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Antioxidant activity of *Mammea longifolia* bud extracts

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Abstract

Mammea longifolia buds (nagkesar) are extensively used in India as a minor spice. The antioxidant activity of its methanol (NM) and aqueous-ethanol (NW) extracts were evaluated by several in vitro experiments, e.g., DPPH, hydroxyl, superoxide radicals and H_2O_2-scavenging assays as well as inhibition of Fe(II)-induced lipid peroxidation of rat liver mitochondria. The extracts were found to possess impressive antioxidant activity in all the tests, the activity of NW being higher than that of NM in most of the assays. The differential activities of NW and NM could be correlated with their respective total phenolic, flavonoid and proanthocyanidin contents.

Keywords: *Mammea longifolia* buds; Antioxidant activity; Spice

1. Introduction

Reactive oxygen species (ROS), including superoxide radicals, hydroxyl radicals, singlet oxygen and hydrogen peroxide, are often generated as by products of biological reactions or from exogenous factors (Cerutti, 1991). In vivo, some of these ROS play a positive role, such as energy production, phagocytosis, and regulation of cell growth and inter-cellular signalling, or synthesis of biologically important compounds (Halliwell, 1997). However, ROS may also be very damaging as they can induce oxidation of lipids and DNA, causing membrane damage, decreasing membrane fluidity, and leading to cancer via DNA mutation (Cerutti, 1994; Pietta, 2000). A potent scavenger of these ROS may serve as a possible preventive intervention of free radical-mediated diseases (Ames, Gold, & Willet, 1995).

Recent studies show that several plant products, including polyphenolic substances (e.g., flavonoids and tannins) and various plant or herb extracts, exert antioxidant actions (Kahkonen et al., 1999; Liu & Ng, 2000; Yokozawa et al., 1998). To this end, medical and nutritional experts, in recent years, have seriously noted the antioxidant properties of food constituents . Several dietary compounds are known to show antioxidant activity, primarily through their radical-scavenging potential. Amongst dietary sources, spices and condiments possibly have the best potential, as these are widely used in traditional recipes as adjuncts to enhance the flavour, colour and taste of food preparations.

The buds of *Mammea longifolia* Planch and Triana syn (Guttiferae) are well known in India as nagkesar and are used as a minor spice. *M. longifolia* is a large tree, found in southwestern India, bearing annual flowers. The flower buds are stimulant, carminative and astringent, and are used in the treatment of dyspepsia and hemorrhoids (Chopra, Chopra, & Varma, 1969). The dried flower buds resemble enlarged clove buds, and are extensively used in culinary preparations, especially in spice blends and *garam masala* powders and as a substitute for cloves in making pan masala, which is a digestive chewing product in India.

Very recently, the methanol extract of *M. longifolia* buds (nagkesar) was found to scavenge DPPH and superoxide radicals (Rao, Yada, Ono, Ohnishi-Kameyama, & Yoshida, 2004). In view of our interests in developing natural antioxidants from dietary sources (Joshi, Adhikari, Patro, Chattopadhyay, & Mukherjee, 2001; Patro et al., 2002), a

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detailed study on the antioxidant property of the methanolic and aqueous-alcoholic extracts of nagkesar was undertaken. The results of the study, which varied from the previous report, are presented in this paper.

2. Materials and methods

2.1. Reagents and solutions

Dry flower buds of *M. longifolia* (nagkesar) were purchased from the local market. Ascorbic acid, ferrous ammonium sulfate, 2-thiobarbituric acid (TBA) and 2-deoxyribose (DR) were obtained from Himedia Lab., India. H_2O_2 (35%) was purchased from Lancaster (England), while Fe(III) chloride and trichloroacetic acid (TCA) were from Thomas Baker, India. Other materials used were EDTA, hydroxylamine, sulfanilic acid, *N*-(1-naphthyl)ethylenediamine dihydrochloride (NEDD) (all from Sarabhai Chemicals, India), butylated hydroxyanisole (BHA) (BDH, England), horseradish peroxidase (HRPO) (SRL, India), xanthine oxidase (type IV, from butter milk, sp. Act. 0.067 U/mg protein), xanthine and 1,1-diphenyl-2-picrylhydrazyl (DPPH) (all from Sigma, U.S.A.). All solutions, including the stock solution of ferrous ammonium sulfate, were freshly prepared in triply distilled water. Stock solutions of test extracts and compounds (3 mg/ml) were prepared in ethanol or 5 mM aqueous NaOH solution, followed by neutralization with aqueous HCl. Appropriate blanks were used for the individual assays.

2.2. Instrumentation

The absorbance spectrophotometry was carried out at 25 °C, using a Jasco V-550 UV–Vis spectrophotometer. Wavelength scans and absorbance measurements were made in 1 ml quartz cells of 1 cm path length.

2.3. Preparation of the plant extracts

The dry flower buds of *M. longifolia* (nagkesar) (20 g) were powdered with a grinder, extracted successively with methanol and aqueous-alcohol (each 150 ml) for four days and the supernatants, in each case, were decanted. The entire process was repeated thrice, each of the combined supernatants was filtered through a nylon mesh and evaporated in vaccuo to obtain the respective extracts. These were designated as nagkesar methanolic (NM) and aqueous extracts (NW), respectively, and stored in a vacuum desicator.

2.4. DPPH scavenging assay

An ethanolic solution of DPPH (100 μM) was incubated with an ethanolic solution of each of the test samples (NM and NW) (0–10 μg/ml) and the absorbance monitored spectrophotometrically at 517 nm. The percentage reduction of the DPPH absorbance during a 30 min period was taken as a measure of its free radical-scavenging activity (Mellors & Tappel, 1966).

2.5. Anti-lipid peroxidation (LPO) assay

Lipid peroxidation of rat liver mitochondria was carried out as reported earlier (Patro et al., 2002) with minor modifications. For preparation of mitochondria, liver of male Wistar rats (250 ± 20 g) were excised and homogenized in a sucrose solution (0.25 M) containing EDTA (1 mM). The homogenate was centrifuged at 3000*g* for 10 min and the supernatant centrifuged thrice at 10,000*g* for 10 min. The sedimental mitochondria pellet was washed thrice with potassium phosphate buffer (pH 7.4, 0.05 M), resuspended in the same buffer at a final concentration of 20 mg/ml and the protein content estimated by Lowry's method. The mitochondrial lipid peroxidation was initiated by addition of Fe(II) (50 μM) and ascorbate (200 μM) to the reaction mixture containing the following components at the final concentration stated: mitochondrial fraction (4.0 mg protein/ml) and test samples in 50 mM potassium phosphate buffer, pH 7.4. The mixture was incubated at 37 °C for 30 min; TCA–TBA–HCl (2 ml, 15% TCA, 0.375% TBA, 0.25 M HCl) solution was added, the mixture boiled at 100 °C for 10 min and the absorbance at 532 nm read.

2.6. Hydroxyl radical-scavenging assay

The assay was performed as described (Halliwell & Gutteridge, 1981). The reaction mixture (1 ml) contained DR (2.8 mM), Fe(III) chloride (20 μM), EDTA (100 μM) [EDTA and Fe(III) chloride were mixed prior to the addition of DR] and H_2O_2 (200 μM) without or with the test extracts (0–1000 μg/ml) in 10 mM potassium phosphate buffer, pH 7.4. The reaction was triggered by adding ascorbate (300 μM) and subsequent incubation of the mixture for 1 h at 37 °C. Solutions of Fe(III) chloride, ascorbate and H_2O_2 were prepared in deaerated water just prior to use. A solution of TBA in 50 mM NaOH (1 ml, 1% w/v) and TCA (1 ml, 2.8% w/v aqueous solution) was added, the mixture heated for 15 min on a boiling water bath and the amount of chromogen produced was spectrophotometrically measured at 532 nm.

2.7. Hydrogen peroxide-scavenging assay

The procedure employed was essentially the same as described earlier (Pick & Keisari, 1980). The buffered phenol red solution (PRS) used in all the assays contained: 140 mM sodium chloride, 5.5 mM dextrose, 0.28 mM phenol red and 8.5 U/ml of HRPO in 10 mM potassium phosphate buffer, pH 7.0. Phenol red and HRPO were added to the buffer shortly before carrying out the experiment. Different concentrations of NM and NW and H_2O_2 (60 μM) were incubated at 25 °C for 30 min and the concentration of H_2O_2 remaining in each case was assayed by adding PRS, followed by NaOH (10 μl, 1 N), and measuring the

absorbance at 610 nm against an appropriate blank. The concentration of the H_2O_2 stock solution was calculated from its absorbance at 230 nm, using as the extinction coefficient 81 mol^{-1} cm^2. α-Tocopherol was used as a reference inhibitor.

2.8. Superoxide radical-scavenging assay

Superoxide radicals were generated enzymatically by xanthine/xanthine oxidase, as described previously (Cos et al., 1998). Test solutions were prepared by adding xanthine (final concentration 50 μM), hydroxylamine (final concentration 0.2 mM), EDTA (final concentration 0.1 mM) and the test samples at various concentrations (0–30 μg/ml). The reaction was initiated by adding a solution (0.2 ml) of xanthine oxidase (6.25 mU/ml) in phosphate buffer (20 mM, pH 7.5). After incubating the mixture (total volume 1 ml) for 30 min at 37 °C, HCl (0.2 ml, 0.1 M) was added to stop the reaction. The amount of uric acid produced was measured spectrophotometrically from the UV absorbance at 295 nm (Noro, Oda, Miyase, Ueno, & Fukushima, 1983). A test mixture without the extracts was prepared to measure the total uric acid production. The concentration of uric acid produced was calculated from the differential absorbance with a blank solution in which xanthine oxidase was replaced by the buffer solution. To measure superoxide, the colouring reagent (final concentration of 300 μg/ml sulfanilic acid, 5 μg/ml of NEDD and 16.7% (v/v) acetic acid) was added at the end of incubation. The mixture was allowed to stand at 25 °C for 30 min and the absorbance at 550 nm was measured (Oyanagui, 1984). BHA was used as a reference inhibitor.

2.9. Estimation of the total phenolic contents in NM and NW

The method of Singleton and Rossi (1965) downscaled to 1 ml final volume was followed to determine the amounts of total phenolics in the extracts. The NM and NW extracts (each 100 μl) were mixed with 500 μl of 1:10 Folin–Ciocalteau's reagent, followed by addition of Na_2CO_3(400 μl, 7.5%). After incubating the reaction mixture at 24 °C for 2 h, the absorbances at 765 nm were recorded. Gallic acid monohydrate was used as the standard. The total phenolic contents of NM and NW were expressed as gallic acid equivalents (GAE) mg/g dry weight of the extracts.

2.10. Estimation of the total proanthocyanidin contents in NM and NW

A known method of Porter and Rossi (1986) was used with minor modifications. The individual extract (0.25 ml) was added to *n*-butanol–HCl (3 ml, 95:5) in stoppered tubes, followed by addition of a 0.1 M solution of $NH_4Fe(SO_4)_2 \cdot 12\,H_2O$ in 2 M HCl. After incubating the reaction mixture at 95 °C for 45 min, the absorbances at 550 nm were read. The relative proanthocyanidin contents in NM and NW were assessed from the respective absorbances.

2.11. Estimation of the total flavonoid contents in NM and NW

Following a known method (Jia, Tang, & Wu, 1999) downscaled to 1 ml, the individual extract (100 μg) was added to 0.4 ml distilled water, followed by $NaNO_2$ (0.03 ml, 5%). After 5 min at 25 °C, $AlCl_3 \cdot 6\,H_2O$ (0.03 ml, 10%) was added. The reaction mixture was treated with NaOH (0.2 ml, 1 M) after 6 min, diluted to volume (1 ml) with water and, after a thorough mixing, the absorbance at 510 nm was read. Epicatechin was used as the standard and the total flavonoid contents of NM and NW were expressed as epicatechin equivalents (ECE) mg/g dry weight of the extracts.

3. Results and discussion

3.1. General

Several epidemiological studies provide evidence of the protective effect of the consumption of fresh fruits and vegetable against pathogenesis. The preventive role of these foods is due to their constituent chemicals, especially the polyphenolic flavonoids, anthocyanins and anthocyanidins (Pietta, 2000). The total intake of these phytochemicals through the food chain can reach up to 1 g/day for humans (Ho, 1995). The spice, nagkesar (*M. longifolia*) is extensively used as a minor spice in India without any reported toxicity, and is also reported to be a rich source of flavonoids, proanthocyanidins and coumarins (Rao, Yada, Ono, & Yoshida, 2002, 2004). Consequently, we studied its antioxidant activity by a series of in vitro protocols using some biologically relevant models. For this, the plant constituents were partitioned as the methanol (NM) and ethanol–water (NW) extracts. We studied their respective antioxidant activities and attempted to correlate the results in terms of their total phenolic, flavonoid and anthocyanidin contents. The results, as discussed below, differed from those of the earlier preliminary reports that were based on chemical systems.

3.2. DPPH-scavenging activities of NM and NW

The bleaching of DPPH absorption (517 nm) by a test compound is representative of its capacity to scavenge free radicals, generated independently of any enzymatic or transition metal-based systems. The DPPH assay results are known to correlate well with the lipid peroxidation inhibitory capacity of a test compound (Rekka & Kourounakis, 1991). Hence, a concentration-dependent assay was carried out with the extracts (NM and NW) and the results are presented in Table 1 and Fig. 1. Among the extracts, NM was a better radical-scavenger (IC_{20} 2.75 ± 0.13 μg/ml, IC_{50} 8.33 ± 0.39 μg/ml) than NW (IC_{20} 3.49 ± 0.13 μg/ml,

Table 1
Comparative DPPH radical-scavenging activities[a] of NM, NW and α-tocopherol

Sample	IC_{20} (μg/ml)[b]	IC_{50} (μg/ml)[b]
NM	2.75 ± 0.13	8.33 ± 0.39
NW	3.49 ± 0.13	9.82 ± 0.16
α-Tocopherol	1.71 ± 0.02	5.23 ± 0.02

[a] The concentrations of the samples causing 20% and 50% reductions in the absorption of the standard DPPH (100 μM) solution are referred to as their respective IC_{20} and IC_{50} values.
[b] The values are mean ± SE ($n = 4$).

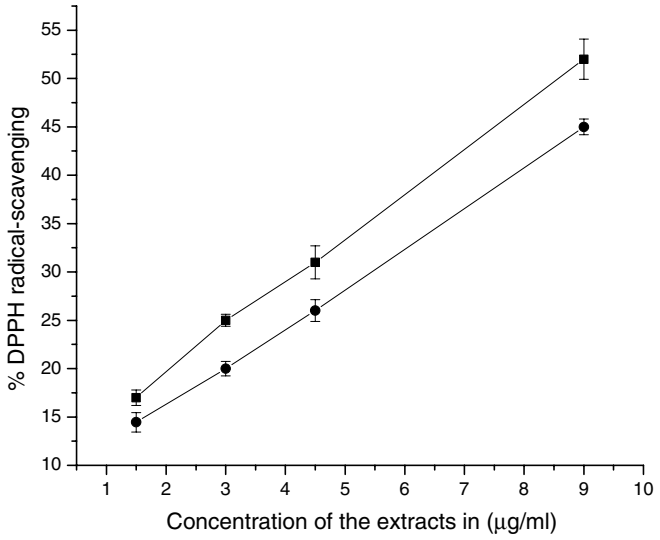

Fig. 1. Concentration-dependent DPPH radical-scavenging activities of nagkesar extracts (NM and NW). ■ – NM; ● – NW. Experimental details are as mentioned in Section 2. The values are means ± SE ($n = 4$).

IC_{50} 9.82 ± 0.16 μg/ml). Under the same conditions, the IC_{20} and IC_{50} values of the positive control, α-tocopherol, were 1.71 ± 0.02 and 5.23 ± 0.02 μg/ml, respectively.

3.3. Anti-lipid peroxidation (LPO) activities of NM and NW

Owing to the high levels of unsaturation and the increased consumption of oxygen, mitochondrial lipids are susceptible to oxidative damage. Lipid peroxidation can inactivate cellular components and plays a major role in oxidative stress in biological systems. Further, several toxic byproducts of the peroxidation can damage other biomolecules, including DNA, away from the site of their generation (Box & Maccubbin, 1997; Esterbauer, 1996). It is well established that transition metal ions, such as iron and copper, stimulate lipid peroxidation through various mechanisms (Halliwell & Gutteridge, 1984). These may either generate hydroxyl radicals to initiate the lipid peroxidation process and/or propagate the chain process via decomposition of lipid hydroperoxides (Braughler, Chase, & Pregenzer, 1987). Hence the anti-LPO activities of NM and NW were studied.

The LPO of rat liver mitochondria was triggered by Fe(II)-ascorbate and the end-products of the process were measured in terms of the thiobarbituric acid-reactive substances (TBARS) formed. In unstimulated experiments, the amount of TBARS was marginal, the absorption at 532 nm (A_{532}) being only 0.083 ± 0.02 ($n = 4$). In control experiments, lipid peroxidation was stimulated by the addition of Fe(II) (50 μM) and ascorbate (200 μM) and the A_{532} value increased to 0.451 ± 0.01 ($n = 4$). Both the test samples, and the positive control, α-tocopherol, inhibited the lipid peroxidation in a concentration-dependent manner (Fig. 2, Table 2). In this case, the order of the activities of the test samples was α-tocopherol (IC_{50} 3.73 ± 0.4 μg/ml) > NW (IC_{50} 11.33 ± 0.4 μg/ml) > NM (IC_{50} 15.04 ± 0.39 μg/ml). The significantly higher potency of α-tocopherol might be due to its better lipophilicity.

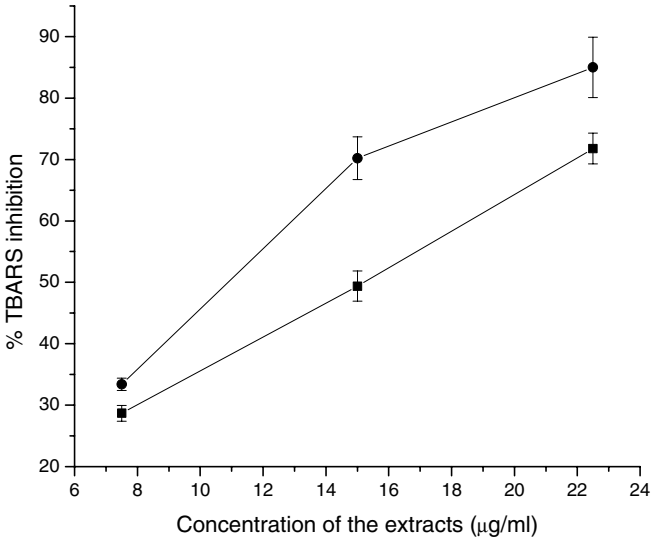

Fig. 2. Concentration-dependent protective activities of NM and NW against Fe(II) (50 μM)-ascorbic acid (200 μM)-mediated lipid peroxidation of rat liver mitochondria, measured in terms of TBARS formed. ■ – NM; ● – NW. Experimental details are as mentioned in Section 2. The values are means ± SE ($n = 4$).

Table 2
Comparative anti-LPO scavenging activities[a] of NM, NW and α-tocopherol

Sample	IC_{50} (μg/ml)[b]
NM	15.0 ± 0.39
NW	11.3 ± 0.4
α-Tocopherol	3.73 ± 0.42

[a] The concentrations of the samples causing 50% inhibitions of lipid peroxidation in rat liver mitochondria are referred to as their respective IC_{50} values. The lipid peroxidation was initiated with Fe(II) (50 μM)–ascorbate (200 μM), and assayed from the TBARS absorbances.
[b] The values are means ± SE ($n = 4$).

3.4. Hydroxyl radical-scavenging activities of NM and NW

Among the ROS, the hydroxyl radicals are the most reactive and predominant radicals generated endogenously during aerobic metabolism (Waling, 1975). Due to the high reactivity, the radicals have a very short biological half-life. Thus, an effective scavenger of them must be present at a very high concentration or possess very high reactivity toward these radicals. Consequently, the abilities of the nagkesar extracts to scavenge these radicals were evaluated by the Fenton-mediated 2-deoxyribose assay.

In a reaction mixture containing Fe (III)-EDTA–H_2O_2 and 2-deoxyribose, very little TBARS were formed ($A_{532} = 0.092 \pm 0.01$, $n = 4$), while addition of ascorbate triggered production of TBARS ($A_{532} = 1.842 \pm 0.08$, $n = 4$). As shown in Fig. 3, both NM and NW inhibited the degradation of 2-deoxyribose efficiently, NW showing a marginally better activity up to a concentration of 700 μg/ml. At a higher concentration, the difference in their potency levelled off. For example, at a concentration of 1.0 mg/ml, the respective 2-deoxyribose protective capacities of NW and NM were 48.8 ± 0.6 and $49.4 \pm 0.32\%$, while the positive control, mannitol (1.02 mM) showed $19 \pm 1\%$ protection under the same conditions.

3.5. H_2O_2-scavenging activities of NM and NW

Although not a radical species, H_2O_2 is an important ROS contributing to oxidative stress. The generation of even low levels of H_2O_2 in biological systems may be important, since naturally occurring iron complexes are believed to react with H_2O_2 in vivo to generate the highly reactive hydroxyl radicals in a superoxide driven Fenton reaction. Hence, the efficacy of NM and NW for degrading H_2O_2 was analyzed, following a reported procedure (Pick & Keisari, 1980). In this case also, the NW extract showed better activity than NM in depleting H_2O_2 with an IC_{50}-value of 40.6 ± 0.2 μg/ml (Table 3, Fig. 4). Under similar conditions, the IC_{50}-values of NM and α-tocopherol in scavenging H_2O_2 were 50.4 ± 0.15 and 29.8 ± 0.45 μg/ml.

Table 3
Comparative H_2O_2-scavenging activities[a] of NM, NW and α-tocopherol

Sample	IC_{50} (μg/ml)[b]
NM	50.4 ± 0.15
NW	40.6 ± 0.20
α-Tocopherol	29.8 ± 0.45

[a] The concentrations of the samples causing 50% reductions in the absorption of H_2O_2 (60 μM) at 610 nm are referred to as their respective IC_{50} values.
[b] The values are means ± SE ($n = 4$).

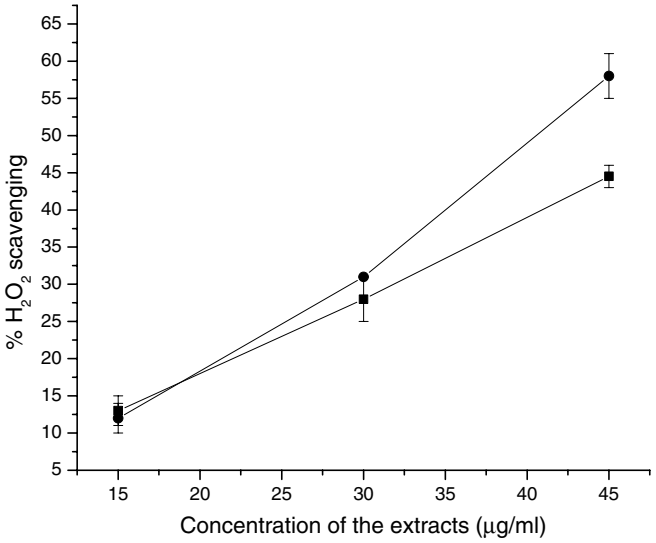

Fig. 4. Concentration-dependent scavenging activities of NM and NW for hydrogen peroxide. ■ – NM; ● – NW. Experimental details are as mentioned in Section 2. The values are means ± SE ($n = 4$).

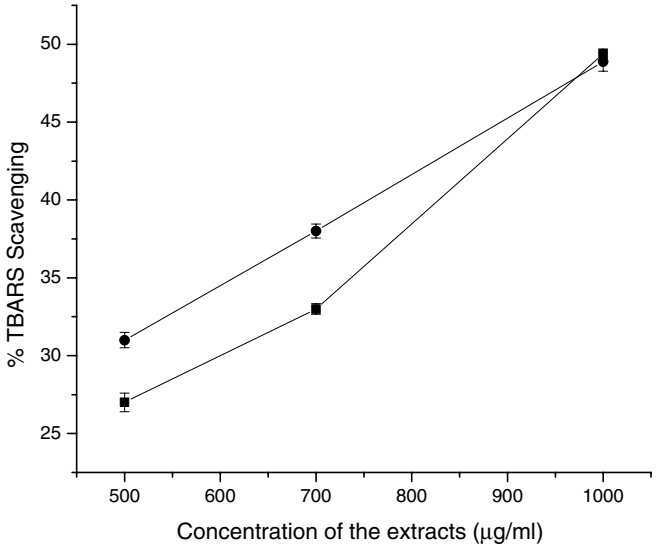

Fig. 3. Concentration-dependent hydroxyl radical-scavenging activities of NM and NW. The assays were carried out by measuring the TBARS formed by the oxidation of 2-deoxyribose (2.8 μM) with Fe(III) (20 μM)–EDTA (100 μM)–ascorbic acid (300 μM)–H_2O_2 (200 μM). ■ – NM; ● – NW. Experimental details are as mentioned in Section 2. The values are means ± SE ($n = 4$).

3.6. Superoxide radical-scavenging activities of NM and NW

Despite its involvement in many pathological processes, superoxide by itself is not as reactive as the well known hydroxyl radicals. But it can give rise to the more toxic hydroxyl radicals, damaging biomacromolecules directly or indirectly (Cotelle et al., 1992) with severe consequences. The superoxide radicals, as such, have been implied to play crucial roles in ischaemia-reperfusion injury (Radi, Beckman, Bush, & Freeman, 1991). Further, their generation in cells, by the oxidation of hypoxanthine or xanthine by xanthine oxidase (XO), produces uric acid, which plays a crucial role in gout (Cos et al., 1998; Tsutomu et al.,

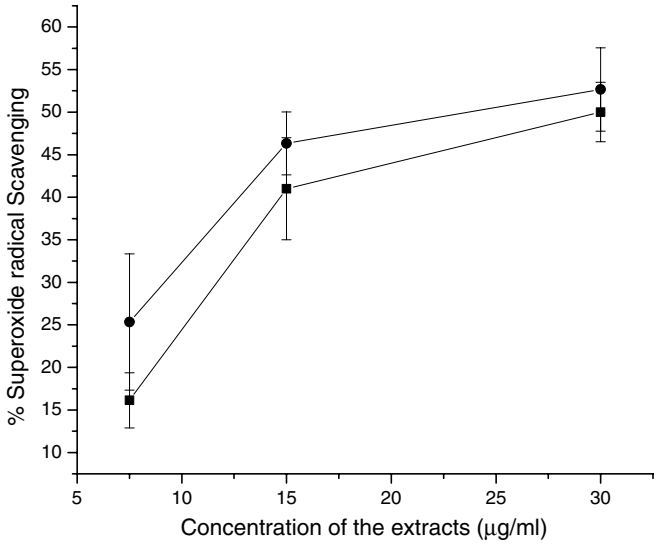

Fig. 5. Concentration-dependent scavenging activities of NM and NW for the superoxide radicals generated by xanthine (50 μM) and xanthine oxidase (0.2 ml, 6.25 mU/ml). ■ – NM; ● – NW. Experimental details are as mentioned in Section 2. The values are means ± SE ($n = 4$).

Table 4
Comparative superoxide radical-scavenging activities[a] of NM, NW and BHA

Sample	IC_{50} (μg/ml)[b]
NM	27.2 ± 0.32
NW	24.7 ± 0.24
BHA	180 ± 0.38

[a] The concentrations of the samples causing 50% reductions in the absorption of the colouring agent (sulfanilic acid–NEDD) at 550 nm are referred to as their respective IC_{50} values.
[b] The values are means ± SE ($n = 4$).

1991). Thus, scavenging of the radicals and/or inhibition of XO would be a promising remedy for these diseases.

Consequently, the scavenging activity of the extracts, NM and NW, for the superoxide radicals generated by xanthine/xanthine oxidase system was carried out by measuring the absorbance at 550 nm (Oyanagui, 1984) in the absence or presence of the extracts (0–30 μg/ml). Fig. 5 shows the scavenging properties of the extracts against superoxide radical anions. The scavenging activities of the extracts as compared to that of BHA are listed in Table 4. The NW extract showed a higher potency than both the NM extract and BHA under the chosen conditions. The comparative IC_{50}-values of NW, NM and BHA were 24.7 ± 0.24, 27.2 ± 0.32 and 180 ± 0.38 μg/ml.

3.7. Xanthine oxidase inhibitory activities of NM and NW

As explained earlier, formulations that can inhibit the enzyme, XO are important in preventive medicine. The XO inhibitory activity of a test sample can be measured from the reduction in the production of uric acid by the xanthine–XO system in its presence. The XO inhibitory activities of NM and NW were studied spectrophotometrically by measuring UV absorbance at 295 nm (Noro et al., 1983) in the presence of the extracts (7.5, 15, 30 μg/ml) and the results are presented in Table 5. Between the two extracts, NM showed better enzyme inhibition than NW at all the test concentrations. For example, at a concentration of 30 μg/ml, the respective inhibitory activities of NM and NW were 42.8 ± 1.04% and 26.2 ± 3.5%. Comparison of the results (Table 5) with those in Table 4 revealed that the superoxide radical-scavenging activity of NM is primarily due to XO inhibition. In contrast, NW can play a dual role of radical scavenger and enzyme inhibitor.

Table 5
Concentration-dependent xanthine oxidase inhibitory activities[a] of NM and NW

Concentration of the extract (μg/ml)	SOD inhibition (%)[b]	
	NM	NW
7.5	12.7 ± 0.28	6.63 ± 0.62
15.0	26.1 ± 0.08	12.8 ± 1.01
30.0	42.8 ± 1.04	26.2 ± 3.50

[a] The inhibition was measured from the amounts of uric acid formed with xanthine oxidase (6.25 mU/ml)/xanthine (50 μM).
[b] The values are means ± SE ($n = 4$).

3.8. Relative polyphenolic, flavonoid and proanthocyanidin contents of NM and NW

It is well known that plant phenolics, in general, are highly effective free radical-scavengers and antioxidants. Consequently, the antioxidant activities of plant/herb extracts are often explained by their total phenolic and flavonoid contents with good correlation. This was also observed in the present study. The total phenolics in NM and NW extracts were determined spectrophotometrically by the Folin–Ciocalteu method and calculated as gallic acid equivalents (GAE). Table 6 shows that the total phenolic content of the NW (250 ± 6.66 mg/g) was higher than that of NM (247 ± 6.66 mg/g).

Likewise, the total flavonoid contents of the NM and NW extracts were also determined by the $AlCl_3$–$NaNO_2$ method and presented as epicatechin equivalents (ECE) in Table 6. In this case also, NW was found to be enriched with the flavonoids (ECE value 203 ± 3.18) compared with NM (ECE value 152 ± 2.29). The proanthocyanidins are a group of biologically active polyphenolic bioflavonoids that have beneficial effects in dermal wound healing, radical scavenging and other relevant redox active properties (Bagchi et al., 1997, 1998; Takahashi, Kamiya, & Yokoo,

Table 6
Comparative total phenolic and flavonoid contents[a] of NM and NW

Sample	GAE (mg/g)[b]	ECE (mg/g)[b]
NM	247 ± 6.66	152 ± 2.29
NW	250 ± 6.66	203 ± 3.18

[a] The total phenolic and flavonoid contents are expressed as gallic acid equivalents (GAE) and epicatechin equivalents (ECE), mg/g dry weights of the samples, respectively.
[b] The values are means ± SE ($n = 4$).

1998). The relative proanthocyanidin contents in NM and NW were assessed from their respective absorbances at 550 nm. It was found that the NW extract had a higher proanthocyanidin content than did NM, the relative ratio being 1.17:1.

3.9. Conclusion

Overall, the methanol and water–ethanol extracts (NM and NW) of nagkesar showed impressive antioxidant activity via their ability to scavenge various biologically relevant ROS and inhibit lipid peroxidation. In accordance with their polyphenolic, flavonoid and proanthocynin contents, NW showed better activity than NM. This is the first exhaustive report on the antioxidant activity of nagkesar, taking into consideration its major constituent chemicals. Compared with the results of a previous report (Rao et al., 2004), the significantly better activity found in the present study might be due to a subtle difference in the composition of the extracts. For example, in contrast to the earlier report, our extracts did not contain free kaempferol and quercetin as revealed from their TLC analyses and comparison with authentic samples. However, acid hydrolysis of the extracts furnished the aglycones which contained the designated flavones, along with catechin, β-sitosterol and 4-hydroxyxoumaric acid. We also subjected the NM extract to preparative TLC (silica gel, 10% methanol:water) to segregate its components in terms of their polarities (Rf : up to 0.15, 0.3–0.45 and >0.55). Analysis of their DPPH scavenging activities revealed that almost all the activity was confined to the most polar fraction, which contained the glycosides. Given that the polyphenolic glycosides are most likely to be extracted in water–ethanol, NW is anticipated to show higher activity. Recently, proanthocyanidins have attracted much attention, in view of their useful medicinal attributes. From this perspective, as well as antioxidant activity, the nagkesar extract appears to be a potential candidate for further in vivo evaluation.

References

Ames, B. N., Gold, L. S., & Willet, W. C. (1995). The causes and prevention of cancer. *Proceedings of National Academy of Sciences USA, 92*, 5258–5265.

Box, H. C., & Maccubbin, A. E. (1997). Lipid peroxidation and DNA damage. *Nutrition, 13*, 920–921.

Braughler, J. M., Chase, R. L., & Pregenzer, J. F. (1987). Oxidation of ferrous ion during peroxidation of various lipid substrates. *Biochimica Biophysica Acta, 921*, 457–464.

Bagchi, D., Garg, A., Krohn, R. L., Bagchi, M., Tran, M. X., & Stohs, S. J. (1997). Oxygen free radical scavenging abilities of vitamins C and E, and a grape seed proanthocyanidin extract in vitro. *Research Communications in Molecular Pathology and Pharmacology, 95*, 179–189.

Bagchi, D., Garg, A., Krohn, R. L., Bagchi, M., Bagchi, D. J., Balmoori, J., et al. (1998). Protective effects of grape seed proanthocyanidins and selected antioxidants against TPA-induced hepatic and brain lipid peroxidation and DNA fragmentation, and peritoneal macrophage activation in mice. *General Pharmacology, 30*, 771–776.

Cerutti, P. A. (1991). Oxidant stress and carcinogenesis. *European Journal of Clinical Investigation, 21*, 1–11.

Cerutti, P. A. (1994). Oxy-radicals and cancer. *Lancet, 344*, 862–863.

Chopra, R. N., Chopra, I. C., & Varma, B. S. (1969). *Supplement to glossary of Indian medicinal plants* (pp. 63–64). New Delhi: CSIR.

Cos, P., Ying, L., Calomme, M., Hu, J. P., Cimanga, K., Poel, B. V., et al. (1998). Structure–activity relationship and classification of flavonoids as inhibitors of xanthine oxidase and superoxide scavengers. *Journal of Natural Products, 61*, 71–76.

Cotelle, N., Bernier, J. L., Henichart, J. P., Catteau, J. P., Gaydou, E., & Wallet, J. C. (1992). Scavenger and antioxidant properties of ten synthetic flavones. *Free Radical Biology and Medicine, 13*, 211–219.

Esterbauer, H. (1996). Estimation of peroxidative damage: a critical review. *Pathologie Biologie Paris, 44*, 25–28.

Halliwell, B., & Gutteridge, J. M. C. (1981). Formation of thiobarbituric-acid-reactive substances from deoxyribose in the presence of iron salts: the role of super oxide and hydroxyl radicals. *FEBS Letters, 128*, 347–352.

Halliwell, B., & Gutteridge, J. M. C. (1984). Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochemistry Journal, 219*, 1–14.

Halliwell, B. (1997). Antioxidants and human diseases: a general introduction. *Nutrition Reviews, 55*, S44–S52.

Joshi, R., Adhikari, S., Patro, B. S., Chattopadhyay, S., & Mukherjee, T. (2001). Free radical scavenging behavior of folic acid: evidence for possible antioxidant activity. Free radic. *Free Radical Biology and Medicine, 30*, 1390–1399.

Ho, C.-T. (1995). Antioxidant properties of flavonoids. *Food Factors Cancer Prevention (Int. Conf.)*, 593–597.

Jia, Z., Tang, M., & Wu, J. (1999). The determination of flavonoid content in mulberry and their scavenging effects on superoxide radicals. *Food Chemistry, 64*, 555–599.

Kahkonen, M. P., Hopia, A. I., Vuorela, H. J., Rauuha, J. P., Pihlaja, K., Kujala, T. S., et al. (1999). Antioxidant activity of plant extract containing phenolic compounds. *Journal of Agricultural and Food Chemistry, 32*, 661–667.

Liu, F., & Ng, T. B. (2000). Antioxidative and free radical scavenging activities of selected medicinal herbs. *Life Sciences, 66*, 725–735.

Mellors, A., & Tappel, A. L. (1966). The inhibition of mitochondrial peroxidation by ubiquinone and ubiquinol. *Journal of Biological Chemistry, 241*, 4353–4356.

Noro, T., Oda, Y., Miyase, T., Ueno, A., & Fukushima, S. (1983). Inhibitors of xanthine oxidase from the flowers and buds of *Daphne genkwa*. *Chemical and Pharmaceutical Bulletin (Tokyo), 31*, 3984–3987.

Oyanagui, Y. (1984). Reevaluation of assay methods and establishment of kit for superoxide dismutase activity. *Analytical Biochemistry, 142*, 290–296.

Patro, B. S., Rele, S., Chintalwar, G. J., Chattopadhyay, S., Adhikari, S., & Mukherjee, T. (2002). Protective activities of some phenolic 1,3-diketones against lipid peroxidation: possible involvement of the 1,3-diketone moiety. *ChemBioChem, 3*, 364–370.

Pick, E., & Keisari, Y. (1980). A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. *Journal of Immunological Methods, 38*, 161–170.

Pietta, P. G. (2000). Flavonoids as antioxidants. *Journal of Natural Products, 63*, 1035–1042.

Porter, L. J., & Rossi, J. A. (1986). The conversion of procyanidins and prodelphinidins to cyanidins and delphinidins. *Phytochemistry, 25*, 225–230.

Radi, R., Beckman, J. S., Bush, K. M., & Freeman, B. A. (1991). Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Archives of Biochemistry and Biophysics, 288*, 481–487.

Rao, L. J. M., Yada, H., Ono, H., & Yoshida, M. (2002). Acylated and non-acylated flavonol monoglycoside from Indian minor spice nagkesar (*Mammea longifolia*). *Journal of Agricultural and Food Chemistry, 50*, 3143–3146.

Rao, L. J. M., Yada, H., Ono, H., Ohnishi-Kameyama, M., & Yoshida, M. (2004). Occurrence of antioxidant and radical scavenging proanthocyanidins from the Indian minor spice nagkesar (*Mammea longifolia* planch and tirana syn). *Bioorganic and Medicinal Chemistry, 12*, 31–36.

Rekka, E., & Kourounakis, P. N. (1991). Effect of hydroxyethyl rutosides and related compounds on lipid peroxidation and free radical scavenging activity. Some structural aspects. *Journal of Pharmacy and Pharmacology, 43*, 486–491.

Singleton, V. L., & Rossi, J. A. Jr, (1965). Colorimetry of total phenolics with phosophomolybdic–phosphotungstic acid reagents. *American Journal of Enology and Viticulture, 16*, 144–158.

Tsutomu, H., Taeko, Y., Rieko, Y., Yukihiko, I., Muneto, M., Kazafumi, Y., et al. (1991). Inhibitory effects of galloylated flavonoids on xanthine oxidase. *Planta Medica, 57*, 83–84.

Takahashi, T., Kamiya, T., & Yokoo, Y. (1998). Proanthocyanidins promote fair follicle cell proliferation and the anagen phase of hair growth. *Acta Dermato–Venereologica (Stockh), 78*, 428–432.

Waling, C. (1975). Fenton's reagent revisited. *Accounts of Chemical Research, 8*, 125–131.

Yokozawa, T., Chen, C. P., Dong, E., Tanaka, T., Nonaka, G. I., & Nishioka, I. (1998). Study on the inhibitory effect of tannins and flavonoids against the 1,1-diphenyl-2-picrylhydrazyl radicals. *Biochemical Pharmacology, 56*, 213–222.