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ORIGINAL ARTICLE

Free radical scavenging activity of *Shirishavaleha* – A Poly Herbal Ayurvedic formulation

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ABSTRACT

Objective: Synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have recently been reported to be hazardous to human health. Thus, the search for effective, non-toxic natural compounds with antioxidative activity has been intensified in recent years. The current attempt aimed to determine the antioxidant activity of two samples of *Shirishavaleha*, one prepared with water and the other with *Kanji* as liquid media, using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and lipid peroxidation (LPO) assay.

Methods: Batches of *Shirishavaleha* with water and *Kanji* were prepared by following standard manufacturing procedures. Quercetin (2 mg/ml) and butylated hydroxyanisole (BHA, 0.8 mg/ml) were used as standard drugs for DPPH and LPO, respectively.

Results: Both the drugs were found to possess DPPH radical and LPO scavenging activity, comparatively higher degrees in the sample prepared with Kanji.

Conclusion: Based on the observations; it can be concluded that both the samples possesses significant antioxidant activities which may open their way for being used as potential natural antioxidants.

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INTRODUCTION

Oxygen free radicals induce damage due to peroxidation to biomembranes and also to DNA, which lead to tissue destruction and thus can initiate disease manifestation. Antioxidants neutralize such free radicals through different ways and prevent the body from diseases. Antioxidants may be synthetic or natural. Synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have recently been reported to be hazardous to human health. Thus, the search for effective, non-toxic natural compounds with antioxidative activity has been intensified in recent years [1].

As sources of natural antioxidants, much attention is being paid towards natural resources like plants; thus interest in natural antioxidant, especially of plant origin, has greatly increased in recent years [2]. *Shirishavaleha* is a classical Ayurvedic formulation possessing significant immuno-modulatory and anti-asthmatic activities [3, 4].

The free radical scavenging activity of numerous individual drugs have been reported [5, 6], but that of *Shirishavaleha* is not evaluated till date. Considering this, two samples of *Shirishavaleha*, *i.e.* one prepared in presence of water and the other in *Kanji* (sour gruel), were investigated for their possible antioxidant activity through 2,2-diphenyl-1-picrylhydrazyl solution (DPPH) and lipid peroxidation (LPO) assay. It was hypothesized that *Kanji* may facilitate more extractions into liquid media [7], and hence may significantly improve the free radical scavenging activity.

MATERIALS AND METHODS

Collection and preparation of test sample

Shirishavaleha is a compound Ayurvedic formulation (Table 1), which comprises powders of nine drugs in equal proportion and *Guda* (jaggery) as sweetening substance [8]. Stem bark of *Shirisha* (*Albizia lebbeck* Benth.) was freshly collected from the botanical garden while other raw materials were procured from the Pharmacy Department of Gujarat Ayurved University, Jamnagar. Jaggery was purchased from a local market of Jamnagar. All the herbal drugs were authenticated at Pharmacognosy Laboratory, Institute for Postgraduate Teaching and Research in Ayurveda (IPGT & RA), Gujarat Ayurved University. These drugs were cleaned and shade dried before usage. As *Kanji* may help in extracting more principles into the finished product, *Shirishavaleha* was prepared in presence of water (SW) and *Kanji* (SK). *Kanji* was prepared by following classical guidelines [9].

Preparation of drugs

Both the drugs were prepared in the department of Rasashastra and Bhaishajya Kalpana, IPGT & RA. *Shirisha Kwatha* (decoction) was prepared in presence of water and *Kanji*, then *Guda* (jaggery) was added to it and treacle was prepared. After obtaining *Avaleha Siddhi Lakshana* [10], *Prakshepa dravya* (adjuvants) were added and packed in air tight containers.

Table 1. Composition of *Shirishavaleha*

	Ingredient	Botanical name	Family	Part	Quantity in gram
1	*Shirisha*	*Albizia lebbeck* Benth.	Mimosoideae	Dried St. Bk.	2,400
2	*Pippali*	*Piper longum* Linn.	Piperaceae	Dried Fr.	48
3	*Priyangu*	*Callicarpa macrophylla* Vahl.	Verbenaceae	Dried Fl.	48
4	*Kushtha*	*Saussurea lappa* C. B. Clarke	Asteraceae	Dried Rt.	48
5	*Ela*	*Elettaria cardemomum* Maton.	Zingiberaceae	Dried Sd.	48
6	*Nilini*	*Indigofera tinctoria* Linn.	Fabaceae	Dried Rt.	48
7	*Haridra*	*Curcuma longa* Linn.	Zingiberaceae	Dried Rz.	48
8	*Daruharidra*	*Berberis aristata* DC.	Berberidaceae	Dried St.	48
9	*Shunthi*	*Zingiber officinale* Roscoe.	Zingiberaceae	Dried Rz.	48
10	*Nagkesara*	*Mesua ferrea* Linn.	Guttiferae	Dried Stmn.	48
11	*Guda*	-	-	-	9600
12	*Jala/Kanji (v/v)*	-	-	-	24,500 l

St. Bk., stem bark; Fr., fruit; Fl., flower; Rt., root; Sd., seed; Rz., rhizome; Stmn., stamen

Qualitative analyses of functional groups and estimation of tannins, saponins and alkaloids

Determination of physico-chemical parameters, *i.e.* total tannin [11], total saponin and total alkaloids [12] estimation were made. Also qualitative analyses of functional groups were done by using methanolic extract of *Shirishavaleha*. Methanol extract was prepared by taking 5 g of drug in 100 ml of methanol, it was shaken for some time; mild heat was provided to it for half an hour and then filtered on cooling. The filtrate is evaporated on water bath to approximately 20 ml and used.

Estimation of total tannins: 2 g of sample was defatted with 25 ml petroleum ether for 12 h. The marc was boiled for 2 h with 300 ml of double distilled water, cooled, diluted up to 500 ml and filtered; 25 ml of this infusion was measured into a 2-liter porcelain dish; 20 ml Indigo solution and 750 ml double distilled water was added. Then titrated it 0.1N potassium permanganate solution, 1 ml at a time, until blue solution changed to green; thereafter added drop-wise until solution becomes golden yellow. Similarly, mixture of 20 ml Indigo solution and 750 ml of double distilled water was titrated and the difference between two titrations in ml was calculated. Each ml of 0.1N potassium permanganate solution is equivalent to 0.004157 g of total tannins.

Alkaloid estimation: The plant material was macerated with 2% acetic acid in water, filtered and concentrated the filtrate under reduced pressure at 450 to one third of the original volume. The pH was adjusted to 2 by 4M hydrochloric acid. The yellow precipitate was separated from the solution A and dissolved in 0.1M to give solution B. Mayer's reagent was added to solutions A and B to give precipitate of alkaloid-Mayer's reagent complex. Again, it was dissolved in acetone-methanol-water (6:2:10) to give solution C. This complex was finally passed through Amberlite IRA 400 anion exchange resin (500 g) to give an aqueous solution of alkaloid chlorides.

Determination of saponins: The saponin content of the samples was determined by the double extraction gravimetric method [12]. In brief, a measured weight (5 g) of the sample was mixed with 50 ml of 20% aqueous ethanol solution in a flask. The mixture was heated with periodic agitation in water bath for 90 min at 55ºC; it was then filtered through Whatman filter paper (No 42). The residue was extracted with 50 ml of 20% ethanol and both extracts were poured together; the combined extract was reduced to about 40 ml at 90ºC and transferred to a separating funnel where 40 ml of diethyl ether was added and shaken vigorously. Re-extraction by partitioning was done repeatedly until the aqueous layer became clear in color. The saponins were extracted, with 60 ml of normal butanol. The combined extracts were washed with 5% aqueous sodium chloride (NaCl) solution and evaporated to dryness in a pre-weighed evaporation dish. It was dried at 60ºC in the oven and reweighed after cooling in a desiccator. The process was repeated two more times to get an average. Saponin content was determined by difference and calculated as a percentage of the original sample.

DPPH assay

Reagents: 22 mg of DPPH was accurately weighed and dissolved in 100 ml of methanol. From this stock solution, 18 ml was taken and diluted to 100 ml using methanol to obtain 100 μM DPPH solution.

Test solutions: 21 mg each of the extracts were dissolved in distilled dimethyl sulfoxide (DMSO) separately to obtain solutions of 21 mg/ml concentrations. Each of these solutions were serially diluted separately to obtain lower concentrations.

Standard solutions: 10 mg each of ascorbic acid and rutin were weighed separately and dissolved in 0.95 ml of DMSO to get 10.5 mg/ml concentrations. These solutions were serially diluted with DMSO to get lower concentrations.

Procedure: The assay was carried out in a 96 well microtiter plate. To 200 µl of DPPH solution, 10 µl of each of the test sample or the standard solution was added separately in wells of the microtiter plate. The final concentration of the test and standard solutions used were 1000, 500, 250, 125, 62.5, 31.25, 15.625 and 7.812 µg/ml. The plates were incubated at 37°C for 30 min and the absorbance of each solution was measured at 490 nm, using a microplate reader [13].

Lipid peroxidation activity

Preparation of egg lecithin: The egg yolk was separated and washed with acetone until yellow color disappeared.. The creamy white powder obtained was used for the procedure by dissolving in phosphate buffer pH 7.4 (3 mg/ml).

Procedure: The reaction mixture containing egg lecithin (1 ml), ferric chloride (0.02 ml), ascorbic acid (0.02 ml) and extract or standard (0.1 ml) in DMSO at various concentrations was kept for incubation for 1 h at 37ºC. After incubation, 2 ml of 15% TCA (thiazolidine-4-carboxylic acid) and 2 ml of 0.37% TBA (thiobarbituric acid) were added. Then the reaction mixture was boiled for 15 min, cooled, centrifuged and absorbance of the supernatant was measured at 532 nm [14].

RESULTS

DPPH radical scavenging activity

Results of the DPPH assay are presented in Table 2. The IC_{50} (half maximal inhibitory concentration) values of the test samples were found to be > 1000 µg/ml, while it was 12.4 ± 0.1 µg/ml for the standard drug quercetin. Percentage scavenging of DPPH radical was found to rise with increasing concentration of the crude extract (Figures 1 and 2).

LPO assay

Results of the LPO assay are given in Table 3. The IC_{50} values of the test samples were found to be 244 ± 5.29 and > 1000 µg/ml for SK and SW, respectively, while it was 27.4 ± 1 µg/ml for the standard drug BHA. Percentage scavenging of LPO was found to rise with increasing concentration of the crude extract (Figures 3 and 4).

Table 2. DPPH radical scavenging assay

Sample	IC_{50} values (µg/ml)
SK	> 1000
SW	> 1000
Standard (quercetin)	12.4 ± 0.1

DPPH, 2,2-diphenly-1-picrylhydrazyl; SK, *Shirishavaleha* prepared in presence of *Kanji*; SW, *Shirishavaleha* prepared in presence of water

DISCUSSION

Many plant constituents scavenge free radicals. Demand for the natural antioxidants is shooting up nowadays as neutraceuticals, bio-pharmaceuticals, as well as food additives due to consumer preference. Dietary intake of antioxidant rich foods decreases the incidence of human diseases [15]. Also, plant-based antioxidants are preferred against the synthetic ones because of their multiple mechanisms of actions and non-toxic nature.

Table 3. Lipid peroxidation assay

Sample	IC_{50} values (µg/ml)
SK	244 ± 5.29
SW	> 1000
Standard (BHA)	27 ± 1

SK, *Shirishavaleha* prepared in presence of *Kanji*; SW, *Shirishavaleha* prepared in presence of water; BHA, butylated hydroxyanisole

Protection from oxidative damages to tissues are provided by endogenous and exogenous antioxidants. The term 'antioxidant' refers to the activity of numerous vitamins, minerals and phytochemicals which provide protection against the damage caused by reactive species and interfere with the oxidative processes by scavenging free radicals, chelating free catalytic metals and by acting as electron donors [16]. The formulation, *Shirishavaleha* is attributed with significant anti-asthamatic activity [4]. *Shirisha* (*Albizzia lebbeck* Benth.) contains alkaloids, tannins, phenols, glycosides, flavanoids, steroids and saponins abundantly in its bark (Table 4) [17]. Bark contains different forms of saponins like libbekenin A, B, C, albiziasaponins A, B, C, and 7-11% condensed tannins that might be responsible for its therapeutic attributes like anti-anaphylactic, anti-asthmatic, anti-diarrheal, anti-spermatogenic, anxiolytic, anti-inflammatory, anti-histaminic, *etc* [18-24]. *Shirishavaleha*, as a compound is reported to have immunomodulatory, anti-tussive and anti-inflammatory activities in experimental models [3, 25, 26]. These multidemnsional attributes may be due to various phytochemical constituents present in the formulation, further may be due to their antioxidant properties. Ancient seers advocated more extractions of the crude drug into *Kanji* when used as liquid media [7]. Thus, while using *Kanji* as liquid media in the preparation of *Shirishavaleha*; it may get more extractions of the crude drug, *i.e. Kanji* may facilitate more phytochemicals; thus may show comparative better results therapeutically.

Flavonoids, one of the polyphenols, are well known pro-inflammatory mediators in the pathogenesis of inflammation and in inhibiting protein oxidation [27-30], as well as are very efficient free radical scavengers [31]. Antioxidant functions of flavonoids are due to their action as metal chelators, reducing agents, scavengers of reactive oxygen species, chain breaking antioxidants and quenchers of singlet oxygen formation [32]. Dietary flavonoid intake is helpful in balancing the weight and reducing the risk of various disease conditions like

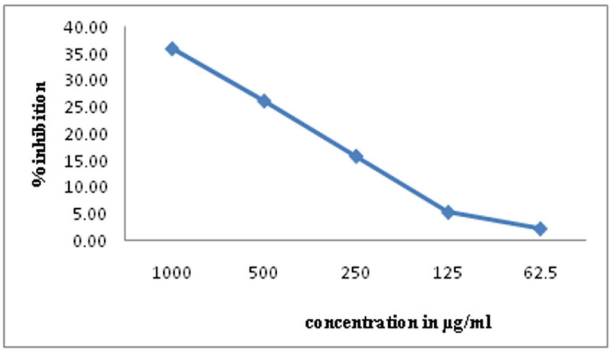

Figure 1. DPPH assay of SW

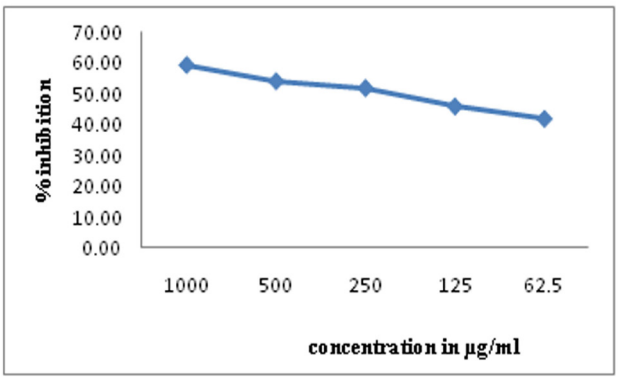

Figure 3. LPO assay of SW

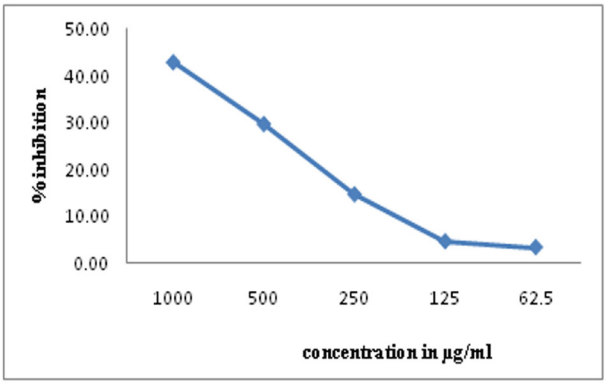

Figure 2. DPPH assay of SK

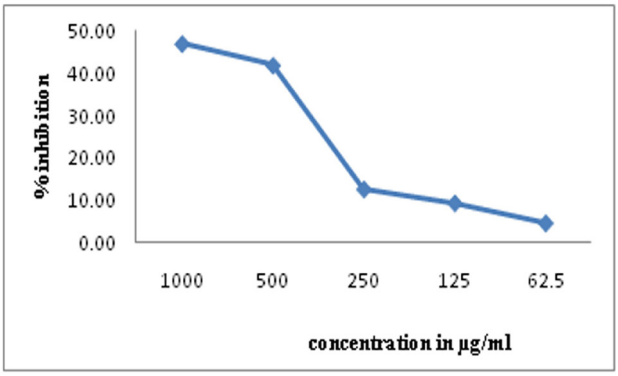

Figure 4. LPO assay of SK

hypertension, diabetes, cardiovascular disease, cancer and mortality [33]. Increased consumption of flavonoids is also reported to be associated with better lung function in asthmatic patients [34, 35].

Flavanoids also reduce asthma inflammation through antioxidant, anti-allergic, and anti-inflammatory properties [36]. Phenolics are widely distributed plant metabolites found virtually in all plants. Many low molecular weight phenolics appear to have specific roles in maintaining health, which inhibits stages of tumor initiation, promotion and progression. These potential benefits of consuming the naturally occurring polyphenolics known as tannins [37]. The tannins are considered as biological antioxidants [38, 39]. Preliminary analytical studies revealed presence of different functional groups in *Shirishavaleha* (Table 4)

Table 4. Qualitative analysis for presence of functional groups in *Shirishavaleha*

	Material	Reagents	Functional groups	Observation	Result
1	*Methanolic extract of *Shirishavaleha*	Dragendorff's reagent	Alkaloids	No Brown ppt.	Present
2		Dilute $FeCl_3$	Tannins	Blue brownish color	Present
3		Neutral $FeCl_3$	Phenols	Violet color	Present
4		Benedict's reagent	Carbohydrates	Yellow ppt.	Present
5		Concentrate H_2SO_4	Glycosides	color change brown color disappears	Present
6		Lead acetate	Flavonoid	Yellow color	Present
7		Chloroform, concentrate H_2SO_4, distilled water	Steroids	Brown ring with rinse	Present
8		Shaking in test-tube	Saponins	Frothing with honeycomb appearance	Present

*Methanolic extract of both SW and SK were made individually

Table 5. Total tannins, alkaloids and saponins in *Shirishavaleha*

	Formulation	Total tannins (%)	Total saponins (%)	Total alkaloids (%)
1	**SW**	2.88	21.47	0.124
2	**SK**	3.26	20.07	0.088

SK, *Shirishavaleha* prepared in presence of *Kanji*; SW, *Shirishavaleha* prepared in presence of water

[8]. Quantitatively, tannins were found to be slightly higher in SK (3.6%) than in SW (2.9%), indicating antioxidant properties of both test samples. Similarly, saponins were present in both samples almost equally, *i.e.* 21.5% in SW and 20.1% in SK (Table 5). Reported studies revealed that the antitumor activity might be mediated through scavenging of free radicals by saponins and their anti-inflammatory activity [40]. Few studies of alkaloids as antioxidant compounds are reported [41, 42]. Total phenolics and flavonoids possess a broad spectrum of chemical and biological activities including radical scavenging properties [43]. Thus antioxidant activity of both samples of this compound formulation may be due to the presence of antioxidant rich phytochemicals. The reason for presenting better antioxidant activity in sample prepared with *Kanji* may be due to a higher extraction grade of these phytochemicals.

In conclusion, the present study presented similar free radical scavenging activity for both drugs in the DPPH, while in the LPO model the activity is highly significant with SK. Thus, a part of the therapeutic effects of *Shirishavaleha* could be attributed to its antioxidant phytochemicals. The formulation may further be used in chronic diseases complementing other needful drugs. This will rationalize the use of natural products in health care. Developing coordinated research collaborations involving traditional health methods seems to be a fruitful step in evaluating the role of antioxidants in health and disease for the coming decades.

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