Indian Journal of Natural Products and Resources
Vol. 6(4), December 2015 pp. 283-287

Isolation of tannin as calcium salt (calcium tannate) and as free tannin from de-oiled seeds of *Shorea robusta* Gaertn. and evaluation of its *in vitro* antioxidant properties

Soma Das[1*], D K Bhattacharyya[2] and Mahua Ghosh[1]

[1]Department of Chemical Technology, University of Calcutta, Kolkata, West Bengal, India.

[2]School of Community Science and Technology, IIEST, Shibpur, Kolkata.

Received 12 June 2014; Accepted 6 October 2015

The present study aimed to develop a novel process which may be commercially feasible for isolating tannin as metallic salt, free from carbohydrates, proteins, minerals, etc. followed by its hydrolysis to free tannin for various potential applications. Accordingly a process was developed which involves separation of the soluble carbohydrates, proteins, minerals, and tannin together in aqueous medium at an appropriate alkaline pH from Sal (*Shorea robusta* Gaertn.) seed de-oiled cake rich in starch and fibre as insoluble constituents. Alkaline aqueous extract's pH was adjusted so as to coagulate the proteins as insoluble components at their iso-electric points. The supernatant obtained from the protein precipitation step containing the soluble carbohydrate, tannin, mineral was subjected to calcium hydroxide treatment which resulted in precipitating the tannin as calcium tannate leaving the supernatant consisting of soluble carbohydrates and minerals. This calcium tannate was hydrolysed by orthophosphoric acid to collect tannin in supernatant part and subjected to dryness for utilization purpose.

Keywords: Calcium tannate, Free tannin, Metal chelating activity, Radical scavenging activity, Reducing activity, Seeds, *Shorea robusta* Gaertn.

IPC code; Int. cl. (2015.01)– A61K 36/00, A61P 39/06

Introduction

Tannins, the derivative of polyhydroxy benzoate are regarded as a biopolymer of plant origin. They are found in tea, red wine, fruits, beverages and various medicinal plants[1-6]. Tannins have been reported to have considerable importance in the field of nutrition, health and medicine mainly due to their physiological activity such as antioxidant activity, antimicrobial effects and anti-inflammatory properties[7-9]. Sal (*Shorea robusta* Gaertn.) seed has been identified as an important source of biopolymers such as starch and tannin. It yields on an average 12 % fat and 88 % de-oiled material known as de-oiled cake (DOC) by weight when extracted with n-hexane at commercial level. Sal fat is a high priced commodity for its composition as well as properties and finds utilization as cocoa butter substitute (CBS) in chocolate formulation. The fat is fractionated to yield sal stearin which is more suitable as high priced CBS product on the other hand huge volume of DOC has very limited commercial value and is either sold at thrown away price or stacked in silos. In the absence of Sal DOC utilization, industries producing sal fat are economically affected. Some preliminary bench-scale investigations have been made for isolation and application of starch and tannins from Sal DOC[10] which contain substantial amount of biopolymers like starch (30-50 %) and tannin (8-17 %). Both have enormous scopes of industrial utilization. Sal DOC contains high amount of tannin and hence unsuitable as animal feed or for human consumption. To make it acceptable for consumption, tannin extraction is very important for which alcohol extraction process has been investigated earlier to isolate mainly tannin[11].

In case of utilizing starch of sal DOC, the only barrier is tannin which is very difficult to remove. Some approaches of removal such as using solvents, ammonia and other chemicals have been investigated. Investigation can also be envisaged to develop a new process technology for the recovery of tannin as metallic salt along with the isolation of other

*Correspondent author
E-mail: ivy.soma@gmail.com

constituents such as starch, protein, etc. but present study is more feasible due to easy process steps, high yield, easy storage and is economic.

Sal tannin has been examined for its use in making product like urea formaldehyde resin and as distinct antioxidant[12]. Since the tannins of plant origins form metallic salts having –OH and –CH groups[13], it could be appropriate to obtain a water insoluble metallic salt derivative of tannin, thereby offering a means of separation of tannin in solid or powdered form after drying. Among the metallic salts, calcium salt of tannin appears to be quite promising for separating tannin from a plant source.

Materials and Methods

The authentic DOC of *Shorea robusta* Gaertn. was provided by M/S Progressive Exim Ltd, Raipur, India. DPPH was purchased from Sigma Aldrich Chemical Co, New Delhi, India and all other chemicals were purchased from Sisco Research Laboratary, Mumbai, India.

Tannin precipitation process as metallic salt (calcium tannate)

Powdered Sal DOC (100 g) was treated with sodium hydroxide solution at pH 8.5 for 1.5 h and centrifuged at 5000 rpm for 15 min to obtain the bulk supernatant. The insoluble matter from the centrifuge was further washed with distilled water and the additional volume of supernatant was collected for further isolating tannin retained in the starch and fibre portion of the DOC as residue. The pH of the total supernatant collected was adjusted to an iso-electric pH 4.5 to precipitate the protein, which was separated by centrifugation. The supernatant part containing tannin was treated with finely powdered calcium hydroxide by stirring while maintaining the pH at 11.5 where by the whole of tannin precipitated out as water-insoluble metallic salt of calcium called calcium tannate. This metallic salt was finally dried under vacuum in a vacuum tray drier and powdered to store. Total tannin content was measured according to the method of Lowenthal's titration method[14].

Process for obtaining free tannin from calcium tannate

The insoluble calcium tannate salt can be decomposed by orthophosphoric acid to obtain equivalent free tannin. In order to release maximum tannin the pH was adjusted to 3-3.5 and centrifuged at 5000 rpm for 15 min. The supernatant containing the free tannin was adjusted to pH 7 to precipitate free phosphate and the residue as calcium phosphate was tested for calcium and phosphate ions and for tannin, if any. The supernatant containing tannin was evaporated to dryness to get the tannin from sal DOC as free tannin.

Antioxidant assays

Estimation of free radical scavenging activity

The free radical scavenging activity of tannin dissolved in distilled water was measured by DPPH. The 0.1 mmol/L solution of DPPH in methanol was prepared and 1 mL of this solution was added to 3 mL solutions of experimental tannin at concentration, 0.125–5.0 µg/mL. After 30 min absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. After running all the analyses in triplicate, results were averaged. Radical scavenging activity was expressed as inhibition percentage and was calculated using the formula of Shyamala *et al*[15]. Inhibition of free radical DPPH in percentage was calculated as follows:

% Radical Scavenging Activity =

(Control OD – Sample OD)/ Control OD × 100.

The radical scavenging activity (%) was plotted against the concentration. All tests and analyses were carried out in triplicate and averaged.

Estimation of reducing activity

Reducing activity was measured according to the method followed by Oyaizu[16]. Increased absorbance of the reaction mixture indicated increased reducing power. Different concentration of tannin sample (0.125- 5.0 µg/mL) of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M/L, pH 6.6) and potassium ferricyanide (2.5 mL, 1 %). The mixture was incubated at 50 °C for 20 min and 2.5 mL of 10 % Trichloroacetic acid was added to the mixture; 2.5 mL of this solution was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1 %), and left to stand for 10 min. The absorbance was measured at 700 nm. All analyses were run in triplicate and averaged.

Determination of metal chelating activity

The ferrous ion-chelating activity of studied samples was estimated based on the decrease in the maximal absorbance of the iron (Fe^{2+})–ferrozine complex according to previously reported methods by Shyamala[15] with some modifications. Briefly, 1 mL solution of test compound at various concentrations dissolved in distilled water was incubated with 0.5 mL of $FeCl_2.4H_2O$ (1.0 mM). The reaction was initiated

by the addition of 0.2 mL of ferrozine (5.0 mM), and then to the total reaction volume 3 mL ethanol was added. After the mixture had reached equilibrium (10 min), the absorbance at 562 nm was read. The control was prepared without the test compound. Fe^{2+}-chelating activity of the test compound was calculated from the following formula:

chelating activity (%) =

(Control OD – Sample OD) / Control OD × 100

The chelating activity (%) was plotted against the concentration. All tests and analyses were carried out in triplicate and averaged.

Results and Discussion

The isolation of tannins as occurring in Sal DOC has been attempted, investigating the condition for the precipitation of tannin as calcium tannate and later for the conversion of calcium tannate to free tannin by evaluating proper condition of decomposition of the calcium tannate salt.

Precipitation of tannin as Calcium tannate

The effect of pH on the precipitation of calcium tannate salt was studied. It was observed that as the pH of the solution increased the quantity of calcium tannate as precipitate also increased and at the pH around 11-11.5 reached the stage of complete precipitation of tannin as calcium tannate which was further confirmed by negative ferric chloride test of the supernatant. At pH 11–11.5 tannin precipitated completely and the supernatant did not respond to ferric chloride test indicating complete precipitation of tannin. The extractable tannin precipitated as calcium tannate was weighed (dry weight) which contained 37.38 % tannin.

Hydrolysis of Calcium tannate to free tannin

The decomposition of calcium tannate to free tannin was based on the pH of the aqueous medium in which the isolated calcium tannate was suspended. Addition of 8.5 % orthophosphoric acid decreased the pH to acidic range (pH 3-3.5) and favored release of free tannin along with precipitation of calcium phosphate. The free tannin accumulated and reached maximum amount at pH 3-3.5. Ferric chloride test indicated that calcium tannate completely decomposed at the pH of 3-3.5 with maximum isolation of free tannin and precipitate calcium phosphate as residue. The amount of tannin recovered by the present process is to the extent of 97.5 % of the extractable tannin content of Sal DOC.

Antioxidant assays

The total antioxidant capacity of the isolated tannin was evaluated using different assays like DPPH radical scavenging activity, reducing activity and metal chelating activity.

Free radical scavenging activity

DPPH is a stable free radical, which has been accepted as a tool for estimating free radical scavenging activity of natural extracts. DPPH assay method was based on the reduction of methanolic solution of coloured free radical DPPH by free radical scavenger. A dose response relationship was found in DPPH radical scavenging activity; the activity increased with an increase in concentration of the extract. The DPPH radical scavenging method was based on the reduction of methanolic DPPH solution in the presence of a hydrogen donating antioxidant, due to the formation of the non-radical form DPPH-H. Monitoring the decrease of the radical in terms of decreasing absorbance values, leads to the assessment of the antioxidant activity of the product. It was observed that the isolated tannins were able to reduce and decolour 1, 1-diphenyl-2-picrylhydrazyl efficiently as concentration increases in the experimental range, via their hydrogen donating ability. Fig. 1 shows the DPPH free radical scavenging activities of extracted tannin at different concentrations (1.25-5.0) µg/ml. *In vitro* assay showed that tannin exhibited higher antioxidant activity at higher concentrations and lower at lower concentration.

Reducing activity

Reducing capacity serve as a significant indicator of the potential antioxidant activity of any bioactive

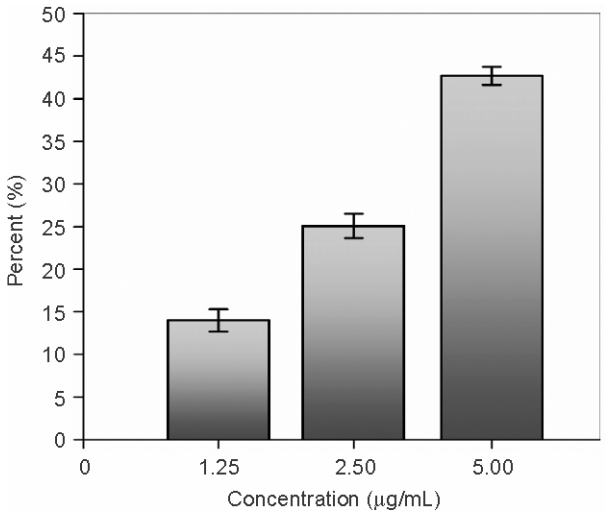

Fig. 1—Estimation of radical scavenging activity of isolated tannin

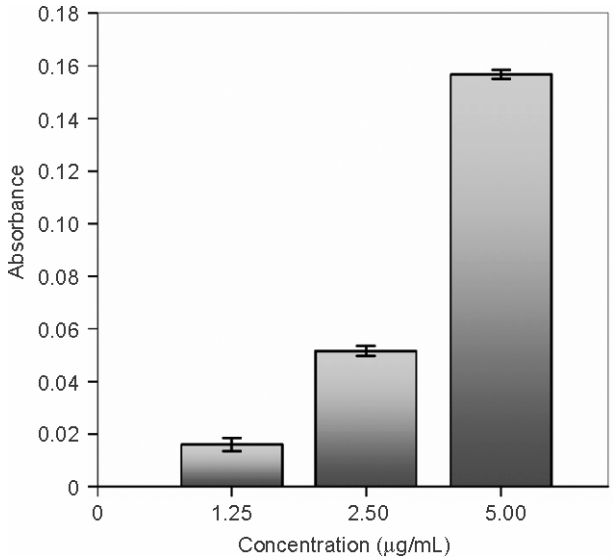

Fig. 2—Estimation of reducing activity of isolated tannin

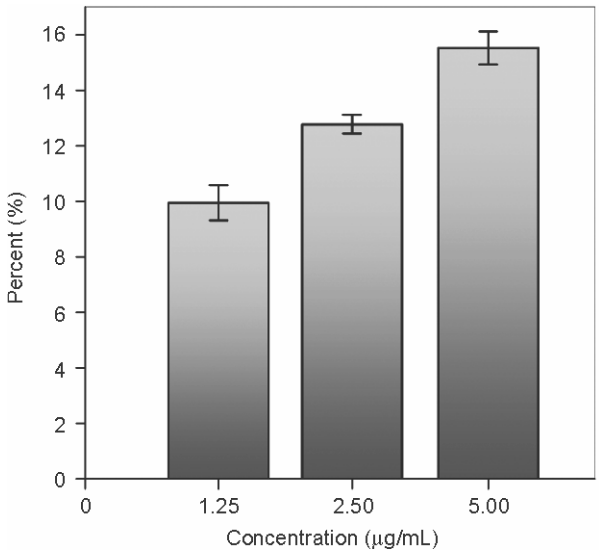

Fig. 3—Estimation of metal chelating activity of isolated tannin

species. Reduction potential bears a proportional dependency to the absorbance measured. Hence the measured absorbance serves to indicate the change in reduction potential of the tested species. The reducing power (transformation of Fe^{3+} to Fe^{2+}) of the antioxidant makes it an efficient electron donor, which can react with free radicals to convert them to more stable products, thereby terminating radical chain reactions. The antioxidant effect increases as a function of the development of reducing power indicating that the antioxidant properties are concomitant with the development of reducing power. Fig. 2 shows dose-response column for the reducing powers of the tannin that increases with concentration.

Metal chelating activity

Metal chelation activity is an example of a complexation reaction where Ferrozine [disodium salt of 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine] is a complex-forming agent of Fe(II) and will form a magenta complex Fe(II)-(Ferrozine)(III) with maximum absorbance at 562 nm. Hence in the presence of a reducing agent the complex formation is hampered resulting in decrease in the colour of the complex and hence a decrease in the absorbance. The isolated tannin showed higher metal chelating activity at higher concentrations as compared to lower concentrations depicted in Fig. 3. Iron chelators like tannin can prevent various processes of oxidative stress *in vivo*; including damage from heart reperfusion and liver injury in chronic iron overload[17].

Conclusion

A new process has been developed for the isolation of the tannin occurring in the de-oiled cake of Sal (*Shorea robusta* Gaertn.) seed in the form of calcium tannate in very high yield and purity. The process offers an advantage that the metallic tannate salt can be decomposed to free tannin readily and the byproduct i.e. calcium phosphate can be used for different purposes. The isolated tannin possesses adequate antioxidant activities such as radical scavenging, reducing and metal chelating activity.

Acknowledgement

The authors acknowledge the financial assistance provided by TRIFED, BESU, Shibpur (TRIFED Ref.No.TFD/HO/R&D/10/08-09/406 dt. 08.05.2009) and TEQIP Phase II, University of Calcutta dated 28.08.2012.

References

1 Hatano T, Constituents of natural medicines with screening effects on active oxygen species-Tannins and related polyphenols, *Nat Med*, 1995, **49**, 357–363.

2 Namiki M, Yamashita K and Osawa T, Active oxygen lipid peroxides and antioxidants, K Yagi, Ed, Japan Scientific Society Press, Tokyo, 1993, 319.

3 Okuda T, Tannins, a new family of bioactive natural organic compounds, *Yakugaku Zasshi*, 1995, **115**, 81–100.

4 Okuda T, Yoshida T and Hatano T, Phenolic compounds, *In*: Food and their effects on health II, Huang M T, Ho C T and Lee C Y, Eds, ACS Symposium Series, American Chemical Society, Washington, DC, 1992, 87.

5 Okuda T, Yoshida T and Hatano T, Antioxidant phenolics, *In*: Oriental medicine, Yagi K, Ed, Japan Scientific Society Press, Tokyo, 1993, 333.

6 Lim Y Y and Murtijaya J, Antioxidant properties of *Phyllanthus amarus* extracts as affected by different drying methods, *LWT-Food Sci Tech*, 2007, **40**, 1664-1669.

7 Sisti M, De Santi M, Fraternale D, Ninfali P, Scoccianti V and Brandi G, Antifungal activity of *Rubus ulmifolius* Schott standardized *in vitro* culture, *LWT-Food Sci Tech*, 2008, **41**, 946-950.

8 Santos-Buelga C and Scalbert A, Proanthocyanidins and tannin-like compounds-nature, occurrence, dietary intake, and effects on nutrition and health, *J Sci Food Agric*, 2000, **80**, 1094-1117.

9 Gulcin I, Oktay M, Kirecci E and Kufrevioglu O I, Screening of antioxidant and antimicrobial activities of anise (*Pimpinella anisum* L.) seed extracts, *Food Chem*, 2003, **83**, 371-382.

10 Bringi N V, Sal seed fat, *In*: Non-traditional Oilseeds and Oils in India, Oxford & IBH Pub Co Pvt Ltd, India, 1987, 1-55.

11 Sengupta S, Ghosh Chowdhury P, Chakraborty M M and Bhattacharyya D, Detoxification of Sal seed (*Shorea robusta*) meal, *Fette Seifen Anstrichmittel*, 1981, **83**, 152.

12 Das S, Bhattacharyya D and Ghosh M, Study on isolation of phytochemicals from sal and mango kernel meals by different solvents and evaluation of *in-vitro* antioxidant activities, *J Int Acad Res Multidiscipl*, 2015, **3**, 269-282.

13 Windhausen O, Bartschat Fr, Bodländer E, Grimme Cl, Großfeld J, van Eck J J and Tillmans J, *Zeitschrift für Untersuchung der Lebensmittel*, 1926, **51**,160-184.

14 Abbhi V, Joseph L and George M, Phytochemical analysis of fruit extract of *Myrsine africana, Int J Pharm Pharmaceut Sci*, 2011, **3**, 427-430.

15 Shyamala B N, Gupta S and Lakshmi A J, Prakash J, Leafy vegetable extract–antioxidant activity and effect on storage stability of heated oils, *Innovat Food Sci Emerg Tech*, 2005, **2**, 239-245.

16 Oyaizu M, Studies on product of browning reaction prepared from glucose amine, *Japanese J Nutr*, 1986, **44**,307-315.

17 George K, Lopes B, Schulman H M and Hermes Lima M, Polyphenol tannic acid inhibits hydroxyl radical formation from Fenton reaction by complexing ferrous ions, *Biochimica et Biophysica Acta,* 1999, **1472**, 142-152.