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Occurrence of antioxidant and radical scavenging proanthocyanidins from the Indian minor spice nagkesar (*Mammea longifolia* planch and triana syn)

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Abstract—Nagkesar (buds of *Mammea longifolia*) is extensively used in culinary preparations especially in spice blend in India. Previously thirteen compounds were identified from the medium polar fractions of methanol extract of buds of *M. longifolia*. In continuation of the study, the polar fraction of methanol extract exhibited stronger antioxidative and radical scavenging activities. An attempt was made to separate and identify the active compounds and found that those were proanthocyanidin oligomers with mean degree of polymerisation ranges from 2 to 10. This is the first report to indicate that *Mammea* buds contain antioxidant and radical scavenging procyanidin oligomers.

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1. Introduction

Buds of *Mammea longifolia* Planch & Triana syn (Guttiferae) are well known in India as Nagkesar (Hindi). The flower buds of *M. longifolia* are extensively used in culinary preparations especially as a minor spice in various spice blends, such as Garam Masala powder, in India for flavoring foods.[1] Dried buds are used as substitutes to cloves in making 'Pan Masala', which is a chewing product in India to improve digestion after heavy meal.

Currently, the use of some naturally occurring antioxidant molecules and their identicals in foods as well as preventive and therapeutic medicine is gaining popularity. It had been found that the methanol extract from the buds of *M. longifolia* showed a strong radical scavenging activity on DPPH radical scavenging assay and xanthine-xanthine oxidase system chemiluminescence assay.[2] Our earlier paper reports the isolation of 13 compounds from the methanol extract, which include two new acylated quercetin monoglycosides.[2] In continuation of our earlier studies, polar fractions from column chromatography of methanol extract showed the highest potential for the above activity.[3] An attempt has been made to isolate and identify the compounds responsible for the radical scavenging activity in these fractions. The results are presented here.

2. Results and discussion

The methanol extract (8.5%) of dried buds (after defatting) on column chromatography over silica gel yielded nine fractions (Table 1). Highly polar fraction eluted with methanol showed the highest antioxidant activity on evaluation using chemiluminescence assay. This fraction had UV absorption maximum at 280 nm (0.001% in methanol) and gave pinkish-red color, when treated with methanolic hydrochloric acid at 60–80 °C, indicating it to contain proanthocynidins.[4–6] Part of the active fraction was subjected to column chromatography on LH-20, using the standard procedure to obtain a pure proanthocyanidin fraction.[7] The fraction obtained using acetone–water (7:3), showed the highest antioxidant activity (Table 2) and was labeled as PA-1 (yield ~0.22% based on dried buds).

Keywords: *Mammea longifolia*; Guttiferae; Nagkesar; Radical scavenging activity; DPPH; Chemiluminescence; Flavonoids; Proanthocyanidins.

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Table 1. Fractionation of methanol extract of buds of *Mammea longifolia* on silica column and chemiluminescence assay of the fractions

Serial no.	Eluting solvent $CHCl_3/MeOH$	Fraction yield (%)	Rutin (1 mg) equiv.
1	100:0	6.59	13.93
2	95:5	7.20	3.79
3	90:10	6.25	5.58
4	85:15	8.74	5.27
5	80:20	10.29	8.11
6	70:30	11.57	5.40
7	60:40	9.55	3.33
8	50:50	7.53	2.93
9	0:100	32.28	1.41

Table 2. Fractionation of most active fraction 9 (Table 1) on Sephadex- LH20

Fraction no.	Solvent	Fraction yield (%)	50%DPPH[a]
1	M/W (1:1)	78.68	0.340
2	M/W (1:1)	1.87	0.055
3	A/W (7:3)	13.21	0.053
4	A/W (1:1)	0.14	0.065
Loss		6.1	

W, water; M, MeOH; A, acetone.

[a] Amount of the fraction (mg) required to scavenge 50% of DPPH.

Table 3. ^1H and ^{13}C NMR data of PA-2

Signal no.	^{13}C δ	Carbon	^1H δ
Solvent	Acetone-d_6 + D_2O (1:1)		
1	155.23	5,7 and 8a	
2	144.44	3′ and 4′	
3	131.30	1′	
4	118.73	6′	6.6–7.0
5	115.79	2′ and 5′	6.6–7.9
6	106.70[a]	8 (substituted)	
7	101.08	4a	
8	96.57	6 and 8 (unsubstituted)	5.8–6.2
9	76.16	2 (2,3-cis)	4.9–5.6
10	71.55	3 (chain extender units)	3.7–4.3
11	66.18	3 (terminal unit)	3.75–4.0
12	36.69	4	4.3–4.8

[a] Major.

Large-scale separations using chromatographic techniques on silica gel is not a viable proposition due to various reasons such as economic feasibility and, in an alternate approach, proanthocyanidins were obtained by precipitation method using acetone and methanol directly from the methanol extract of buds of *M. longifolia*. Initially, insoluble material and sugars were precipitated and removed. Later by addition of excess amount of acetone to the filtrates, proanthocyanidins got precipitated along with remaining sugars. The filtrate contained mostly low polar molecules and flavonol glycosides. Sugars included in the precipitate were rinsed away by water and the remaining precipitate was labeled as PA-2 (~0.78% based on dried buds). PA-1 and PA-2 had UV absorption maxima at 280 nm and gave pinkish-red color, when treated with methanolic hydrochloric acid at 60–80 °C. The antioxidant activity of both these conserves (PA-1 and PA-2) was screened using both chemiluminescence and DPPH assays, and their activity was found to be very high, when compared to standard rutin as well as original methanol extract.

PA-2 was subjected to ^{13}C NMR analysis. ^{13}C NMR spectrum of PA-2 strongly supported the presence of proanthocynidins (Table 3). Assignments of the resonance observed were made using those reported in the literature for model compounds and isolated polymer preparations.[8–10] Resonances centered at δ155.2 (150–159) ppm correspond to C-5, C-7 and C-8a of the aromatic A-ring of the flavon-3-ol units (Fig. 1). Unsubstituted carbons in the A-ring, C-8 and C-6, are assigned to the signal centered at δ 96.6 (94–98) ppm. The typical interflavonoid linkage of C-4 to C-8 is represented by the resonance centered at δ 106.7 (105.5–107.5) ppm assigned to substituted C-8 (Fig. 1). The ^{13}C NMR spectra revealed that the proanthocyanidins are predominantly of the procyanidin type. The signal centered at δ 144.4 (142-146.5) ppm is attributed to C-3′ and C-4′ in the B-ring of the procyanidin flavan-3-ol units. Other aromatic resonances are assigned as follows: δ 101.08 ppm, C-4a; 115.79 (112.5–117.5) ppm, C-2′ and 5′; 118.7 (117.5–120.5) ppm, C-6′; 131.3 (129–133) ppm, C-1′. Signal centered at δ 76.2 (75–78) ppm was assigned to C-2 and indicating 2,3-cis chain extender units. Resonance centered at δ 36.7 (35.0–38.5) ppm is assigned to C-4. The signals centered at δ 71.6 (69.0–73.5) ppm and 66.2 (65.5–67.0) ppm correspond to C-3 chain extender and terminal units, respectively. These assignments clearly supported that the purified conserves contained the mixture of proanthocyanidins.

Final confirmation of the structures for these purified conserves was obtained from thiolysis degradation.[11–16] The thiolysis condition, at 55 °C for 30 min, was suitably optimized after conducting at various temperatures. Under these conditions, the conversion of catechin to epicatechin, epicatechin to catechin and epicatechin to its benzylthioether are minimum. By thiolysis reaction, proanthocyanidin terminal units are released as flavan-3-ols, where extension units are released as their benzylthioether derivatives. These are identified as epicatechin and benzylthioether of epicatechin by comparison of retention times on HPLC (Fig. 2) with that of the authentic sample of epicatechin and the thiolysis products obtained from authentic procyanidin B-2. It confirmed the proanthocaynidin mixture was made up of epicatechin linked from C-4 to C-8. The mean degree of polymerization was determined by the calculating the ratio of peak areas of epicatechin and its benzylthioether divided by the peak area ratio of the same peaks obtained from thiolysis of procyanidin B-2. The mean degree of polymerization (mDP) was found to be 4.44 and 7.91, respectively, for PA-1 and PA-2. Hence an attempt was made to further fractionation of PA-2 using sea sand[14] and LH-20.[7] The obtained fractions were subjected to thiolysis after removal of solvents by evaporation under vacuum and water by lyophylization. Using of the sea sand did not make a chromatographic separation but only a fractionated solubilization of condensed proanthocyanidins.[14] The

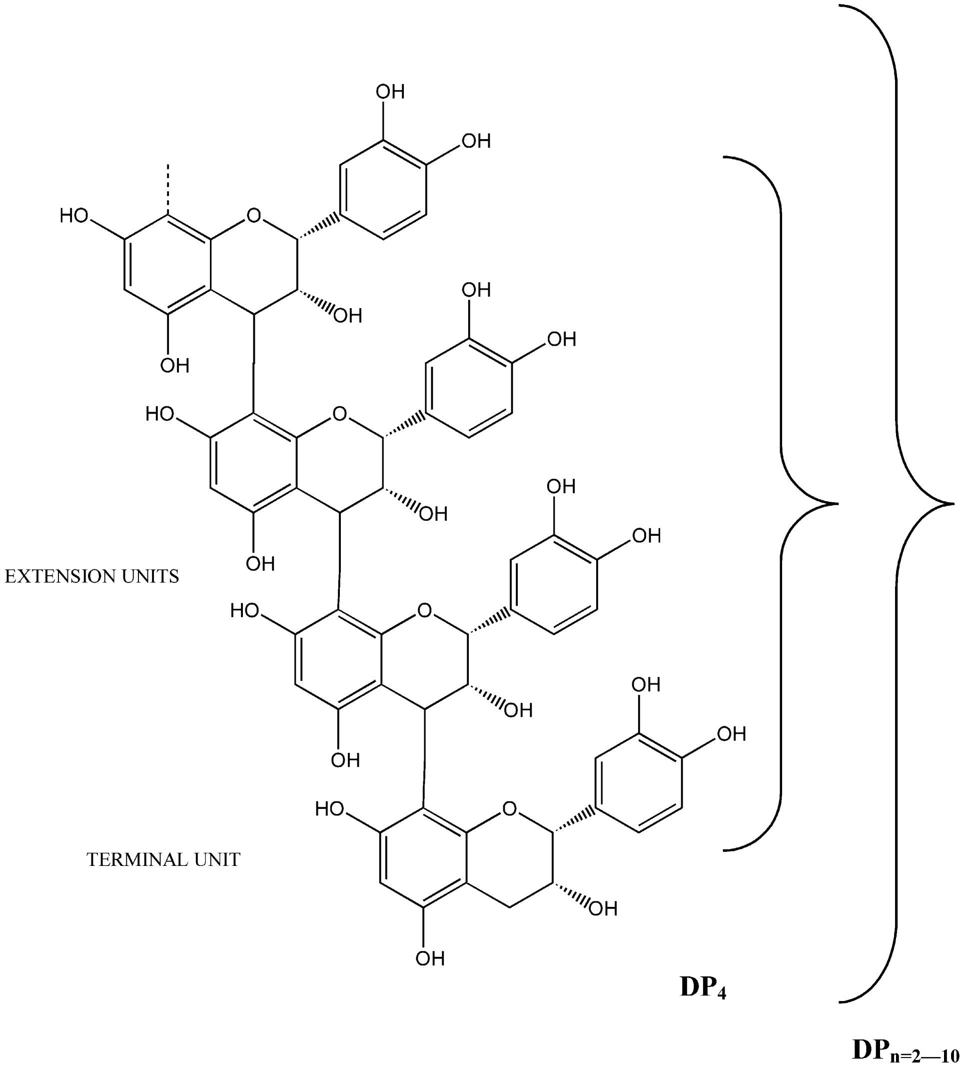

Figure 1. Structure of procyanidin type proanthocyanidin with C4–C8 linkage.

Table 4. Fractionation of PA-2 on sea sand

Fraction no.	Solvent	Fraction yield (%)	MDP
S-1	C/M (8:2)	4.4	2.59
S-2	C/M (7:3)	1.3	3.81
S-3	C/M (6:4)	4.4	5.78
S-4	C/M (1:1)	29.95	7.04
S-5	C/M (4:6)	27.8	8.32
S-6	C/M (3:7)	18.2	8.78
S-7	M	12.75	9.56
Loss		1.0	

C, chloroform; M, MeOH.

Table 5. Fractionation of PA-2 on Sephadex- LH20

Fraction no.	Solvent	Fraction yield (%)	MDP
LH-1	W	8.55	9.32
LH-2	W/M (8:2)	13.20	9.09
LH-3	W/M (6:4)	23.85	8.82
LH-4	W/M (4:6)	10.40	8.30
LH-5	W/M (2:8)	7.45	6.63
LH-6	M	18.15	7.65
Loss		18.40	

W, water; M, MeOH.

thiolysis results from sea sand fractionated fractions indicated that mDP varied from 2.6 to 9.6 (Table 4). Gradual increase of mDP was observed with the increase of methanol content in the chloroform elute, because separation based on the solubility was occurred. The thiolysis result indicated mDP of LH-20 chromatographic fractions varies from 9.3 to 6.6 (Table 5). In this case, gradual decrease of mDP was observed with the increase of methanol content in the water elute because LH-20 chromatography is based on gel filtration with absorption process. Finally, MDP in PA mixture is concluded as 10.

3. Conclusion

In the present work it was found that the buds of *M. longifolia* possess antioxidant and radical scavenging

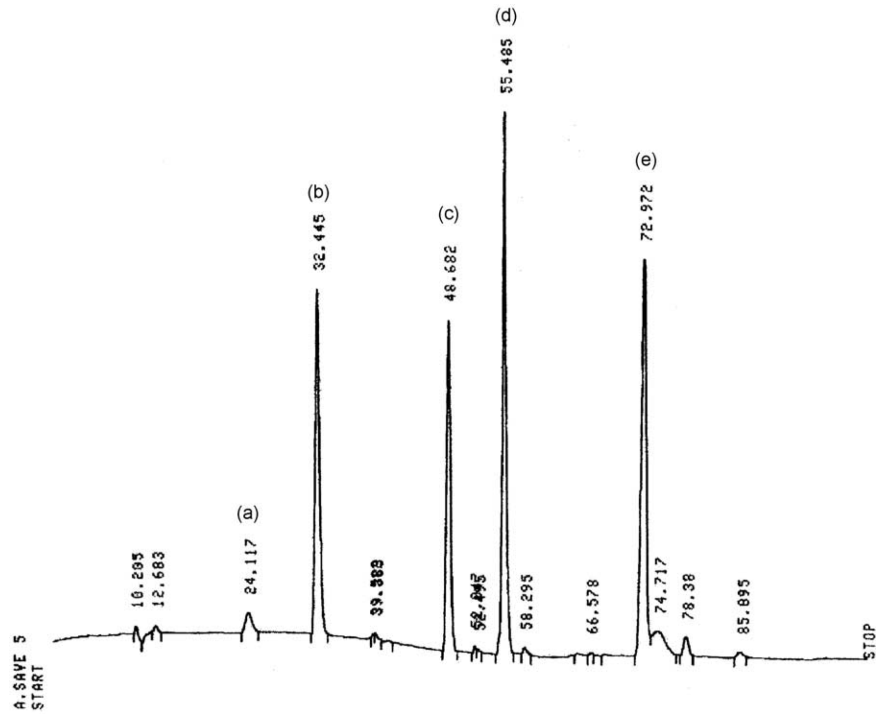

Figure 2. HPLC chromatogram of thiolysis products: (a) Catechin; (b) *epi*-Catechin; (c) *p*-hydroxy benzoic acid methyl ester; (d) benzyl thioether of *epi*-catechin; (e) benzylmercaptan.

activities. The most active fraction was isolated systematically in first attempt and later it was isolated by precipitation method basing on solvent-solvent partition that could be useful for large-scale operations. The most active fraction was subjected to spectral analysis and chemical degradation and found to contain proanthocyanidin oligomers with degree of polymerisation ranges from 2–10. This is the first report of proanthocyanidins from *M. longifolia*. Plant proanthocyanidins are known as the functional food factors that possess a variety of physiological activities such as antioxidant, antimicrobial, anti-allergy, hair-growth promotion, anti-caries, anti-hypertensive and inhibition against activities of some enzymes and receptors.[17] Proanthocyanidins have attracted increasing attention recently, due to the rapidly growing body of evidence associating these compounds with a wide range of potential health benefits as mentioned above. Further, these groups of compounds are associated with potential cardiovascular benefits, including the reduction of platelet aggregation, and reduction of tumor multiplicity in laboratory mice.[18]

4. Experimental

4.1. Plant material

Dried samples of plant material were purchased from a local market in Mysore, India. Samples were identified and voucher specimens were deposited in the herbarium of the Botany Department, University of Mysore, India.

4.2. Extraction of plant material

Powdered dried buds (250 g) were extracted with methanol after defatting with hexane, and the solvent was evaporated to yield 8.5% by weight of extract. This extract was subjected to chemiluminescence assay and DPPH assay for radical scavenging activity.

4.3. DPPH (1,1-diphenyl-2-picrylhydrazyl radical) assay

An aliquot (15 μL) of methanol solution of sample was transferred to the first column of wells of a microplate, and wells in the other columns were filled with serial dilution of the sample (15 μL) with dimethylsulfoxide. One hundred micro molar DPPH solution in ethanol–water (1:1) (185 μL) was added to each well, and absorbance at 550 nm was measured after standing for 5 min.

4.4. Chemiluminescence assay

The chemiluminescence assay for radical scavenging activity was performed as previously reported.[2] One millimolar xanthine solution was prepared by dissolving 1.52 mg of xanthine (Sigma, St. Louis, MO,USA) in 10 mL of 0.5 M Tris–HCl buffer containing 100 μM EDTA. The xanthine solution (1.5 mL) was mixed with the 0.5 M Tris–HCl buffer (13.5 mL) and 3 mM 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*] pyrazin-3- one hydrochloride (MCLA) (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) aqueous solution (5 μL). Five units·mL^{-1} suspension of xanthine oxidase (Wako Pure Chemical Industries) (1.5 μL) was mixed with the 0.5 M Tris–HCl buffer (2 mL) and diluted to

20 mL with water. The xanthine-MCLA solution (100 μL) and xanthine oxidase solution (100 μL) were added to sample solution (10 μL) in each well on a microplate, and chemiluminescence was measured after 19–20 s on an AB-2000 (Atto, Tokyo, Japan).

4.5. Chromatography of active extract

Methanol extract of *M. longifolia* buds was subjected to column chromatography on silica gel (10 times w/w) using $CHCl_3$–MeOH with increasing polarity. Among the nine fractions, the fraction eluted with methanol, showed highest activity in chemiluminescence assay (Table 1).

4.6. Separation of active fraction [proanthocyandin (PA) mixture] by Sephadex-LH20 chromatography

Part of the methanol-eluted fraction was dried and dissolved in methanol (M)–water (W) (1:1). Insoluble (yield in weight 40%) was separated, and soluble (60%) was subjected to a Sephadex-LH20 column (3 cm × 15 cm). The fractions were eluted as follows: Fr 1 M–W (1:1, 300 mL, yield in weight, 78.7%); Fr 2 M–W (1:1, 300 mL, 1.9%); Fr 3 Acetone (A)-W (7:3, 200 mL, 13.2%); Fr 4 A–W (1:1, 200 mL, 0.1%). Solvent was removed by evaporation at 35 °C under vacuum, and water was removed by freeze-drying. Chromatographic fractions were lyophilized by FDU-810 (Tokyo Rika Kikai Co). Fraction 3 (Table 2; 2.56% of methanol extract) showed the highest antioxidant activity and was labeled as **PA-1**.

4.7. Direct separation of active fraction by precipitation method from methanol extract

Methanol extract of *M. longifolia* buds was dissolved in methanol (17.5 times w/v). Insoluble material (9% of methanol extract) was separated by filtration. Equal amount of acetone was added to the filtrate, and the mixture was kept over night in a cold room (7–10 °C). Precipitate was removed by filtration. The filtrate was concentrated and kept over night at lower temperature (7–10 °C). The supernatant was concentrated after removal of precipitates formed by addition of excess amount of acetone. The procedures were repeated three times. The final precipitate (23% of methanol extract) was washed by water and labeled as **PA-2** (9.4% of methanol extract).

4.8. NMR

^{13}C NMR spectrum of PA-2 was recorded on DRX600 spectrometer (Bruker, Karlsruhe, Germany) at a resonance frequency of 150.90 MHz and assignments are provided in Table 3.

4.9. Thiolysis of proanthocyanidin mixtures and HPLC analysis of the products

One mg of the sample was dissolved in 1 mL of absolute methanol, and 1 mL of thiolysis reagent (5% solution of phenylmethanethiol (Wako Pure Chemical Industries, Osaka, Japan) in 0.2 M HCl was added. The reaction mixture was stirred at 55 °C for 30 min. *p*-Hydroxy benzoic acid methyl ester (Wako Pure Chemical Industries) (0.005% in reaction mixture) was used as an internal standard. The products were analyzed by HPLC using a high-pressure gradient dual pump system with LC-6AD pumps (Shimadzu, Kyoto, Japan) and a Cosmosil $5C_{18}$-MS (6 × 250 mm) column (Nacalai Tesque, Kyoto, Japan). Elution was at 0.5 mL/min flow rate under linear gradient from 15 to 75% acetonitrile–water–formic acid (80:18:2 v/v/v) in water–formic acid (98:2, v/v) over 100 min. Detection was at 280 nm on an 875-UV Intelligent UV/Vis detector (Jasco, Tokyo, Japan). Products were identified by comparison with an authentic sample of epicatechin and thiolysis products from procyanidin B-2 [(−)epicatechin-(4β→8)-(−)epicatechin] on the HPLC.

4.10. Fractionation of PA-2 depending on solubility

4.10.1. Using sea sand. Two hundred milligrams of PA-2 was dissolved in 2 mL of methanol and loaded on the top of sea sand (30–50 mesh, 140 g) in a glass column, which had been pre-equilibrated with 125 mL of chloroform–methanol (8:2). PA was precipitated on the top of the column by adding 8 mL of chloroform. Seven fractions were eluted using chloroform–methanol (8:2; 7:3; 6:4; 1:1; 4:6; 3:7—100 mL each) and methanol (200 mL) for the last fraction. During the increase of methanol ratio in the eluting solvent, PA got dissolved and eluted through the column. Solvent was removed by vacuum evaporation using rotary evaporator, and yield of each fraction was shown in Table 4. These fractions were subjected to thiolysis and mean degree of polymerization (mDP) values were calculated.

4.10.2. Using Sephadex-LH 20 with MeOH-H_2O. PA-2 (200 mg) was dissolved in minimum amount of methanol and loaded on the top of Sephadex LH-20 (2 cm × 5 cm) equilibrated with water. Six fractions were eluted using water-methanol (10:0; 8:2; 6:4; 4:6; 2:8–20 mL each) and methanol (100 mL) for the last fraction. PA was precipitated on the column when water was added for the first elution solvent. During the increase of methanol ratio in the eluting solvent, PA got dissolved and eluted through the column. All six fractions were freeze-dried after removal of solvent by vacuum evaporation, and yield of each fraction was shown in Table 5. These fractions were subjected to thiolysis, and mDP values were calculated.

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