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Phytochemical screening and evaluation of antioxidant potentials of some Indian medicinal plants and their composite extract

Brahm Kumar Tiwari, A.B. Abidi, Syed Ibrahim Rizvi* and Kanti Bhooshan Pandey**

Department of Biochemistry and Biochemical Engineering, SHIATS, Allahabad-211007, Uttar Pradesh, India
Present Address : Dr. Brahm Kumar Tiwari, College of Biotechnology, I.T.S Paramedical College, Ghaziabad-201206, Uttar Pradesh, India
**Department of Biochemistry, University of Allahabad, Allahabad-211002, Uttar Pradesh, India*
***CSIR-Central Salt and Marine Chemicals Research Institute, Gijubhai Badheka Marg, Bhavnagar-364002, Gujarat, India*

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Abstract

Four medicinal plants, *Azadirachta indica* A.Juss., *Aegle marmelos* Correa ex Roxb., *Ocimum sanctum* Linn. and *Murraya koenigii* (Linn.) Spreng, have been selected, based on their use in traditional systems of medicine. The comparative phytochemical screening and antioxidant potential evaluation of leaf extracts of individual plants and of their composite extract were done. The results obtained from the study showed the presence of amino acids, anthraquinones, glycosides, saponin, steroids, tannin, triterpenoids, phenolic compounds and flavonoids in significant amount in the leaf extracts which were correlated with their antioxidant capacities. The composite extract of leaves of four selected medicinal plants, elicited higher antioxidant potential as evidenced by enhanced ferric reduction and greater 1, 1-diphenyl-2-picrylhydrazyl radical inhibition, irrespective to the content of phytochemicals and polyphenols. We propose that synergic effect of active phytochemicals may be responsible for improved antioxidant as well as radical scavenging abilities of composite extract. Study concludes that using cocktail of herbal formulation may be an advance theme to develop phytomedicines with improved efficacy.

Key words : Medicinal plant, composite extract, flavonoid, antioxidant, FRAP, DPPH

1. Introduction

Plant-derived natural products are known for their medicinal properties since ancient. Many plant species have been reported to contain active ingredients that have significant potential against management of chronic human diseases including cancer, diabetes and cardiovascular disorders (Modak *et al.* 2007; Tiwari *et al*., 2013). Furthermore, many components of herbal remedies are documented to have the capacity of being combined with other substances and making them active in context of biological properties (Kennedy and Wightman, 2011; Shariff, 2001). In addition to this, minimum side effect, easy availability and less expensive in comparison to synthetic drugs, make plant based therapies as a frequently adopted cure for many pathological events throughout the world (Harnafi and Amrani, 2008). It has been estimated that about 80% of individuals from developed countries use traditional medicines, prepared from plants (Patwardhan *et al*., 2004; Biradar, 2015).

Murraya koenigii (Linn.) Spreng. (Family: Rutaceae), commonly known as 'curry', is used as a spice in India for its characteristic flavour and aroma. It is also used as traditional medicine against diarrhoea, diabetes, fatigue, stress and microbial infections (Ningappa *et al*., 2008; Ajay *et al*., 2011). *Azadirachta indica* A.Juss. (Family: Meliacae), is predominantly found in the Asian sub region and other parts of Africa, where it is functionally used as an antidiabetic, antimicrobial natural agent (Anyaehie, 2009). *Ocimum sanctum* Linn. (Family: Lamiaceae), commonly known as 'holy basil', found throughout the south Asian region, is used medicinally as antihyperglycaemic, antibronchitis, antianalgesic and fungicide (Khan *et al*., 2010; Hannan *et al*., 2006). *Aegle marmelos* Correa ex Roxb., (Family: Rutaceae), known as 'bael', its fruit is frequently used in medicinal cause in many developing countries (Gandhi *et al*., 2012). It possesses broad range of therapeutic effects including antibacterial, antiviral, antidiarrheal, gastroprotective, hepatoprotective, antidiabetic and cardioprotective effects (Kumar *et al*., 2012; Tiwari *et al*., 2013).

However, in current scenario of prevalence of life threating new health issues, phytodrugs with enhanced efficacy are badly needed. Keeping this rationale in mind, present study was designed to analyse the bioactive components and antioxidative potentials of the mathonolic extracts of *M. koenigii, A. indica, O. sanctum, and A. marmelos* leaves and composite leaf extract of these four medicinal plants.

2. Materials and Methods

2.1 Plant materials and their extraction

A. indica, A. marmelos, O. sanctum and M. koenigii leaves were collected from Sam Higginbottom Institute of Agriculture, Technology and Sciences (SHIATS) orchard, Allahabad, India. The leaves of *A. indica, A. marmelos, O. sanctum,* and *M. koenigii* were collected, dried at room temperature and powdered in warring grinder and passed through 40 mesh sieve. Obtained mixture was kept in

Author for correspondence: Dr. Kanti Bhooshan Pandey
CSIR-Central Salt and Marine Chemicals Research Institute, Gijubhai Badheka Marg, Bhavnagar-364002, Gujarat, India
E-mail: kantibiochem@gmail.com
Tel.: +91-9913811711

filter and processed in Soxhlet apparatus at 60^0C where 80% methanol solvent was used for extraction. Extraction was done for 10 h. for maximum yield. Finally, the extract was evaporated to dryness in a boiling water bath and lastly preceded for measurement. The yields of extracts were about 13% for *A. marmelos*, 7% for *A. indica*, 6% for *M. koeingii*, and 9% for *O. sanctum* leaves.

2.2 Phytochemical screening

1 g of the methanolic crude plant extracts of *M. koenigii*, *A. indica*, *O. sanctum* and *A. marmelos* leaves were dissolved in 100 ml of methanol to obtain a stock sample of conc. 1% (v/v). The preliminary phytochemical screening of these samples was done by following the methodologies of Harborne (1998) and Kokate (2001).

2.2.1 *Test of amino acids and anthraquinones*

1 ml of the crude stock extract was added with a few drops of ninhydrin reagent. The purple colour appearance showed the presence of amino acids. To test anthraquiones, 5 ml of the crude stock extract solution was hydrolysed with diluted conc. sulfuric acid (H_2SO_4) extracted with benzene and dilute ammonia solution was added to it. Appearance of rose pink colouration suggested the presence of anthraquinones.

2.2.2 *Test of glycosides, saponins and tannins*

The crude extract was hydrolysed by hydrochloric acid (HCl) for few hours on water bath. 1 ml of pyridine was added to the hydrolysate and a few drops of sodium nitroprusside solutions were added and then it was made alkaline with NaOH solution. Transformation of the pink to red showed the presence of glycosides. To test the presence of saponins and tannins, the crude extract stock solution was diluted with 20 ml of distilled water and was agitated in a graduated cylinder for 15 min. The formation of 1 cm foam layer showed the presence of saponins. 3 ml of the extract and a few drops of 1% lead acetate were added. A yellow precipitate was formed, indicated the presence of tannins.

2.2.3 *Test for steroids and triterpenoids*

1 ml of the crude plant extract was dissolved in 10 ml of chloroform and to it, an equal volume of concentrated H_2SO_4 was added from sides of the test tube. The upper layer turns into red and the H_2SO_4 layer showed yellow with green fluorescence. This indicated the presence of steroids. 5 ml of the crude plant extract was dissolved in 1 ml of chloroform and then 1 ml of acetic anhydride was added following the addition of 1 ml of conc. H_2SO_4. Formation of reddish violet colour indicated the presence of triterpenoids.

2.3 Determination of phenolic content

The total phenolic content (TPC) in extracts was determined, using the Folin-Ciocalteu colorimetric method (Singleton *et al*., 1999), with some modifications. Diluted extracts were used to obtain readings within the standard curve ranges of 0.0-600.0 µg gallic acid/ml. Then 125 µl of the standard gallic acid solution or diluted extracts were mixed with 0.5 ml of distilled water in a test tube, followed by addition of 125 µl of Folin-Ciocalteu reagent. The samples were mixed well and then allowed to stand for 6 min, before 1.25 ml of a 7% sodium carbonate aqueous solution was added. Water was added to adjust the final volume to 3 ml. Samples were allowed to stand for 90 min at room temperature before measurement at 760 nm versus the blank using a spectrophotometer in comparison with the standards prepared similarly with known gallic acid concentrations. All values are expressed as mean (mg gallic acid equivalents (GAE) /100g extract) ± SD for triplicate replications.

2.4 Determination of total flavonoid content

Total flavonoid content was determined by using the method described by Dewanto *et al*. (2002). Briefly, 0.25 ml of diluted extract was mixed with 1.25 ml of distilled water and subsequently with 0.075 ml of 5% sodium nitrite solution and allowed to react for 5 min. After that, 0.15 ml of 10% aluminium chloride was added and allowed to further react for 6 min before 0.5 ml of 1 M sodium hydroxide was added. Distilled water was added to bring the final volume of the mixture to 3 ml. The absorbance of the mixture was immediately measured at 510 nm wavelength against a prepared blank using a spectrophotometer. The flavonoid content was determined by a quercetin as standard and expressed as mean (mg of quercetin equivalents per g of extract) ±SD for the triplicate extracts.

2.5 Evaluation of free radical scavenging activity by DPPH· method

Free radical scavenging activity (FRSA) of extracts was measured by slightly modified method as suggested by Brand-Williams *et al.* (1995). 100 ml of diluted methanolic extract was added to 3 ml DPPH· (1, 1-dipheny l-2-picrylhydrazyl) solution (0.6×10^{-5} M). Mixture was incubated for 15 min at 21°C and then the decrease in absorption was measured at 515 nm. Absorption of blank sample containing the same amount of methanol and DPPH· solution was prepared. The experiment was carried out in triplicate. FRSA was calculated by the following formula:

$$\% \text{ Inhibition} = \frac{\text{A Control} - \text{A Sample}}{\text{A Control}} \times 100$$

where 'A' stands for absorption

2.6 Determination of antioxidant power

The Ferric Reducing Antioxidant Power (FRAP) was determined by the method of Benzie and Strain (1996). Working FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), (10 mM in 40 mM HCl) solution and $FeCl_3.6H_2O$ (20 mmol/l) solution in 10:1:1 ratio, respectively. FRAP reagent (3 ml) was mixed with 100 µl of extracts and the content was mixed vigorously. The absorbance was read at 593 nm at the interval of 30 sec for 4 min. Aqueous solution of known Fe^{2+} concentration in the range of 100 - 1000 µmol/l was used for calibration. The antioxidant activity in the samples was calculated as mmol Fe^{2+}/g extract.

2.7 Statistical analysis

Statistical analyses were performed using Prism version 5.00 for Windows, GraphPad Software, San Diego California USA. Values are shown as Mean ± standard deviation (SD) for three replicate in each experiment.

3. Results

3.1 Phytochemical screening

Positive results obtained by all the extracts confirmed presence of amino acids, anthraquinones, glycosides, saponin, steroids, tannins and triterpenoids.

3.2 Total phenolic content

The tested plant extracts showed wide range of phenolic concentrations varies from 71.38 to 82.870 mg GAE /g of extract (Figure 1). The total phenolic content was 78.680 mg GAE /g in composite extract, 82.870 mg GAE /g in *M. koenigii*, 79.260 mg GAE /g in *A. indica*, 79.230 mg GAE /g mg in *A. marmelos* and 71.380 mg GAE /g in *O. sanctum*. The highest phenolic content was measured in *M. koenigii* extract and lowest was in *O. sanctum*.

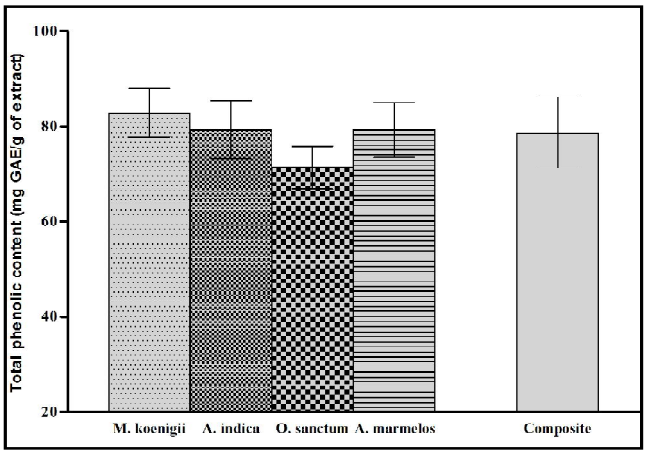

Figure 1: Phenol content in plant extracts. Phenolic content is reported in terms of mg GAE/100g. Values are expressed as Mean ± SD of the 3 independent experiments

3.3 Total flavonoid content

Total flavonoid content in tested plant extracts varied from 21.32 to 34.61 mg quercetin equivalents/g of extract. *M. koenigii* showed the highest flavonoid content (34.61 mg quercetin equivalents/g extract), followed by composite extract 30.52 mg quercetin equivalents/ g extract, *A. indica* 29.73 mg quercetin equivalents/ g extract and *A. marmelos* (24.56 mg quercetin equivalents/ g extract). In this assay, *O. sanctum* 21.32 mg quercetin equivalents/ g extract showed the lowest flavonoid content (Figure 2).

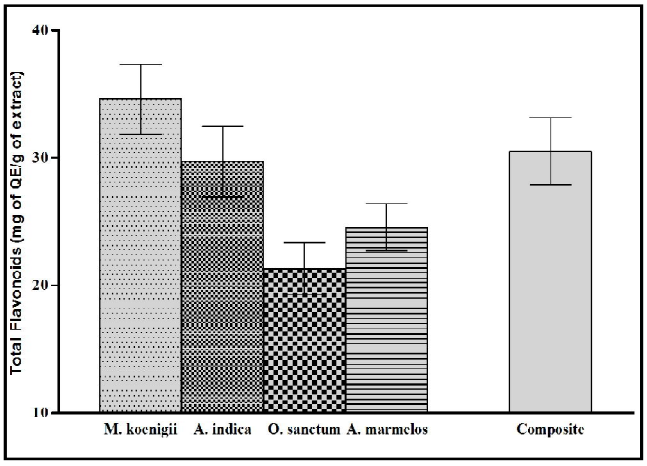

Figure 2: Flavonoid content in plant extracts. Flavonoid content is reported in terms of mg of quercetin equivalents/ g of extract. Values are expressed as Mean ± SD of the 3 independent experiments

3.4 Free radical scavenging activities

All the plant extracts showed very significant radical scavenging activities when determined by DPPH· assay. The radical scavenging activities of plant extracts was varied from 72.34 to 96.71 %. Here again, composite extract showed the highest antioxidant capacity (96.71% of DPPH· inhibition), followed by *M. koenigii*, *A. marmelos*, and *A. indica* which were 86%, 80% and 82% of DPPH· inhibition, respectively. *O. sanctum* extract showed the minimum (~ 72%) DPPH· inhibition (Figure 3).

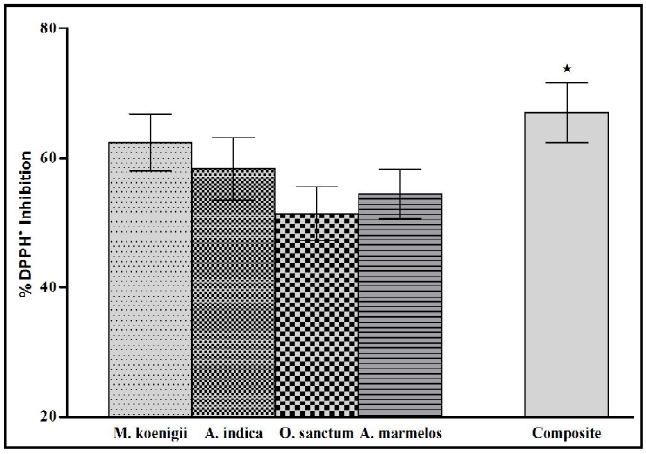

Figure 3: % DPPH· free radical inhibition of plants extracts. Values are expressed as Mean ± SD of the triplicate experiments.

3.5 Ferric reducing antioxidant potentials

All the tested plant extracts showed effective antioxidant effects when determined by FRAP assay. The antioxidant potentials were 3.860 mmol Fe^{2+}/g for *M. koenigii*, 3.510 mmol Fe^{2+}/g for *A. indica*, 3.210 mmol Fe^{2+}/g for *A. marmelos* and 2.760 mmol Fe^{2+}/g for *O. sanctum*. Composite extract showed very strong ferric ion reducing activity (6.220 mmol Fe^{2+}/g) (Figure 4).

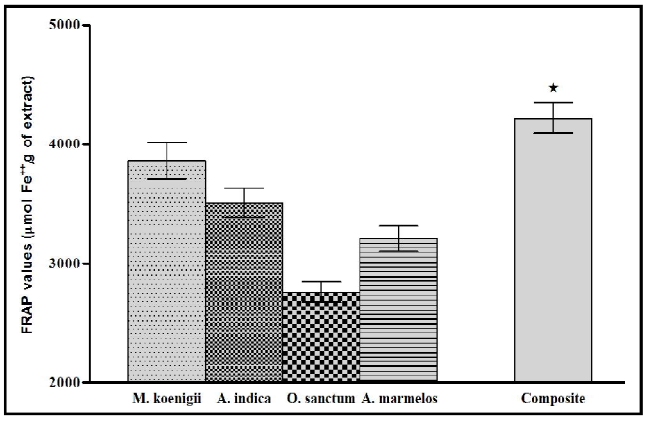

Figure 4: FRAP antioxidant potency of plant extracts. FRAP value expressed as µmol Fe^{2+}/g extract, Mean ± SD of the triplicate experiments

4. Discussion

The medicinal actions are unique to a particular plant species or group, consistent with the concept that the combination of secondary products in a particular plant is taxonomically distinct (Parekh *et al*., 2005). In this study, leaves of *A. indica*, *A. marmelos*,

O. sanctum, M. koenigii and the composite extract of all showed the presence of phytochemicals such as phenols, tannins, flavonoids, saponins, glycosides, steroids, anthroquinones and amino acids. Researchers have revealed that all these active constituents exhibit medicinal properties and play very significant role in health promotion (Craig, 1999). These active constituents are reported to reduce the chance of development of many oxidative damage associated diseases including cancer, cardiovascular diseases, cataracts, atherosclerosis, diabetes, arthritis, immune deficiency diseases and ageing (Sofowra, 1993; Bharti *et al*., 2012). The presence of phenols in tested extracts of plants advocates their homeostasis maintaining abilities since phenols are known as hydrogen donors and singlet oxygen quenchers due to their redox properties (Rice-Evan *et al*., 2005).

Flavonoids are reported as strong antioxidant bioactive compounds against free radicals (Pandey and Rizvi, 2009). It has been reported that efficiency of flavonoids to act as antioxidant depends upon the number of hydroxyl substitutions in their backbone structure. Flavonoids stabilize the free radicals by providing them reducing equivalents from their hydroxyl groups (Nijveldt *et al*., 2001).

The DPPH$^{\cdot}$ is widely used to evaluate the free radical scavenging activity of hydrogen donating antioxidants (Kumar *et al*., 2011). Antioxidants, on interaction with DPPH$^{\cdot}$, either transfer electrons or hydrogen atoms to DPPH$^{\cdot}$, thus neutralising free radical character (Naik *et al*., 2003). In our study, leaf extracts of *M. koenigii, A. marmelos, A. indica and, O. sanctum* showed significant scavenging activity against free radicals which may be correlated with the presence of phenolic compounds in extensive amount in these extracts. Our proposal is in accordance with the studies of Dehpour *et al*. (2009); Uddin *et al*. (2015) and Pongianta *et al*. (2016) in which they have reported that the presence of phenolic compounds including flavonoids is responsible for DPPH$^{\cdot}$ scavenging activity in plant extract.

FRAP assay is a sensible and authentic indicator to evaluate the total antioxidant capacity (Vassalle *et al*., 2004). This assay offers a putative index of antioxidant or reducing potential of biological fluids within the reach (Benzie and Strain, 1996). FRAP assay is an electron-transferred-based method that uses Fe (III)- 2,4,6-tri (2-Pyridyl)-S-triazine (TPTZ) as an oxidant. Our results revealed that the leaf extracts of *M. koenigii, A. marmelos, A. indica and O. sanctum* possess strong antioxidant potentials as evidenced by significant FRAP values. Phenolic compounds content dependent FRAP values, represent direct relationship of antioxidant potential and presence of polyphenols. Our results are in continuation with other studies that suggested that concentration of phenols in plant extracts including flavonoids is responsible for strong antioxidant capacity (Firuzi *et al*., 2005).

In our study, interestingly the composite extract elicited higher free radical scavenging as well as higher ferric reducing abilities than all the four studied plant extracts individually, irrespective of contents of phenolics and flavonoids, they possessed. We hypothesize this may be due to the synergic effects of the polyphenolic compounds present in that extract. Number of studies have documented that many phenolic compounds in the plant extracts exist in their inactive form, however, on mixing/combining with other plant extract, they become active and, thus, overall effect of this composite formulation exceeds than individual one (Mukherjee *et al*., 2011; Adwan and Mhanna, 2008).

Hartmann and colleagues have reported that, combination of nettle (*Urtica ioica*) and pygeum bark (*Pygeum africanum*), which are recommended against benign prostate hyperplasia, inhibited 5-reductase and aromatase more significantly than the sum of either alone. Experimental evidence of interaction between herbs was also provided by investigations into a clinically successful formulation of Chinese herbs, used to treat eczema (Hartmann *et al*., 1996; Sheehan and Atherton, 1992). We propose that higher DPPH• scavenging activity and enhanced FRAP values of composite extract of four select plant leaf extracts are due to synergic effects of different phenolic compounds present in these extracts.

5. Conclusion

The finding of present study suggests that the presence of biologically active phytochemicals in leaf extracts of *M. koenigii, A. marmelos, A. indica and O. sanctum* are responsible for their antioxidant and radical scavenging properties and their synergy reflected in enhanced potential of composite extract of leaves of these plants. We hypothesize that instead of individual plant, recommendation of composite formulation may be a better strategy against those events where oxidative stress prevails.

Conflict of interest

We declare that we have no conflict of interest.

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