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Original Research

In vitro antioxidant potential and radical scavenging activity of polyherbal drug Shrishadi

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$ABTS^+$ assay; *Albizzia lebbeck*; *Cyprus rotundus*; *Solanum xanthocarpum*

Abstract
Free radicals are atoms or molecules or ions with one or more unpaired electrons on an open shell configuration. This electron imbalance causes high reactivity creating other free radicals by chain reactions. This will lead to tissue damage and oxidative stress. Aim of the present study was to identify antioxidant potential of the polyherbal formulation Shrishadi as a safe and inexpensive antioxidant. The plants *Albizzia lebbeck*, *Cyprus rotundus* and *Solanum xanthocarpum* (ingredients of Shrishadi compound) were extensively used in ayurvedic medicine and were investigated together in the form of a polyherbal compound. Hydroalcholic extract was prepared from the plant samples and was tested for total reducing power and *in vitro* antioxidant activity; evaluated by 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) $(ABTS)^+$ assay, superoxide anion scavenging activity assay and lipid peroxidation assay. Reducing power showed dose-dependent increase in concentration maximum absorption of 0.716 ± 0.015 at 0.8 mg/ml; $ABTS^+$ assay showed maximum inhibition of 82.27 ± 2.69 with EC_{50} 462.72 ± 4.56; superoxide free radicals showed maximum scavenging activity of 68.54 ± 1.78 with EC_{50} 658.51 ± 6.24; anti-lipid-peroxidation free radical scavenges maximum absorption of 72.91 ± 1.68 with EC_{50} 640.75 ± 4.45. As flavonoids are the major constituents responsible for antioxidant activity, free radicals such as superoxide anions and lipid peroxides were scavenged in a concentration-dependent manner suggesting the probable role of the polyherbal formulation to be a good source for antioxidant activity.

INTRODUCTION

An antioxidant is defined as a molecule capable of inhibiting the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions [1]. They do so by being oxidized themselves; so antioxidants act often as reducing agents such as thiols, ascorbic acid or polyphenols. Oxidative-process is the most common route for producing free radicals in food, drugs and even in living systems [2]. Free radicals finally contribute to different human disorders like atherosclerosis, arthritis, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS [3]. The majority of free radicals that damage biological systems are oxygen radicals, the main byproducts formed in the cells of aerobic organisms [4].

Antioxidants also act as radical scavengers, hydrogen donors, electron donors, peroxide decomposers, singlet oxygen quenchers, enzyme inhibitors and metal-chelating agents [5]. Due to effect on immune system, there is a need for natural antioxidants (safe and non-toxic) as compared to synthetic antioxidants (toxic for human). Beside this, liver toxicity and carcinogenesis has already reported by the accumulation of butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) [6].

Shrishadi is a polyherbal formulation used in the Ayurvedic medicine for management of respiratory disorders. It contains plants bark of *Albizzia lebbeck* (Shirisha), rhizome of *Cyprus rotundus* (Nagarmotha) and whole plant of *Solanum xanthocarpum* (Kantakari). *Albizzia lebbeck* has been reported to present anti-allergic [7], anti-asthmatic [8], antiinflammatory [9], analgesic [10], anxiolytic and antidepressant effects; in Ayurveda it is generally named as *Vishaghana*, *i.e.* destroying the toxins present in body. *Solanum xanthocarpum* known as Kantakari is a very effective hepatoprotective [11], antidiabetic [12], antioxidant [12, 13], anti-hyperglycemic [13], and larvicidal [14]. *Cyprus rotundus* or Nagarmotha is primarily responsible for treating inflammation and also as anti-pyretic and analgesic [15]; it is effective to manage fevers and digestion related disorders (diarrhea, vomiting, indigestion, *etc*).

We have recently reported the presence of phenolic and flavonoid content in the Shrishadi extract [16]. So the plan of this current study was to carry out the total antioxidant activity potential of this polyherbal formulation by using different standard tests.

MATERIALS AND METHODS

Plant collection

Albizzia lebbeck, Cyprus rotundus and Solanum xanthocarpum plants were collected from local market of Varanasi (India). The identification of the plants was done by Prof. A.K. Singh, Department of Dravyaguna, Sampurnanand Sanskrit University (SSU), Varanasi, Uttar Pradesh, India. The plant materials were air dried in shade at room temperature (26ºC) and were cleaned and powdered for further extraction process.

Preparation of the plant extract

A hundred grams of plant powder was subjected to hydroalcoholic extraction (distilled water:ethanol, 2:1) by hot percolation method through soxhlet apparatus. The extract was first filtered through a Whatmann filter paper No.42 (125 mm) and then through cotton wool. Then extract was concentrated using rotary evaporator with hot water bath at 40ºC and dried extract was put to the process of standardization. The percentage yield of the extract was determined and found to be 12%.

Total reducing potential

A 2.5 ml solution of extract (100-800 lg/ml) was mixed with equal volume of phosphate buffer (0.2 M, pH 6.6) and 1% potassium ferricyanide and placed in water bath at 50ºC for 20 min. Then it was cooled rapidly and 2.5 ml of 10% trichloroacetic acid was added and vortexed. This incubation mixture was centrifuged at 3,000 rpm for 10 min and its 5 ml supernatant was mixed with equal volume of distilled water and 1 ml of 0.1% ferric chloride. It was further incubated at room temperature for 10 min and absorbance was read at 700 nm. The reducing property of test sample was standardized against quercetin and expressed as difference in optical density (OD) from control as well as test as 0.1, and expressed as lg/ml [17].

$ABTS^+$ assay/total antioxidant activity

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) $(ABTS)^+$ radicals were pre-generated/activated by adding 5 ml of a 4.9 mM potassium persulfate solution to 5 ml of a 14 mM $ABTS^+$ solution and kept for 16 h in the dark. This solution was suitably diluted with distilled water to yield an absorbance of 0.7 at 734 nm and then used for antioxidant assay. Solution of vitamin C (50 µg/ml) was used as standard. It was added to 950 µl of $ABTS^+$ solution, vortexed for 10 seconds and after 6 min reduction in absorbance was recorded at 734 nm, using distilled water as a blank, on ELICO SL-150 UV-visible spectrophotometer (Sanathnagar, Hyderabad, Andhra Pradesh, India). Same volume of test solutions of each extract was also taken in similar manner [18].

Superoxide radical-scavenging property

This activity was measured by the reduction of nitro-blue tetrazolium (NBT) as it was based on the capacity of the extract to inhibit the photochemical reduction of NBT [19, 20]. In brief, each 3 ml reaction mixture contained 0.01 M phosphate buffer (PBS, pH 7.8), 130 mM methionin, 60 mM riboflavin, 0.5 mM EDTA, 0.75 mM NBT and 0.5 ml of test sample solution or standard solution. The tubes were kept in front of a fluorescent light and absorbance was taken after 6 min at 560 nm against blank (0.01 M PBS). Identical tubes were kept in dark which served as controls. The non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system generates superoxide radicals, which reduce NBT to a purple formazan. The percentage inhibition of superoxide generation was measured by comparing the absorbance of the control tube with that of test material or standard containing tubes.

Lipid peroxidation assay

A modified thiobarbituric acid reactive substances (TBARS) assay [21] was used to measure the lipid peroxide in egg-yolk homogenates [22]. Here, malondialdehyde (MDA), a secondary product of the oxidation of polyunsaturated fatty acids, reacts with two molecules of thiobarbituric acid (TBA), yielding a pinkish red chromogen with an absorbance maximum at 532 nm [23]. The egg yolk homogenate was prepared in distilled water, (10%, v/v) and 0.1 ml of extract was mixed in a test tube and the volume was made up to 1 ml, by adding distilled water. Finally, 0.05 ml $FeSO_4$

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(0.07 M) was added to the above mixture and incubated for 30 min to induce lipid peroxidation. Thereafter, 1.5 ml of 20% acetic acid (pH adjusted to 3.5 with NaOH) and 1.5 ml of 0.8% TBA (w/v) (prepared in 1.1% sodium dodecyl sulphate) and 0.05 ml 20% trichloroacetic asit (TCA) were added, vortexed and then heated in a boiling water bath for 60 min. After cooling, 5 ml of 1-butanol was added to each tube, vortexed properly and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532 nm against blank, where 0.1 ml of distilled water was used in place of the extract.

Statistical analysis

The data were subjected to correlation coefficient by using SigmaStat statistical analysis software (version 3.1). The correlation of the data was determined by Pearson's test. $P < 0.05$ was considered as statistically significant.

RESULTS

In-vitro antioxidant assay of the polyherbal compound revealed the presence of antioxidant potential. The percentage of inhibition was observed that free radicals were scavenged by the test compounds in a concentration dependent manner up to the given concentration in all the models.

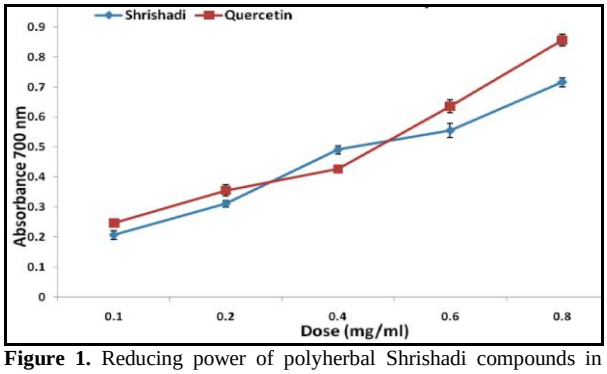

Figure 1. Reducing power of polyherbal Shrishadi compounds in hydroalchoholic extract and comparison with quercetin.

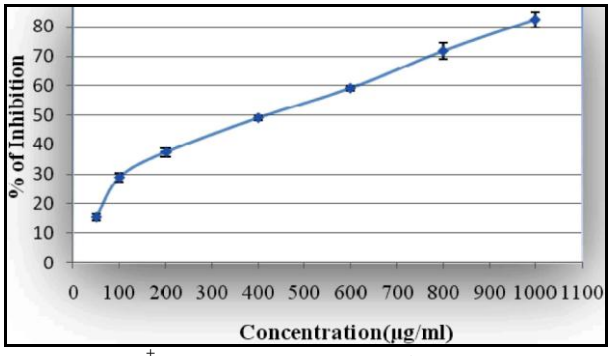

Figure 2. $ABTS^{+}$ radical scavenging activity of Shrishadi polyherbal

Reducing potential of the polyherbal drug as a function of concentration dose dependend increase of Fe^{3+} to Fe^{2+} was observed and shown in Fig.1. Amongst different concentration of extracts tested 0.1, 0.2, 0.4, 0.6 and 0.8 mg/ml presented maximum absorption of 0.716 ± 0.015 at 0.8 mg/ml compared with standard quercetin as 0.856 ± 0.020.

$ABTS^{+}$ radicals' inhibition, shown in Fig.2, presented maximum inhibition of 82.27 ± 2.69 at 1000 µg/ml among different concentrations of 50, 100, 200, 400, 600, 800, and 1000 µg/ml with EC_{50} 462.72 ± 4.56. Superoxide free radicals showed maximum inhibition of 68.54 ± 1.78 at concentration of 1000 µg/ml with EC_{50} value of 658.51 ± 6.24 proving again the better antioxidant activity.

Anti-lipidperoxidation free radicals were scavenged with maximum inhibition of 72.91 ± 1.68 at the concentration of 1000 µg/ml with EC_{50} 640.75 ± 4.45.

DISCUSSION

In general, it can be concluded that the radical scavenging property and antioxidant activity of polyherbal extracts is positively correlated with their total phenolics content [16]. However, some other antioxidant compounds may also be present in this particular polyherbal compound Shrishadi; thus, in the present study different chemical aspects were evaluated for the *in vitro* antioxidant assay of the polyherbal formulation.

In living systems, free radicals are constantly generated causing extensive damage to tissues and biological molecules which may lead to various diseases [24]. Many synthetic antioxidant drugs are available but, because of their adverse side effects an alternative solution to this problem is to develop such as natural antioxidants through food supplements and traditional medicines [25]. There are a lot of medicinal plants having potent free radical scavenging activity reported. Though contemporary medicines are useful in the prevention and management of diseases but wide range of toxic side effects and, on the other hand, resistances to antibiotics compel us to search some new natural compounds. The hydro-ethanolic extracts of Shrishadi and its phytochemical analysis have already been reported. Phytochemical screening of the extracts showed that Shrishadi compound has significant phenolic and flavonoid content. Literature search of individual plants shows the presence of tannins, glycosides, alkaloids, flavones, *etc* are the prominent phytochemicals in the three plants of this formulation.

These phytochemicals are structurally polyphenolic in nature and associated with free radical scavenging properties. They have good antioxidant potential,

wound healing and nutritive value [26]. These plant extracts and there pure phytochemicals exert their beneficial health effects mainly through their antioxidant activity by preventing the first step in the chain reaction of lipid peroxidation, *i.e.* the initiation step. They scavenge the existing free radical species such as hydroxyl, superoxide radicals or by binding metal ions (metal chelation) responsible for hydroxyl radical generation through Fenton's reaction. It may interrupt the chain reaction by decomposing the primary products of oxidation to non-radical species [27].

Previous studies have suggested that the total polyphenolic content of the plant extracts are positively correlated to the scavenging activities and reducing potential of an extract. Our result shows that Shrishadi scavenges pre-generated $ABTS^{+}$ radicals; here blue chromophore produced by the reaction between ABTS and potassium persulfate [28] is reduced in the concentration dependent manner (Fig.2). These results were compared with those obtained with gallic acid which indicates that the extract is a potent concentration-dependent antioxidant. Similar results were seen with lipid peroxide scavenging by the drug (Fig.3). Again, a similar trend of scavenging superoxide anions indicates that Shrishadi is a potent superoxide scavenger in correlation with its concentration (Fig.4).

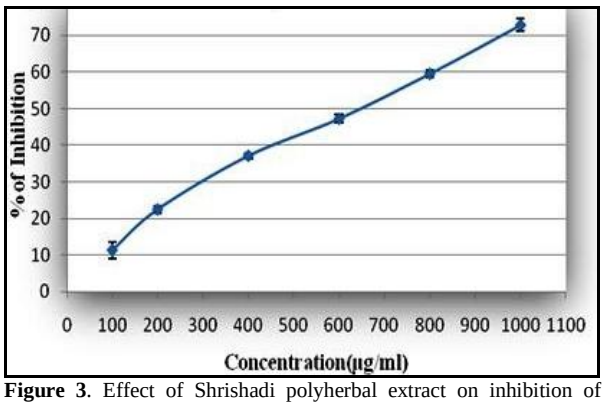

Figure 3. Effect of Shrishadi polyherbal extract on inhibition of $FeSO_4$ induced lipid peroxide

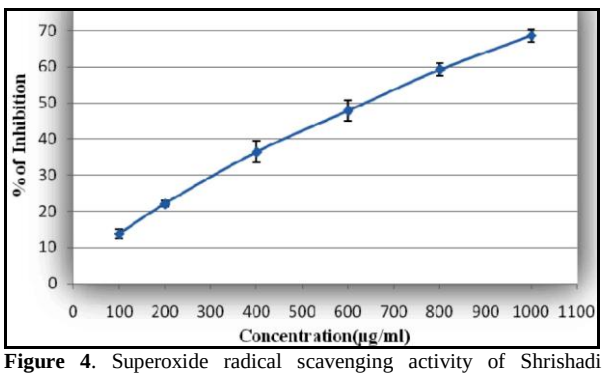

Figure 4. Superoxide radical scavenging activity of Shrishadi polyherbal

Our results show that this extract inhibits the $FeSO_4$ induced lipid peroxidation in egg yolk, which is the net result of iron-mediated hydroxyl radicals. This can be achieved either by scavenging the hydroxyl radicals or by chelating the iron ions, which is responsible for initiation of Fenton's reaction. Earlier studies with the plant of Shrishadi indicate metal chelating and hydroxyl radical scavenging properties [29, 30], So inhibition of lipid peroxidation may be immediate by both the properties as described earlier. Our qualitative test for various groups of phytochemicals show the presence of tannins in Shrishadi reportedly to have metal chelating and hydroxyl radical scavenging properties [31].

In conclusion, the data obtained in the present investigation suggest that polyherbal compounds may be good sources of antioxidants for radical scavenging. The highly positive correlation of radical scavenging activity and total polyphenolic content in polyherbal compounds indicates that phenols and flavonoids are important components which are mainly responsible for the free radical scavenging activity.

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