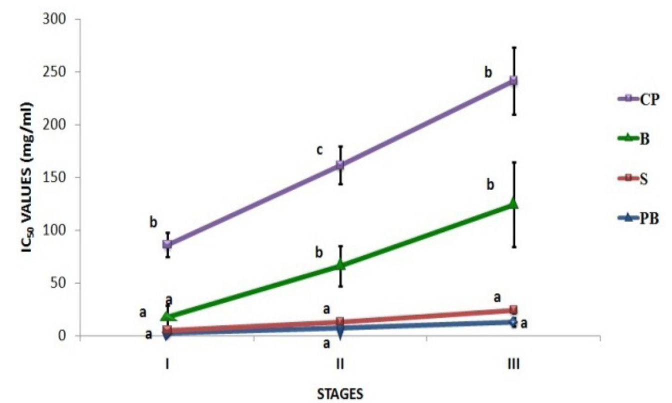

Figure 6: Antilipid Peroxidation Activity

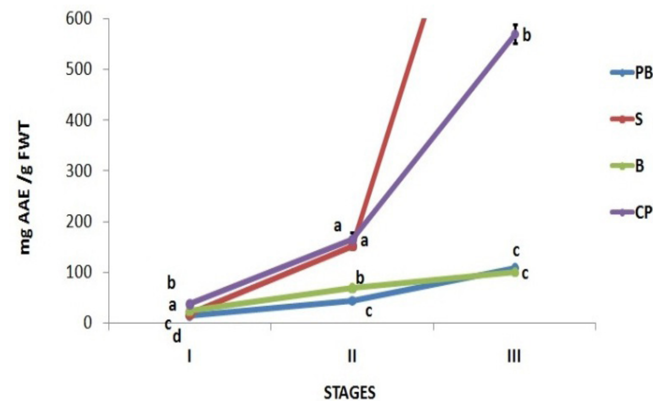

Figure 7: Ferric Reducing Antioxidant Potential

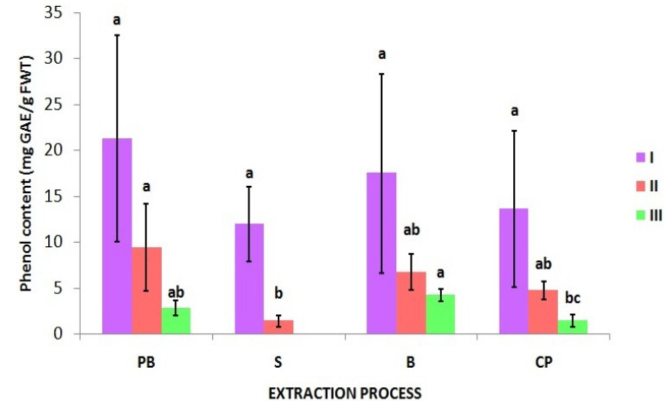

Figure 8: Total phenol content

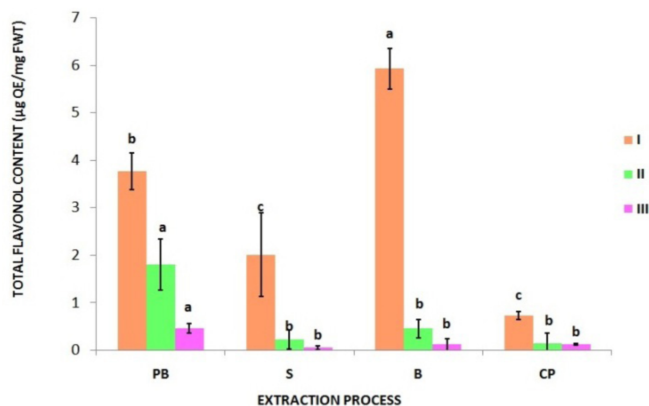

Figure 9: Total flavonol content

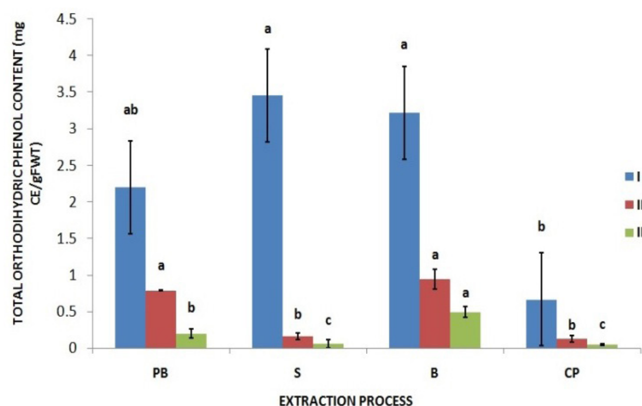

Figure 10: Total orthodihydric phenol content. I, II and III represents first, second and third successive stages of extraction. Values with different letters (a, b, c & d) are significantly ($p < 0.05$) different from each other by Duncan's multiple range test (DMRT).

RESULTS

From the results obtained, it might be stated that the bark of *F. floribunda* contains potentially active antioxidants. The radical scavenging activity of bark of *F. floribunda* obtained from different extraction procedures at three successive stages are presented in figure 1-6. In free radical scavenging assays such as DPPH (Figure 1), $ABTS^+$ (Figure 2), nitric oxide (Figure 5) and anti-lipid peroxidation activity (Figure 6), it was found that the extracts obtained from pressure boiling have shown lowest IC_{50} values in all cases as well as it has a good ferric oxide reducing potential (Figure 7) thus high antioxidant activity. But there are some cases where boiling has proved to be a better extraction method than other methods for superoxide scavenging activity (Figure 3) while cold percolation method has shown superior restoration of metal chelating capacity (Figure 4). Along with the free radical scavenging activities, some bioactive phytochemicals were also estimated such as total phenol content, total flavonol content and total ortho dihydric phenol content. The highest phenol content was exhibited by extracts from pressure boiling (Figure 8) while elevated flavonol content (Figure 9) was obtained by extracts from boiling and optimum (Figure 10) orthodihydric phenol content was obtained from extracts through soxhletion. Along with four different extraction methods, the bark was extracted in three successive stages in each method. This was done to observe the retention of antioxidant properties at different successive stages. From the graphs, it is clear that the antioxidant activity of the bark of the plant was retained till third stage except in soxhletion method as the antioxidant activity was negligible in the third stage. From the Pearson correlation test (Table 1), insignificant negative correlation was obtained between phenolics content and antioxidant activity assayed by different free radicals scavenging assays while significant positive correlation was observed between various radical scavenging assays such as $ABTS^+$, DPPH, superoxide and nitric oxide. Similarly phytochemical contents (phenol, flavonol and ortho dihydric phenol) were found to have significant positive correlation among them. Significant positive correlation was also observed between nitric oxide assay and metal chelation as well as between

Table 1: Correlations between the IC_{50} values of antioxidant activities, phenolics, flavonoids and orthodihydric phenol content of bark of *F. floribunda*

	DPPH	ABTS	TPC	TFC	TOPC	FRAP	SO	MC	NO
ABTS	0.985**	-	-	-	-	-	-	-	-
TPC	-0.431	-0.381	-	-	-	-	-	-	-
TFC	-0.301	-0.251	0.800**	-	-	-	-	-	-
TOPC	-0.226	-0.167	0.842**	0.674*	-	-	-	-	-
FRAP	0.279	0.132	-0.555	-0.432	-0.448	-	-		-
SO	0.870**	0.839**	-0.576	-0.431	-0.377	0.380	-	-	-
MC	0.481	0.510	-0.544	-0.375	-0.418	0.137	0.532	-	-
NO	0.975**	0.962**	-0.490	-0.331	-0.297	0.317	0.908**	0.629*	-
ALP	-0.095	-0.215	-0.378	-0.460	-0.528	0.595*	-0.071	0.248	-0.025

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

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

DPPH1: 1-diphenyl-2-picrylhydrazyl; ABTS: 2,2´-azinobis-3-ethylbenzo-thiozoline-6-sulphonic acid; TPC: Total phenol content; TFC: Total flavonol content; TOPC: Total orthodihydric phenol content; FRAP: Ferric reducing antioxidant power; SO: Superoxide; MC: Metal chelation; NO: Nitric oxide; ALP: Anti-lipid peroxidation.

anti-lipid peroxidation and reducing power.

DISCUSSION

Plants are the main source of natural antioxidants, which are greatly important for human health.[12] Several natural antioxidants have already been isolated from various plant materials such as leafy vegetables, fruits, seeds, cereals[13] and had showed ROS (reactive oxygen species) scavenging activity and prevention of lipid peroxidation.[14,15] The decoction of aqueous extracts of fruits of Dilleniaindica used by the Assamese people in their cuisine was found to be an ideal source of antioxidants.[16] This protection could have been acquired due to the presence of antioxidant molecules in plants and the products of secondary metabolites such as phenolics, flavonoids and polypropanoids which have the capacity to scavenge free radicals due to their ability to donate proton.

Extraction procedure is one of the crucial points for the extraction of various bioactive compounds. The influence of variation in extraction solvents and techniques on the content of natural antioxidants in extracts has been reported by many researchers.[17,18] In the present study, the bark of *Fraxinus floribunda* has been extracted through four different methods including normal/conventional boiling, pressure boiling by using autoclave, soxhlet extraction and cold percolation. From the results, it was revealed that the bark of *F. floribunda* contains potentially active antioxidants and free radical scavenging activity. It was observed that the sample extracted through pressure boiling has exhibited highest free radical scavenging activity, reducing antioxidant power as well as highest total phenol content. Similar result was obtained in case of ginseng seeds where total amount of phenolic compounds were about three times higher when processed under high pressure through autoclaving at 1300 C than in seeds that were not treated.[19] Autoclaving for a longer time enhanced the diffusion of phenols from seed coats to cooking water and from there to cotyledons which caused increased levels of all tocopherols and tocotrienols along with the increase in vanillin and ferulic and p-coumaric acids.[20] But in case of total flavonol content and superoxide radical scavenging assay, the sample extracted through normal boiling have showed the best result. Soxhletion has proved to be a good extraction procedure for the isolation of total ortho dihydric phenol content while cold percolation method has showed moderate result in all the assays.

A statistically insignificant negative correlation was obtained between phenolic contents and various free radical scavenging activities. Hence this data indicated that the phenolic compounds might not be contributing for the high antioxidant activities in the aqueous bark extract of *F. floribunda*. Similar result was obtained in the aqueous extract of Brazilian mushroom and button mushroom as their aqueous extract showed no correlation between the phenolic compounds and antioxidant potential.[21] Thus, it is reasonable to state that phenolics may not act as the most important antioxidant components in the aqueous extracts of bark of *Fraxinus floribunda*. There might be some active non-phenolic constituents that can be extracted in aqueous system. The presence of non-phenolic compounds like uronic acids and amino acids might also be suggested that can produce higher antioxidant capacity than those generated by polyphenols.[22,23]

CONCLUSION

The purpose of the present experiment was to compare changes in phytochemical content and antioxidant activities from the bark of *Fraxinus floribunda* and measuring alteration of bioactivity of the samples extracted through process variation (boiling at atmospheric pressure, pressure boiling, soxhlet decoction and cold percolation). Results indicated that various extraction methods have significant influence on the antioxidant property as well as phytochemical content of extracts. Based on the above presented results, various extraction methods might be applied to isolate pharmacologically active components from *F. floribunda*, especially the extracts obtained through pressure boiling exhibited the most significant antioxidant activities, and therefore it seemed to be the more efficacious method for acquiring antioxidants. The results and findings of this study support this view that the bark of *Fraxinus floribunda* is a promising source natural antioxidant which might help in preventing the progress of various oxidative stress mediated disorders.

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REFERENCES

1. Gurung B. The medicinal plants of the Sikkim Himalaya, Subash publication, Gangtok, Sikkim; 2002.
2. Blois MS. Antioxidant determinations by the use of a stable free radical. Nature 1958; 181: 1199-200.
3. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med. 1999; 26(9-10): 1231-7.
4. Nishikimi M, Appaji N, Yagi K. The occurrence of superoxide anion in the reaction of reduced phenazinemethosulfate and molecular oxygen. Biochem Biophys Res Commun. 1972; 46(2): 849-54.
5. Dinis TC, Madeira VM, Almeida LM. Action of phenolic derivates (acetoaminophen, salycilate, and 5-aminosalycilate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. Arch Bio chem Biophys. 1994; 315(1): 161-9.
6. Oyaizu M. Studies on products of browning reactions: antioxidative activities of products of browning reaction prepared from glucoamine. Jpn J Nutr. 1986; 4(6): 307-15.
7. Marcocci L, Packer L, Droy-Lefaix MT, Sekaki A, Gardes-Albert M. Antioxidant action of *Ginkgo biloba* extract EGb 761. Methods Enzymol 1994; 234: 462-75.
8. Bouchet N, Barrier L, Famconneau B. Radical scavenging activity and antioxidant properties of tannins from *Guiera senegalensis* (Combretaceae). Phytother Res. 1998; 12(3): 159-62.
9. Chandler SF, Dodds JH. The effect of phosphate, nitrogen and sucrose on the production of phenolics and solasidine in callus cultures of *Solanum laciniatum*. Plant Cell Rep. 1983; 2(4): 205–8.
10. Sultana B, Anwar F, Ashraf M. Effect of extraction solvent/technique on the antioxidant activity of selected medicinal plant extracts. Molecules 2009; 14(6): 2167-80.
11. Kim DO, Jeong SW, Lee CY. Antioxidant capacity of phenolic phytochemicals from various cultivars of plums. Food Chem. 2003; 81(3): 321- 6.
12. Parasuraman S, Thing GS, Dhanaraj SA. Polyherbal formulation: Concept of ayurveda. Phcog Rev. 2014; 8(16): 73-80.
13. Pokorny J. Natural antioxidants for food use. Trends Food Sci Technol. 1991; 2: 223-7.
14. Parasuraman S, Kumar E, Kumar A, Emerson S. Free radical scavenging property and diuretic effect of triglize, a polyherbal formulation in experimental models. J Pharmacol Pharmacother. 2010; 1(1): 38-41.
15. Aqil F, Ahmad I, Mehmoud Z. Antioxidant and free radical scavenging properties of twelve traditionally used Indian medicinal plants. Turk J Biol. 2006; 30: 177-83.
16. Das M, Sarma BP, Ahmed G, Nirmala CB, Choudhury MK. In vitro antioxidant activity and total phenolic content of *Dillenia indica* and *Garcinia penducalata*, commonly used fruits in Assamese cuisine. Free Rad Antiox. 2012; 2(2): 30-6.
17. Grigonisa D, Venskutonis PR, Sivik B, Sandahl M, Eskilsson CS. Comparison of different extraction techniques for isolation of antioxidants from sweet grass (*Hierochl oeodorata*). Journal Super crit Fluids 2005; 33(3): 223-33.
18. Michiels JA, Kevers C, Pincemail J, Defraigne JO, Dommes J. Extraction conditions can greatly influence antioxidant capacity assays in plant food matrices. Food Chem. 2012; 130(4): 986-93.
19. Bae HM, Kim SS, Cho CW, Yang DC, Ko SK, Kim KT. Antioxidant activities of ginseng seeds treated by autoclaving. J Ginseng Res. 2012; 36(4): 411-7.
20. Bryngelsson S, Dimberg LH, Kamal-Eldin A. Effects of commercial processing on levels of antioxidants in oats (*Avena sativa L.*). J Agric Food Chem. 2002; 50(7): 1890-96.
21. Gan CH, Nurul Amira B, Asmah R. Antioxidant analysis of different types of edible mushrooms (*Agaricus bisporous* and *Agaricus brasiliensis*). Int Food Res J. 2013; 20(3): 1095-2.
22. Chye FY, Wong JY, Lee JS. Nutritional quality and antioxidant activity of selected edible wild mushrooms. Food Sci Technol Int. 2008; 14(4): 375-84.
23. Perez-Jimenez J, Saura-Calixto F. Antioxidant capacity of dietary polyphenols determined by ABTS assay: a kinetic expression of the results. Int J Food Sci Technol. 2006; 43(1): 185-91.