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Antioxidant activities of three Indian commercially available Nagakesar: An *in vitro* study

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ABSTRACT

The objective of the present study was to evaluate the in vitro antioxidant activity of the methanolic extract of various commercial Nagakesar available in the Indian market. The three commercially available Nagakesar used in the present study are Mesua ferrea Linn., Ochrocarpus longifolius Benth & Hook.f, and Cinnamomum wightii Meissn. The in vitro antioxidant models studied are the total antioxidant capacity by phosphomolybdenum method, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging, superoxide anion radical scavenging and hydrogen peroxide scavenging activity. The radical scavenging activities of the three Nagakesar extracts were compared to the standard ascorbic acid and rutin incorporating the statistical linear regression equation. Among all the three Nagakesar flowers, Cinnamomum wightii was found to exhibit the highest antioxidant and potent radical scavenging activity as compared to Mesua ferrea and Ochrocarpus longifolius. A positive, significant linear relationship between antioxidant activity and total phenolic content (R^2= 0.652) showed that phenolic compound were the dominant antioxidant components in the tested flower extract. In addition to the antioxidant activity the thin layer chromatography (TLC) fingerprinting and the quantitative estimation of the total phenolic (Folin-Ciocalteau method), flavonoid and flavonol content were also performed.

Keywords: Nagakesar, antioxidant activity, phenolics, flavonoids

INTRODUCTION

Phenolic compounds are abundantly present in human diet and acts as antioxidants and are widespread constituents of fruit, vegetables, cereals, olive oil, dry legumes, chocolate and beverages [1]. Also they are found in both edible and non-edible plants. They may exert antioxidant effects as free radical scavengers, as hydrogen donating sources or as singlet oxygen quenchers and metal ion chelators [2]. Phenolic compounds are known to counteract oxidative

stress in the human body by helping maintaining a balance between oxidant and antioxidant substances [3, 4].

The indigenous market of Indian traditional medicine is full of herbal products mainly from Indian medicinal plants. 'Nagakesar' is a well known plant in Ayurveda. Various plants of different genus and family are sold under the same common name Nagakesar. In our market survey five medicinal plants viz. *Mesua ferrea* Linn. (Guttiferrea), *Ochrocarpus longifolius* Benth & Hook.F (Guttiferrea), *Cinnamomum wightii* Meissn. (Lauraceae), *Calophyllum inophylum* Linn. (Guttiferrea) and *Dillenia pentagyna* Roxb. (Dilleniaceae) were found to be sold in the Indian market under the similar common name 'Nagakesar'.

The literature review reveals their uses as antibacterial, antifungal, anti-inflammatory and cytotoxic. Though antioxidant activity of *Ochrocarpus longifolius* was reported but there is no comparative antioxidant activity reported till date for all the commercially available Nagakesar present in the Indian market. As all these plants are sold under the same common name, so there was an inquisitiveness to know whether these plants are related or totally unrelated in their antioxidant potential. The comparative evaluation of antioxidant activity will establish the quality and pharmacological standard for these plants; also it will identify the plants having maximum activity. This will also help in their commercial gradation. Here we have undertaken to evaluate the *in vitro* antioxidant activity of *Mesua ferrea*, *Ochrocarpus longifolius* and *Cinnamomum wightii* by different antioxidant assay that are total antioxidant capacity, DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging assay, scavenging of superoxide radical (Alkaline Dimethyl Sulfoxide method) and scavenging of hydrogen peroxide assay. Furthermore, the estimation of total phenolic contents as well as total flavonoid and flavonol content and their correlation with the antioxidant activities were ascertained.

EXPERIMENTAL SECTION

Plant material and preparation of extract

The dried flowers of *Mesua ferrea* Linn. and the dried flower buds of *Ochrocarpus longifolius* Benth & Hook.f were brought out from the Gola market of Varanasi, Uttar Pradesh, India, whereas the dried buds of *Cinnamomum wightii* Meissn. were brought out from Chennai, Tamil Nadu, India. The authentication of all the three commercial Nagakesar was done by Prof. S. D. Dubey, Department of Dravyaguna, Faculty of Ayurveda, Institute of Medical Science, Banaras Hindu University, Varanasi, India. The crude drugs were cleaned, spread under shade in room temperature and were powdered. The extraction was done by the use of soxhlet extractor (250 ml capacity). 100 g of the powdered crude drugs was taken and extracted with 500 ml methanol for two days (12 hours each day). The final extracts obtained were concentrated under vacuum evaporator and the dried extract was investigated for the antioxidant study.

Phytochemical screening and Thin layer chromatographic fingerprinting

The methanolic extract obtained by soxhlet extraction was screened for the presence of various phytoconstituents using various qualitative reagents [5]. Pre-coated aluminium silica gel TLC plates 60 F_{254} was used as a stationary phase. Spraying reagent used for detection was 5% ferric chloride (for phenolics) and 2% ethanolic aluminium chloride (for flavonoids).

Antioxidant and radical scavenging assay

Determination of Total antioxidant capacity

The determination of total antioxidant activity was done as per the phosphomolybdenum method with some modifications [6]. The basic principle of the assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate Mo (V) complex at acidic pH. 0.3 ml extract was combined with a mixture of 3ml of reagent solution (0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes containing the reaction solution were then capped and incubated at 95 °C for 90 minutes. After the samples had cooled to room temperature, the absorbance of the solution was then measured at 695 nm against blank. Methanol (0.3 ml) in the place of extract is used as the blank. The antioxidant activity is expressed as the number of equivalents of ascorbic acid.

DPPH radical scavenging activity

The radical scavenging activity was determined by the used of DPPH free radical assay with some modifications [7]. 50 µl of the various concentrations of plant extracts in methanol were added to 5 ml of 100 µM solution of DPPH in methanol. After 30 minutes incubation absorbance was read against blank taken as methanol at 517 nm and the percentage inhibition activity was calculated from the following equation:

$$\% \text{ Inhibition} = [(A_o - A_1)/A_o] \times 100 \ldots\ldots\ldots\ldots \text{(i)}$$

Where A_o is the absorbance of the control, and A_1 is the absorbance of the extract/ standard. Here rutin was used as a standard. All readings were performed in triplicates.

Scavenging of superoxide radical by alkaline DMSO method

Superoxide scavenger inhibits the formation of a red dye formazan. In alkaline DMSO method, superoxide radical was generated by the addition of sodium hydroxide to air saturated dimethyl sulfoxide (DMSO) [8]. To the reaction mixture containing 0.1 ml of NBT (1 mg/ml solution in DMSO) and 0.3 ml of the various concentration of the extract, 1 ml of alkaline DMSO was added to give a final volume of 1.4 ml and the absorbance was measured at 560 nm. The same procedure was repeated for the standard ascorbic acid. The percentage inhibition was calculated using equation (i).

Scavenging of hydrogen peroxide

A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffered saline (PBS, pH 7.4). 1ml of various concentrations of the extracts or standards in methanol was added to 2ml of hydrogen peroxide solution in PBS. Then finally the absorbance was measured at 230nm after 10 minutes [9]. All readings were performed in triplicates and the percentage inhibition was calculated using equation (i).

Determination of total phenolics, flavonoids and flavonol content

The determination total phenolic content of crude drug extract was done using Folin-Ciocalteu reagent with some modifications [10]. For total phenolic estimation, 100 mg of the methanolic extracts of the Nagakesar was weighed accurately and dissolved in 100 ml of triple distilled water (TDW). 1 ml of this solution was transferred to a test tube, then 0.5 ml 2N of the Folin-Ciocalteu reagent and 1.5 ml 20% of Na_2CO_3 solution was added and ultimately the volume was

made up to 8 ml with TDW followed by vigorous shaking and finally allowed to stand for 2 hours after which the absorbance was taken at 765 nm. These data were used to estimate the total phenolic content using a standard calibration curve obtained from various diluted concentrations of gallic acid.

For the determination of the total flavonoid content, the aluminium chloride method is incorporated using rutin as the standard [11]. The method is based on the formation of the flavonoid-aluminium complex which has an absorptivity maximum at 415nm. 100µl of the plant extracts in methanol (10 mg/ml) was mixed with 100 µl of 20 % aluminum trichloride in methanol and a drop of acetic acid, and then diluted with methanol to 5ml. The absorption at 415 nm was read after 40 minutes. Blank samples were prepared from 100 ml of plant extracts and a drop of acetic acid, and then diluted to 5ml with methanol. The absorption of standard rutin solution (0.5 mg/ml) in methanol was measured under the same conditions. All determinations were carried out in triplicates. The amount of flavonoids in plant extracts in rutin equivalents (RE) was calculated by the following formula:

$$X = (A \, . \, m_o)/(A_o \, . \, m) \ldots\ldots\ldots\ldots\ldots \text{(ii)}$$

Where 'X' is the flavonoid content, mg/g plant extract in RE, 'A' is the absorption of plant extract solution, 'A_o' is the absorption of standard rutin solution, 'm' is the weight of crude drug extract in mg and 'm_o' is the weight of rutin in the solution in mg.

The total flavonols content was also determined as per the aluminium chloride method [11] with some modifications using rutin as a reference compound. This method is also based on the formation of complex with maximum absorption at 440 nm. 1 ml of each methanolic plant extract (10 mg/ml) was mixed with 1 ml aluminum trichloride (20 mg/ ml) and 3ml sodium acetate (50 mg/ ml). The absorbance at 440 nm was read after 2.5 hours. The absorption of standard rutin solution (0.5 mg/ml) in methanol was measured under the same conditions. All determinations were carried out in triplicates. The amount of flavonols in plant extracts in rutin equivalents (RE) was calculated using the equation (ii).

Statistical analysis

All the results were carried out in triplicates and were expressed as Mean ± S.E.M. The amount of extract needed to inhibit free radical concentration by 50% (IC_{50}), was graphically determined by a linear regression method using MS Windows based Graphpad Instat (Version 3) software.

RESULTS AND DISCUSSION

Phytochemical screening and Thin layer chromatographic fingerprinting

From the results obtained from the preliminary phytochemical screening (Table 1) it was observed that all the three methanolic extracts of the Nagakesar contains phenolics in a high amount and moderate flavonoids along with other phytoconstituents. This depicts that the crude drugs may have antioxidant effect due to its polyphenolic property which is to be further investigated. Polyphenols and flavonoids are used for the prevention and cure of various diseases which is mainly associated with free radicals [12].

Table 1: Preliminary phytochemical screening of the different commercially available Nagakesar

Phytoconstituents	*M.ferrea*	*O.longifolius*	*C.wightii*
Alkaloids	+	-	-
Steroids/ triterpenoids	++	+	+
Phenolics and tannins	++	+++	+++
Flavonoids	++	+++	+++
Glycosides	+	+	+
Saponins	+++	++	++
Reducing sugars	+	+	++
Volatile oils	++	+	+

(-) absent; (+) present in a negligible quantity; (++) present in moderate quantity; (+++): present in a considerable quantity

Since the preliminary phytochemical screening showed the presence of a considerable amount of polyphenolics which are the major phytoconstituents behind the antioxidant activity, so it is dual necessary to perform thin layer chromatography for their further confirmation. Phenolics gave blue color when sprayed with 5% $FeCl_3$ whereas flavonoid gave a clear yellow color when sprayed with 2% ethanolic aluminium chloride. The solvent system used was $CHCl_3$: MeOH (9:1). A densely blue band was seen both in *O.longifolius* and *C.wightii* having an R_f of 0.18 and 0.21 respectively. For flavonoids *O.longifolius* was seen to possess three yellow bands with one band (R_f 0.95) having a dense yellow color after spraying. *C.wightii* also showed the presence of two densely yellow bands with R_f values 0.27 and 0.43. The results of TLC corroborate the presence of phenolics and flavonoids which comply with the results of preliminary phytochemical screening. The intensity of color obtained was comparatively more for *Cinnamomum wightii* for phenolics and flavonoids. So it is understood that more amounts of phenolics and flavonoids are contained in *Cinnamomum wightii.*

Antioxidant and radical scavenging assay

The total antioxidant capacity in all the three Nagakesar methanolic extracts was determined using the linear regression equation of the calibration curve ($y=0.002x+0.012$, $r^2 = 0.940$) and was expressed as ascorbic acid equivalent. Table 2 showed that the potent antioxidant capacity was seen with that of *C.wightii* extract followed by *O. longifolius* and the least occurs with that of *M. ferrea*. Nevertheless, *C. wightii* also shows potent scavenger of DPPH radical, superoxide and hydrogen peroxide radicals which is quite comparable to that of the standard rutin and ascorbic acid. This can be explicate by the capability of *C. wightii* extract to reduce the purple DPPH radical to yellow color and also the maximal reduce level of red formazan in DMSO method as well as the reduction of hydrogen peroxide radical in the solution. Thus this comparatively depicts its flawless antioxidant activity when compared to the other two Nagakesar.

Correlation results between phenolics and its antioxidant effect

In our present investigation, it was found that out of all the three commercial Nagakesar, *C.wightii* has the highest phenolic and moderate flavonoid content with a value of 156.6 mg/g and 30 mg/g crude drug extract equivalent to gallic acid and rutin respectively Table 3. The content of the total phenolics in all the Nagakesar extracts was determined using the linear regression equation of the calibration curve ($y= 0.093x+0.033$, $R^2 = 0.995$) and is expressed as gallic acid equivalent.

Table 2: Total antioxidant and radical scavenging activity of standards and Nagakesar methanolic extracts [IC_{50} expressed as Mean ± S.E.M.[a]]

Standards & Extracts	Total antioxidant capacity Equivalent to Ascorbic acid	DPPH free radical scavenging activity (µg/ml)	Scavenging of superoxide radical (µg/ml)	Scavenging of hydrogen peroxide (µg/ml)
(Standards)				
Ascorbic acid	-	-	34.79±0.64	16.00±0.19
Rutin	-	54.76 ± 0.58	-	-
(Crude drug extract)				
C.wightii	205±1.44	108.35±0.19	80.89±0.73	21.70±0.63
O.longifolius	108.33±1.59	168.46±1.03	148.30±0.77	30.47±0.71
M.ferrea	91.67±2.16	300.01±5.91	273.66±3.27	47.46±2.10

(a): average of three determinations

Table 3: Yield, total amount of phenolic, flavonoid and flavonol content of the methanolic extract of Nagakesar [Mean ± S.E.M.[a]]

Sample	Yield (%)	Total phenolics mg/g plant extract (in GAE)	Total flavonoid mg/g plant extract (in RE)	Total flavonol mg/g plant extract (in RE)
C.wightii	28.63	156.6 ± 2.85	30 ± 0.52	3.60 ± 0.25
O.longifolius	16.03	138.3 ± 4.58	41 ± 1.27	0.57 ± 0.04
M.ferrea	20.53	92.4 ± 3.45	23 ± 1.37	0.49 ± 0.03

(a): average of three determinations

Some authors claims that the antioxidant activity is mainly due to the high phenolic content [13, 14]. It is not always easy to judge the antioxidant property only through the presence of a high amount of phenolics, but to have better judgement, a comparison of the correlation coefficient R^2 (obtained by plotting the graph between the total antioxidant capacity versus total phenolic content) is made to correlate with the R^2 of total antioxidant capacity ($y=0.002x+0.012$, $R^2 = 0.940$). The graph obtained after plotting the total antioxidant activity versus total phenolics was found to have a promising linear regression value of $y=0.436x+70.15$, where $R^2=0.652$. So from this we can justify that the correlation coefficient R^2 of the total phenolic content versus total antioxidant capacity ($R^2=0.652$) is quite significant when compared with the R^2 of the total antioxidant activity ($R^2 = 0.940$). From this we can conclude that the antioxidant property of the methanolic extract of *C.wightii* might be verily due to the presence of phenolic components.

CONCLUSION

In our present investigation it was found that the three commercially available Nagakesar was having varying antioxidant activity. Methanolic flower bud extract of *C.wightii* is having maximum antioxidant activity which may be due to total phenolic content. Further *in vivo* studies will be carried out to confirm the antioxidant activity methanolic bud extracts of *C.wightii*. Moreover, the isolation, identification and antioxidant studies of different phenolic phytoconstituents may be carried out to identify the specific phenolic phytoconstituents having a major role in antioxidant activity.

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