Available online at www.ijpsdr.com
International Journal of Pharmaceutical Sciences and Drug Research 2012; 4(2): 143-146

Research Article ISSN 0975-248X

Evaluation of DPPH Radical Scavenging Activity and Reducing Power of Four Selected Medicinal Plants and Their Combinations

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ABSTRACT
Reactive oxygen species [ROS] cause oxidative damage to the tissues and protection from such damages are provided by endogenous and exogenous antioxidants. Plant based antioxidants are preferred due to the multiple mechanisms of actions and of the phytochemicals present in them. 80% alcoholic extract of leaves of *Aloe vera, Bacopa monniera, Moringa oleifera* and rhizome of *Zingiber officinale* was tested individually and in combination in equal proportion of each extract for DPPH scavenging activity and reducing power. The results indicate that the combination of the extract has better DPPH scavenging action and reducing power compared to the individual plant extract indicating synergistic and supra additive effect of phytochemicals present in the extract.

Keywords: Reactive oxygen species [ROS], antioxidants, phytochemicals, DPPH scavenging activity, reducing power, synergistic effects.

INTRODUCTION

Reactive oxygen species [ROS], sometimes called as active oxygen species, are various forms of activated oxygen, which include free radicals such as superoxide ions ($O_2^{\cdot-}$) and hydroxyl radicals ($OH^{\cdot}$) as well as non-free radical species such as hydrogen peroxide (H_2O_2). [1] These ROS play an important role in degenerative or pathological processes, such as aging, cancers, coronary heart diseases, Alzheimer's disease, neurodegenerative disorders, atherosclerosis, cataracts and inflammations. [2] Living organisms have antioxidant defence systems that protects against oxidative damage by removal or repair of damaged molecules. [3] The term 'antioxidant' refers to the activity of numerous vitamins, minerals and phytochemicals which provide protection against the damage caused by ROS. [4] Antioxidants interfere with the oxidative processes by scavenging free radicals, chelating free catalytic metals and by acting as electron donors. [5] The natural antioxidant mechanisms maybe insufficient in variety of conditions and hence dietary intake of antioxidant compounds are important. [6] The therapeutic effects of several medicinal plants are usually attributed to their antioxidant phytochemicals. It has been suggested that there is an inverse relationship between dietary intake of antioxidant rich foods and incidence of human diseases. [1] Plant based antioxidants are preferred to the synthetic ones because of their multiple mechanisms of actions and non-toxic nature. These facts have inspired widespread screening of plants for possible medicinal and antioxidant properties; the isolation and characterization of diverse phytochemicals and the utilization to antioxidants of natural origin to prevent the diseases. [7]

Aloe vera (L.) Burm f. (*Aloe barbadensis* Miller) or commonly called as 'khorpad' in Marathi; is a perennial succulent xerophytes, which develops water storage tissues in their leaves to survive in dry areas of low or erratic rainfall. It has been reported by several authors that different fractions of *Aloe vera* as well as unfractionated whole gel have antioxidant effects. Glutathione peroxidase, superoxide dismutase enzyme and phenolic antioxidants were found to be present in *Aloe vera* gel which may be responsible for its antioxidant effects. [8]

Bacopa monniera Linn (Scrophulariaceae) commonly known as 'brahmi'; is a component of several popular drugs of Ayurvedic system of medicine. The whole plant of brahmi has shown presence of phytochemicals like alkaloids, saponins, D-mannitol, betulic acid, β-sitosterols and stigma sterols showing health beneficial effects. [9]

Zingiber officinale or ginger belongs to Zingiberaceae family .The rhizomes of ginger have been used as medicine from Vedic period and is called ''maha aushadhi" means the great medicine. [10] The antioxidant properties of [6]-gingerol, which is very effective therapeutic agent present in ginger, studied in both *in-vitro* and *in-vivo* against UV induced skin disorders. [11]

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Moringa oleifera Lam commonly known as "drumstick", belongs to Moringaceae family. The derivatives of caffeic, p-coumaric and ferulic acids are dominant phenolic constituents which are present in Moringa *oleifera* leaves extract. [12]

The different plant extracts will have different modes of action for curing diseases and in mixture form may exhibit enhanced activity than that of individual plants, which is known as 'synergistic action'. A particular principle in the pure form may have only a fraction of the pharmacological activity than it has in its plant matrix. This highlights the importance of using the plant as a whole or a mixture of plants for treating a disease. [13] The objectives of the present study were to quantify total phenolics, flavonoids and flavonols in the combination of the four medicinal plant extracts selected; that included leaves of *Aloe vera, Bacopa monniera, Moringa oleifera* and rhizome of *Zingiber officinale.* It was also aimed to observe the antioxidant properties in terms of DPPH scavenging activity and reducing power of the individual plant extracts and their combinations for existences of any synergistic property of the four medicinal plant extracts selected.

MATERIALS AND METHODS

Leaves of *Aloe vera, Bacopa monniera, Moringa oleifera* and rhizome of *Zingiber officinale* were collected from Loni and adjoining areas, Maharashtra. These four individual plants were identified and authenticated by a Professor of Botany, Loni. The four medicinal plants were shade dried and powdered. The selected part of the medicinal plants was individually extracted in 80% ethanol by hot extraction in Soxhlet apparatus till colourless solvent was obtained. Individual extracts obtained were allowed to dry till constant weight was obtained. The percentage yield of extract of each plant was *Aloe vera* 28.62%, *Bacopa monniera* 16.18%, *Moringa oleifera* 14.90% and *Zingiber officinale* 12.69%.

Concentration of combination of herbal preparation

Combination of plant extracts was prepared by mixing 25 mg each of individual extract and dissolved in 10 ml methanol (that is 100mg/10ml), boiled and cooled. It was, then centrifuged at 2500 rpm for 10 minutes. The supernatant thus obtained was named as "Herbal Preparation" (HP-4) and this combination was used in further experiments.

Phytochemical Analysis

The herbal preparation was subjected to preliminary phytochemical analysis using standard procedures to estimate the phytochemicals present.

Estimation of total phenolic compounds

Total phenolic content was determined by the Folin Ciocalteu method by Folin *et al* 1927. [14] To 0.5 ml of 1-5 mg/ml of herbal preparation made up with 0.5 ml of distilled water, 0.5 ml of Folin Ciocalteu reagent was added and gently mixed. After 2 minutes 0.5 ml of 100mg/ml sodium carbonate was added. The contents were mixed and allowed to stand for 2 hours. The optical density of the blue coloured samples was measured at 765 nm spectrophotometrically. Standard gallic acid of concentration 100-500µg/ml was used. The concentration of total phenolics is expressed as milligram of gallic acid /g of mixture. All determinations were carried out in triplicate.

Estimation of flavonoids

The method used by Chang *et al* 2002 [15] with slight modifications in total volume of reagents used; was followed for estimation of flavonoids. 0.5 ml of concentration 100-500µg/ml of herbal preparation was mixed with 1 ml aluminium trichloride in ethanol (20g/l) and diluted with ethanol to 25 ml. The absorbance was read after 40 minutes incubation at 37°C spectrophotometrically at 415nm. Rutin (a citrus flavonoids glycoside) of concentration 0.5mg/ml, 1.0 mg/ml, 1.5 mg/ml, 2.0 mg/ml and 2.5 mg/ml was used as a reference compound and absorbance was measured under the same conditions. All determinations were carried in triplicate. The amount of flavonoids in herbal preparation was calculated as milligram of rutin/g of mixture.

Estimation of flavonols

The content of flavonols was determined by the method of Yermakov *et al* 1987 [16] with slight modifications like reduction in total volume of reagents used. 0.05 ml of various concentrations (100-500µg) was treated with 1ml of 2% aluminium trichloride in ethanol and 1ml of 5% sodium acetate. The absorption was read at 400nm was read after 2.5 hours at 37°C. The same procedure was carried out for 2ml of reference compound rutin for concentration 0.2mg/ml, 0.4 mg/ml, 0.6 mg/ml, 0.8 mg/ml and 1.0 mg/ml. All determinations were carried out in triplicate .The content of flavonols was calculated in terms of milligram of rutin /g of mixture.

Method for DPPH radical scavenging assay

Radical scavenging activity of plant extracts against stable 2, 2 diphenyl 2 picryl hydrazyl hydrate (DPPH) was determined by the slightly modified method of Brand-Williams *et al* 1995. [17] DPPH reacts with an antioxidant compound, which can donate hydrogen, and reduce DPPH. The change in colour (from deep violet to light yellow) was measured at 517 nm on a UV visible light spectrophotometer. The solution of DPPH in methanol 6×10^{-5} M was prepared fresh daily before UV measurements. Three ml of this solution was mixed with 100 microgram/ml concentration of individual plant extracts as well as herbal preparation. The samples were kept in the dark for 15 minutes at room temperature and the decrease in absorbance was measured. The experiment was carried out in triplicate. Radical scavenging activity was calculated by the following formula.

$$\% \text{ Inhibition} = [(A_B - A_A)/A_B] \times 100$$

Where A_B = absorption of blank sample (t= 0 min)

A_A = absorption of test extract solution (t=15 mins) [18]

Table 1: Preliminary phytochemical analysis

Phytochemicals	Contents
Total Phenolic compounds (mg gallic acid/g)	29.53 ± 0.42
Flavonoids (mg rutin/g)	24.05 ±0.57
Flavonols (mg rutin/g)	17.9 ±0.24

Table 2: DPPH scavenging activity

S. No	Concentration (µg/ml)	% Inhibition*
Aloe vera	100	38.47 ± 1.94
Bacopa monniera	100	37.98 ± 0.77
Moringa oleifera	100	38.28 ± 3.26
Zingiber officinale	100	43.02 ± 3.47
Herbal Preparation	100	63.75 ± 0.73**
Std BHT	100	44.70 ± 11.8
Std Vitamin C	100	80.38 ± 0.05

*Mean ± SD of three determinations. **Statistically significant ($p<0.05$), when compared with individual extract

***In-vitro* Reducing Power**

The reducing power of the individual plant extracts as well as their mixture was determined according to the method of Oyaizu 1986. [19] The experiment was carried out in triplicate.

Pre-determined concentration of 100µg/ml of individual plant as well as herbal preparation was mixed into the mixture of 2.5 ml of 0.2 M phosphate buffer (pH 7.4) and 2.5 ml of 1% potassium ferricynide. The mixture was then incubated at 50^{o}C for 20 minutes.

After incubation, 2.5 ml of trichloroacetic acid was added to the mixture, which was then centrifuged at 3000 rpm for 10 minutes. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml, 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Statistical methods: The results of all experiments performed were expressed as Mean ± SD of three determinations, the test of significance was applied wherever necessary and values obtained as $p<0.05$ were considered as statistically significant.

Table 3: *In- vitro* reducing power

S. No	Concentration µg/ml	Absorbance*
Aloe vera	100	0.045 ± 0.004
Bacopa monniera	100	0.038 ± 0.015
Moringa oleifera	100	0.111 ± 0.032
Zingiber officinale	100	0.119 ± 0.086
Herbal Preparation	100	0.185 ± 0.062**
Std BHT	100	0.702 ± 0.076
Std Vitamin C	100	1.315 ± 0.030

*Reducing power in terms of absorbance, mean ± S.D of three determinations measurements.

** Statistically significant (p<0.05), when compared with individual extract.

RESULTS

The Table 1 depicts the results of phytochemical analysis.

The DPPH radical scavenging activity was recorded in terms of % Inhibition as shown in Table 2. It was observed from Table II that *Bacopa monniera* has minimum DPPH scavenging activity (37.98 ± 0.77%) and *Zingiber officinale* has maximum DPPH scavenging activity (43.02 ± 3.47%) among individual plant extracts. The Results obtained were comparative to standards used that BHT and Vitamin C. Higher % Inhibition indicates better scavenging activity or antioxidant potential. HP-4 has shown better DPPH scavenging activity as compared to individual plant extracts. The results obtained were statistically significant with $p<0.05$.

As observed from Table 3, *Bacopa monneira* has minimum reducing power (0.038 ± 0.015) and *Zingiber officinale* has maximum reducing power (0.119 ± 0.086) amongst individual plant extracts. Higher absorbance indicates more reducing power. HP-4 has shown more reducing power in comparison to individual plant extracts. The results obtained were statistically significant with $p< 0.05$. It is reported that antioxidant activity and reducing power is one and the same. [8]

DISCUSSION

The phytochemical analysis of the combination of four selected medicinal plants, HP-4 is rich in total phenolic compounds, flavonoids and flavonols. Tannins [20], polyphenols [21] and flavonoids [22] are reported to have significant antioxidant properties .Accordingly; these compounds have shown to have antioxidant activity. [23-24] Total phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators hence it was reasonable to detect their amount in the herbal preparation. Flavonoids are the most widespread group of natural compounds and probably the most important natural phenolics. Total phenolics and flavonoids possess a broad spectrum of chemical and biological activities including radical scavenging properties. Such property is especially distinct for flavonols .The medicinal effects of plants are often attributed to the antioxidant activity of phytochemical constituents mainly phenolics, flavonoids and flavonols. [25] It is claimed that phenolic compounds are powerful chain breaking antioxidants. [26] The scavenging activity of phenolic group is due to its hydroxyl group. [27] The antioxidant activity has been reported to be concomitant with the development of reducing power. [7] Herbal preparation revealed synergistic effects both in DPPH scavenging and reducing power in comparison with the individual plant extracts selected for the study. The crude extracts of plants are pharmacologically more active than their isolated active principles due to the synergistic effects of various components present in the whole extract. [28] Synergistic effects with respect to antioxidant properties and blood glucose lowering effect in diabetic mice were reported by Triphala (*T. belerica, E. offcinalis* and *T. chebula*) than the individual constituents of the triphala formulation. [29] A synergistic relationship amongst phytochemicals has been adduced to be responsible for the overall beneficial effects derivable from constituent plants. The synergy of phytochemicals may make up for the apparent low values for individual classes of phytochemicals. [30] Similar effect on antioxidant activity was reported by Licovin, a polyherbal formulation of Lyka Labs Limited, Mumbai compared with the individual components of Licovin. [31] The administration of saffron (*C. sattivus*), garlic (*A. sativum)* and curcumin (*C. longa*) together showed increase in antigenotoxic effects against cyclophosphamide induced genotoxicity in mice against administration of individual components separately. [32]

The synergistic and supra additive effects of herbal formulation, HP-4 in our study may be due to the increased possibility of the interaction that allows more co operative effects involved in herbal formulation which is unison with studies by Liu. [33-34] Therefore the present study highlights the synergy of phytochemicals in the herbal preparation, HP-4 as compared to individual extracts. However, it is also possible to study the *in-vitro* antioxidant potential of herbal preparation HP-4 in terms of various other experimental models such as 2, 2' azino-bis (3-ethylbenzothiazoline-6-sulphonate) radical cation assay, superoxide scavenging assay, hydroxyl scavenging activity, nitric oxide scavenging assay, *in-vitro* antioxidant activity and total antioxidant activity in order to confirm the synergistic effect of the combination of individual extracts compared to single plant extract.

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