ORIGINAL ARTICLE

Standardization and antioxidant activity of an *Ayurvedic* formulation "*Kushavleha*"

Vineet Sharma, Kritika Hem, Seth Ankit, Santosh Kumar Maurya

Department of Ayurvedic Pharmacy, Ayurvedic Pharmacy Laboratory, Rajiv Gandhi South Campus, Banaras Hindu University, Barkachha, Mirzapur, Uttar Pradesh, India

Abstract

Background: *Kushavleha*, an Ayurvedic polyherbal formulation is an effective medicine for urinary calculi and many more. The present study was carried out to standardize the formulation and established its antioxidant activity *in vitro*. **Methods:** The formulation was prepared in the laboratory with authenticated plant drugs. The standardization procedure was conducted on the basis of pharmacognostical and phytochemical parameters as directed by World Health Organization guidelines. **Results:** Powder study of *Kushavleha* shows several diagnostic characters such as starch with concentric hilum, pitted vessel, stone cells, fiber with oil globules, pollen grains, lignified fiber epidermis of testa, unicellular trichome and stellate trichome. However some major phytoconstituents like flavonoids, phenolics, tannins, steroids, glycosides, alkaloids and amino acids were found to be positive in preliminary phytoconstituent screening of formulation. Total phenolic, tannin, flavonoid and flavonol content were found to be 144.60 ± 0.41 mg/g in gallic acid equivalent, 123 ± 0.53 mg/g in tannic acid equivalent, 69 ± 0.12 mg/g in rutin equivalent and 0.61 ± 0.27 mg/g in rutin equivalent respectively. Moreover, total solid content, fat content, sugar content, reducing sugar and non reducing sugar were found to be 69 % (w/w), 3.08% (w/w), 68.70% (w/w), 14.21% (w/w) and 54.49% (w/w) respectively. The formulation also exhibited potential antioxidant activity in *in vitro* DPPH scavenging screening with an IC50 of 63.80 µg/mL. **Conclusion:** The present result will help in the quality control standardization tool for the manufacturing and processing of *Kushavleha*.

Key words: Antioxidant, *Ayurveda*, *Kushavleha*, pharmacognostical

INTRODUCTION

Ayurveda is a curative and health maintaining science.[1] The maintenance of health is the primary aim of *Ayurveda,* which later on gained popularity as preventive and promotive health science. It emphasizes not only on medicines but also on the diet and lifestyle along with the stress reducing yogic practices. Since many centuries, *Ayurvedic* medicines have been used by people due to its ability to enhance immunity and prevent diseases.[2] Due to lack of scientific standards for the *Ayurvedic* medicines, *Ayurveda* does not gain its glory worldwide.[3] Hence, in the current scenario, its a major challenge in front of researchers from this field.[4] Standardization of *Ayurvedic* formulations can be achieved by pharmacognostic identification, physical, chemical, biochemical estimation and determination of active phytoconstituents in the plant as well as in formulations.[5] Standardization of any drug needs laborious effort because many factors directly influence the quality and purity of the drugs.[6] *Ayurvedic* medicine is available in a variety of dosages form such as *Avaleha* (electuary), *Asava-Arishta* (alcoholic preparations), *Ghrita* (fat based medicine), *Taila* (oil based medicine), *Churna* (powder), *Swarasa* (juice), *Vati* (tablet), *Kwath* (decoction), and much more.[7] *Avaleha*, a semi-solid dosage form is well-known for its acceptability and palatability. *Kushavleha* is a popular *Avaleha*, recommended for all types of *Prameha* (diabetes), *Agnimandya* (digestive impairment), *Aruci* (tastelessness), *Mutraghata* (urinary problem), and *Ashmari*

Address for correspondence:
Santosh Kumar Maurya, Department of Ayurvedic Pharmacy, Ayurvedic Pharmacy Laboratory, Rajiv Gandhi South Campus, Banaras Hindu University, Barkachha, Mirzapur - 231 001, Uttar Pradesh, India.
E-mail: dravyapharma@gmail.com

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(renal calculi). The formula composition and therapeutic indications of *Kushavleha* are documented in the *Bhaishajya Ratnavali*.[8] In the present work, an attempt was made to standardize *Kushavleha.*

MATERIALS AND METHODS

All the ingredients [Table 1] were procured from authentic shop at Gola Dinanath, Varanasi. Taxonomical authentication and identification of the crude drugs were done by Prof. A. K. Singh, Department of Dravyaguna, Faculty of Ayurveda, Institute of Medical Science, Banaras Hindu University Varanasi. A voucher specimen (APRL/HERB/14-15/13-30) of the each drug has been placed at the Ayurvedic Pharmacy Laboratory, Rajiv Gandhi South Campus, Banaras Hindu University, Mirzapur, Uttar Pradesh, India for further reference.

Method of Preparation

The formulation was prepared as per classical method mentioned in *Sharangadhara Samhita*.[9] The dried *kwath dravyas* were made into coarse powder separately in the mechanical grinder and passed through 20#. The *praksepa dravya* was converted into a fine powder with the help of mechanical grinder and 85# separately. A mixture of coarse powder of *kwath* drug (each 460 g) was soaked in 23 L water for overnight. Then, it was heated over 450°C until total water content was reduced up to 1/4th of its initial quantity. The contents were filtered with muslin cloth. Semisolid jaggery was added to the decoction and boiled again at 450°C with continuous stirring. Heating was continued until the desired characteristic of *Avaleha* (consistency of two threads when pressed between two fingers) was obtained. After 15 min, fine powders of *praksepa dravya* were added and mix gently as well as uniformly until a homogeneous mixture was obtained. The prepared *Kushavleha* formulation was kept in air tight container.

Determination of Physicochemical

The prepared *Kushavleha* was used for the determination of different physicochemical parameters such as crude fiber, different ash values, extractive values, total solid content, fat content, acidity, pH, sugar content, reducing sugar, nonreducing sugar according to standard procedures.[10,11] Organoleptic characters were described according to the method stated by PLIM.[12]

Phytochemical Evaluation

The preliminary phytochemical screening of the methanolic as

Table 1: Ingredients of *Kushavleha*

Drug	Botanical name*	Family*	Part used	Quantity
Kusa	*Desmostachya bipinnata* Linn.	Poaceae	Root	460 g
Kasa	*Saccharum spontaneum* Linn.	Poaceae	Root	460 g
Khasa ushira	*Chrysopogon zizanioides* Linn.	Poaceae	Root	460 g
Kala ikshu	*Saccharum officinarum* Linn.	Poaceae	Root	460 g
Ramasar	*Saccharum bengalense* Retz.	Poaceae	Root	460 g
Jaggery				746 g
Water				23 L
Mulethi	*Glycyrrhiza glabra* Linn.	Fabaceae	Root	11.5 g
Kakadi	*Cvcitmis sativus* Linn.	Cucurbitaceae		11.5 g
Kohda	*Cucurbita maxima* Duchesne	Cucurbitaceae		11.5 g
Khira tripush	*Cucumis sativus* Linn.	Cucurbitaceae		11.5 g
Vanslochana	*Bambusa bambos* Linn.	Poaceae		11.5 g
Amalaki	*Phyllanthus emblica* Linn.	Phyllanthaceae	Fruit	11.5 g
Tejpata	*Cinnamomum tamala* Buch. -Ham.	Lauraceae	Leaves	11.5 g
Dalchini	*Cinnamomum zeylanicum* Blume	Lauraceae	Stem bark	11.5 g
Ela	*Elettaria cardamomum* Linn.	Zingiberaceae	Seed	11.5 g
Nagkesar	*Mesua ferrea* Linn.	Calophyllaceae	Stamen	11.5 g
Varun	*Crataeva nurvala* Buch-Ham	Capparidaceae	Stem bark	11.5 g
Guduchi	*Tinospora cordifolia* (Willd.) Miers	Menispermaceae	Stem	11.5 g
Priyangu	*Callicarpa macrophylla* Vahl.	Meliaceae		11.5 g

*The botanical names and family are according to http://www.theplantlist.org

well as aqueous extracts was carried out according to standard procedure.[10,13] Further the presence of a different class of phytochemical was confirmed by thin layer chromatography (TLC).[14] The TLC plates used for the stationary phase are the pre-coated aluminum silica gel plates 60 F254. Various mixtures of solvents having varying polarities were used as mobile phase in chromatography. For identification of the different classes of phytoconstituents, spraying reagents used were: 5% ferric chloride (for phenolics), 2% ethanolic aluminum chloride (for flavonoids), Liebermann–Burchard reagent (for triterpenes and steroids), Dragendorff reagent (for alkaloids) and benzidine sodium metaperiodate reagent (for glycosides). Quantitative estimation of various phytoconstituents, *viz*. total phenolic, tannin,[15] flavonoid and flavonol[16] was done as per the methods.

Microbial Contamination

Microbial contamination and total viable aerobic count were determined in 1 month old sample using MacConkey and soybean-casein digest mediums as per method described by WHO.[11]

Heavy Metal Analysis and Pesticide Residue Evaluation

Wet digestion procedure was followed for sample preparation. The heavy metal analysis was carried out with the help of atomic absorption spectroscopy (Shimadzu-AA6300). Each sample was tested thrice. The limits of quantification were 3 ppm for mercury, arsenic, cadmium and 10 ppm for lead. Pesticide residue determination was done according to a guideline issued by WHO.[11]

Antioxidant Activity

20 g of *Kushavleha* was macerated with 300 ml of n-hexane for 24 h to remove fat and waxe then the supernatant was decanted. The solid mass was macerated with 300 ml of methanol for 24 h. The methanolic fraction was dried under reduced pressure. The dried extract was dissolved in methanol to a final concentration of 100 µg/mL. Five solutions of different concentration (20, 40, 60, 80 and 100 µg/mL) were prepared from the stock solution. A 0.1 mM (3.95 mg) 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution was prepared in methanol. 3 ml of the DPPH solution was mixed with 1 ml of sample solution and standard solution separately. These solution mixtures were kept in dark for 30 min (28°C), and absorbance was measured in 517 nm using the double beam ultraviolet-spectrophotometer (Varian carry 100, India) spectrophotometer. Methanol (1 ml) with 3 ml DPPH solution was used as a control. Methanol was used as blank. The antioxidant activity was compared with the reference drug ascorbic acid. The absorbance was recorded, and % inhibition was calculated using the formula. A plot was constructed between concentrations versus % reduction in absorbance of DPPH and calculated the inhibitory concentration 50% (IC_{50}) value.[17]

$$\text{Percent inhibition of DPPH activity} = A-B/A\times 100$$

Where, A = Absorbance of the control,
B = Absorbance of the sample.

Hydrogen Peroxide Scavenging Assay

The hydrogen peroxide scavenging ability of extract was estimated by the method of Ruch *et al*.[18] A 40 mM solution of hydrogen peroxide was prepared in phosphate buffer at pH 7.4. 500 µg/mL of extracts are dissolved into distilled water and added to a 0.6 ml, 40 mM hydrogen peroxide solution. The reaction mixture was kept for incubated on at 25°C for 10 min. The absorbance value of the reaction mixture was taken at 230 nm phosphate buffer without hydrogen peroxide serve as blank solution. The percentage of hydrogen peroxide scavenging was calculated by formula as above.

Nitric Oxide Radical Scavenging Activity

The nitric oxide radical scavenging activity of the extract was calculated by the method of Sreejayan and Rao.[19] 10 mmol/L sodium nitroprusside solution in phosphate buffered saline and it was mixed with extracts at various concentrations. The whole reaction mixture was kept for incubation at temperature of 25°C for 150 min. After incubation time, 0.5 ml of Griess reagent which is containing 1% sulphanilamide, 2% H_3PO_4 and 0.1% N-ethylene diamine dihydrochloride was added in the reaction mixture. The absorbance was recorded at 546 nm. Ascorbic acid and reaction mixture without extracts were employed as the positive and negative control. The inhibition percentage of nitric radical generation was calculated by above formula.

RESULT AND DISCUSSION

Organoleptic Character and Powder Microscopy

The prepared *Kushavleha* were semisolid, smooth in touch and brownish in color. It possesses pleasant odor, spicy sour taste. Diagnostic microscopic characters of *Kushavleha* are starch with concentric hilum, cork in surface view, sclereids of cortex, pitted vessel from *Mulethi*; starch grain, annular vessel, siliceous crystals from *Vanslochana*; tannin and stone cells from *Dalchini*; fiber with oil globules from *Tejpatra*; starch grain, sclereids and fibers from *Amalaki*; brownish red coloring, pollen grains from *Nagkesar*; fibers, bordered pitted vessel, lignified fiber from *Guduchi*; parenchymatous cell from *Priyangu*; pitted vessels, epidermis of testa composed

of prosenhymatous cells with pitted walls and globules of volatile oil with underlying hypodermis and epidermis from *Ela*; unicellular trichome from *Khira* stone cell, crystal from *Varun*; stellate trichome from *Kohda* [Figure 1].

Physicochemical Evaluation

Results of different physicochemical parameters were enumerated in Table 2. Estimation of total ash represents the total amount of material that remains after ignition includes both the "physiological and non-physiological" ash. The ash value also provide information regarding a number of inorganic compounds, heavy metals and other extraneous matter (e.g. sand and soil) that adheres to the plant surface.[20,21] The acid insoluble ash measures the amount of silica present especially in the form of sand or siliceous earth material whereas water soluble ash is used to detect the presence of material exhausted by water.[22] The percentage of loss on drying is quite high which indicates that the formulation might contains an extensive amount of moisture.[23] Extractive values are useful to assess the amount of active chemical constituents present in the drug.[24] The water-soluble extractive and methanol soluble extractive values were found to be 84.85% and 66.95% respectively, indicating a considerable amount of polar

Figure 1: Microscopy of *Kushavleha.* (a) Starch with concentric hilum (*Mulethi*); (b) cork in surface view (*Mulethi*); (c) sclereids of cortex (*Mulethi*); (d) vessel pitted (*Mulethi*); (e) starch grain (*Vanslochan*); (f) annular vessel (*Vanslochan*); (g) siliceous crystals (*Vanslochan*); (h) tannin contents (*Dalchini*); (i) fiber with oil globules (*Tejpatra*); (j) stone cells (*Dalchini*); (k) starch grain (*Amalaki*); (l) sclereids of *Amalaki*; (m) fibers (*Amalaki*); (n) brownish red coloring matter (*Nagkesar*); (o) pollen grains (*Nagkesar*); (p) bordered pitted vessels (*Guduchi*); (q) lignified fibers (*Guduchi*); (r) fibers (*Guduchi*); (s) parenchymatous cell (*Priyangu*); (t) pitted vessels (*Ela*); (u) epidermis of testa composed of prosenhymatous cells with pitted walls and globules of volatile oil with underlying hypodermis and epidermis (*Ela*); (v) unicellular trichome (*Khira*); (w) stone cell (*Varun*); (x) crystal (*Varun*); (y) stellate trichome (*Kohda*)

compounds in the sample. Determination of crude fiber shows the presence of excessive woody material in the drug.[25] The pH conventionally represents the acidity and alkalinity. pH of *Kushavleha* was showing slightly acidic nature which may be because of acidic salts present with-in the formulation.[26]

Table 2: Physicochemical evaluation

Parameter	Results
Loss on drying (% w/w)	Not more than 21.96
Ash values	
Total ash (% w/w)	Not more than 3.20%
Water soluble ash (% w/w)	Not more than 0.38±0.02%
Acid insoluble ash (% w/w)	Not more than 0.81±0.01%
Extractive values	
Water	Not <84.85% (w/w)
Methanol	Not <66.95% (w/w)
pH value (5% aqueous solution)	4.39
Acidity	0.70%
Quantitative estimation	
Total solid content	69% (w/w)
Fat content	3.08% (w/w)
Sugar content	68.70% (w/w)
Reducing sugar	14.21% (w/w)
Nonreducing sugar	54.49% (w/w)
Crude fiber	Not <13.10 (w/w)
Total phenolic (mg/g) (in GAE*)	144.60±0.41
Total tannin (mg/g) (in TAE*)	123±0.53
Total flavonoid (mg/g) (in RE*)	69±0.12
Total flavonol (mg/g) (in RE*)	0.61±0.27

GAE: Gallic acid equivalent, TAE: Tannic acid equivalent, RE: Rutin equivalent

Table 3: Phytochemical screening of *Kushavleha*

Phytochemicals	Methanol extract	Aqueous extract
Steroids	+	−
Glycosides	+	+
Alkaloid	+	−
Tannins	+	−
Triterpenoids/steroids	+	−
Saponin	−	−
Flavonoid	+	+
Carbohydrate	+	+
Proteins	+	+
Amino acids	+	+

+: Present, −: Absent

Phytochemical Screening

Results of phytochemical screening were shown in Table 3. Phytochemical screening of methanolic and aqueous extract of *Kushavleha* indicates the presence of some major constituents which are which are flavonoids, phenolics, tannins, steroids, glycosides, alkaloids and amino acids. Saponin was found to be absent in the extract. Such phytochemical screening is assist in identification of phytoconstituents class present in the tested drugs since phytochemicals are considered to be responsible for the activity of the drugs.[27] The presence of these phytochemicals was further confirmed with the help of TLC. It depicts the separation of the individual phytoconstituents which can be easily identified from the chromatogram. The identification of major phytoconstituents in the different extract of *Kushavleha* by the use of TLC was also studied to further confirm the presence of the possible phytoconstituents, and this was represented in Figure 2. The solvent system for developing the chromatogram contains the mixture of chloroform and methanol (9:1). The TLC chromatogram for the different phytoconstituents can be identified clearly after spraying with their respective spraying reagent. Light orange color indicates after spraying of Dragendorff's reagent shows the presence of alkaloids having an Rf value of 0.78. Flavonoids give two bright yellow spots after spraying with alcoholic aluminum chloride with an Rf value of 0.47 and 0.63. Steroids (Rf = 0.12) gives

Table 4: Safety profile

Parameter	Results
Pesticide residue	
Chlorinated pesticide residue	
TS1 (first elute)	Not more than 0.0012 mg/kg
TS 2 (second elute)	Not more than 0.011 mg/kg
Phosphated pesticide residue	
TS1 (first elute)	Not more than 0.019 mg/kg
TS 2 (second elute)	Not more than 0.010 mg/kg
TS 3 (third elute)	Not more than 0.005 mg/kg
Heavy metals	
Lead (Pb)	Not more than 0.010 ppm
Cadmium (Cd)	Not more than 0.0002 ppm
Zinc (Zn)	Not more than 0.052 ppm
Mercury (Hg)	Not more than 0.110 ppm
Microbial load	
Total plate count	75 cfu/g
Yeast and Mould	6 cfu/g
E. coli negative	Negative
Salmonella	Negative
Staphylococcin	Negative

ppm: Parts per million, CFU: Colony forming unit per gram, *E. coli: Escherichia coli*

purplish color when sprayed with Liebermann–Burchard reagent. Phenolics (Rf = 0.67) gives a bluish color when sprayed with ferric chloride reagent. For glycosides detection a single cream to yellow color spots (Rf = 0.66) were seen after spraying with benzidine sodium metaperiodate reagent.

Quantitative Estimation

Quantitative estimation of total phenolics, tannins, flavonoids and flavonol components present in the extracts of *Kushavleha* were enumerated in Table 2. Literatures revealed that phenolics and flavonoids are the two main phytoconstituents that are mainly responsible for the antioxidant property.[28] Thus, extract of *Kushavleha* can be acts as a potential candidate for anti-oxidative property due to its high phenolic content.

Safety Profile

Table 4 shows the outcome of microbial load, pesticide residue and heavy metals screening of the prepared *Avaleha*. Since, the sample was in the form of *Avaleha* containing a significant quantity of sugar; hence, sugar estimation was considered as an important parameter. Total sugar was found to be 68.70% suggesting the presence of considerable amount of sugar in the sample which may act as a preservative.[29] It was further confirmed by microbial load in 1 month old sample. The microbial growth after 1 month was negligible as it was falls within the permissible limit given in WHO guidelines.[11] Quantitative determination of pesticide residue and heavy metals in herbal drugs are very important in the present scenario as high quantity of these can lead to a number of health hazards. These heavy metals are usually accumulated in the plant through soil, contaminated water or air pollution.[30] Consumption of such contaminated plant products may lead to various consequences in human's physiological system like renal damage, high blood pressure, changes in heart rhythm or paralysis and possibly death.[31] Hence, it was recommended by WHO that every herbal products or mineral based drugs should be examined for the heavy/toxic metals.[32] Moreover, a number of pesticides are used in the cultivation of medicinal plants. The remnants of these pesticides also cause many health problems in human beings.[20] In the present study, heavy metals (Zn, Pb, Cd, Hg) and pesticide residue were found to be within the permissible limit prescribe by WHO guidelines, indicating that the formulation is free from any unwanted contaminations and safe for consumption.[24]

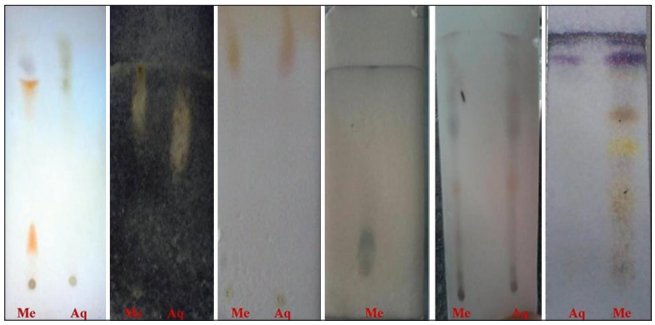

Figure 2: Thin layer chromatography of methanolic and aqueous extract of *Kushavleha*: (a) For alkaloid, (b) for glycosides, (c) for amino acids, (d) for steroids, (e) for flavonoid, (f) for tannin

Antioxidant Activity

The percentage inhibition in the different antioxidant screening of herbal preparation was presented in Figure 3. The methanolic extract of herbal preparation exhibited a maximum DPPH scavenging activity of 69.11% at 100 µg/ml whereas for ascorbic acid (standard) was found to be 84.91% at 100 µg/ml. The IC_{50} values of the methanolic extract of herbal preparation and ascorbic acid were 63.80 µg/ml and 47.67 µg/ml, respectively. The methanolic extract of *Kushavleha* showed maximum activity of 70.12% at 100 µg/ml in nitric oxide radical scavenging model, whereas ascorbic acid at the same concentration exhibited 91% inhibition. Whereas maximum hydrogen peroxides scavenging activity (65.01%) was showed inhibition at 100 µg/mL. The IC_{50} values of the methanolic extract of herbal preparation was found to be 71.71 µg/mL and 74.40 µg/mL respectively in nitric oxide radical scavenging and hydrogen peroxides scavenging model. Hence, the results show that *Kushavleha* possesses antioxidant potential which may help in treating *Prameha* and *ashmari*.

Standardization of herbal medicines is a valuable issue at present because herbal medicines are very prone to contamination, deterioration and variation in composition due to biodiversity as well as careless collection. The quality control testing of completely finished products may lead to the production of standardized and therapeutically effective herbal formulations. This can be achieved with the help of modern techniques.[5,6,11] Thus, the present work was carried out on *Kushavleha* to prepare ideal monograph of the formulation and will serve in establishing its authenticity, quality, safety, and reproducibility. On the basis of the pharmacognostical studies, it was observed that there are various diagnostic features present in the *Kushavleha* which can serve as useful information in maintaining standards of the formulation.

CONCLUSION

Kushavleha was traditionally prepared and standardized by the intervention of modern scientific quality control measures. Pharmacognostical evaluation, preliminary phytochemical studies and safety profile of the *Kushavleha* were provided in the present article. The analytical data generated here may be considered as the standard parameter for this formulation and may help in preserving the quality of the drug.

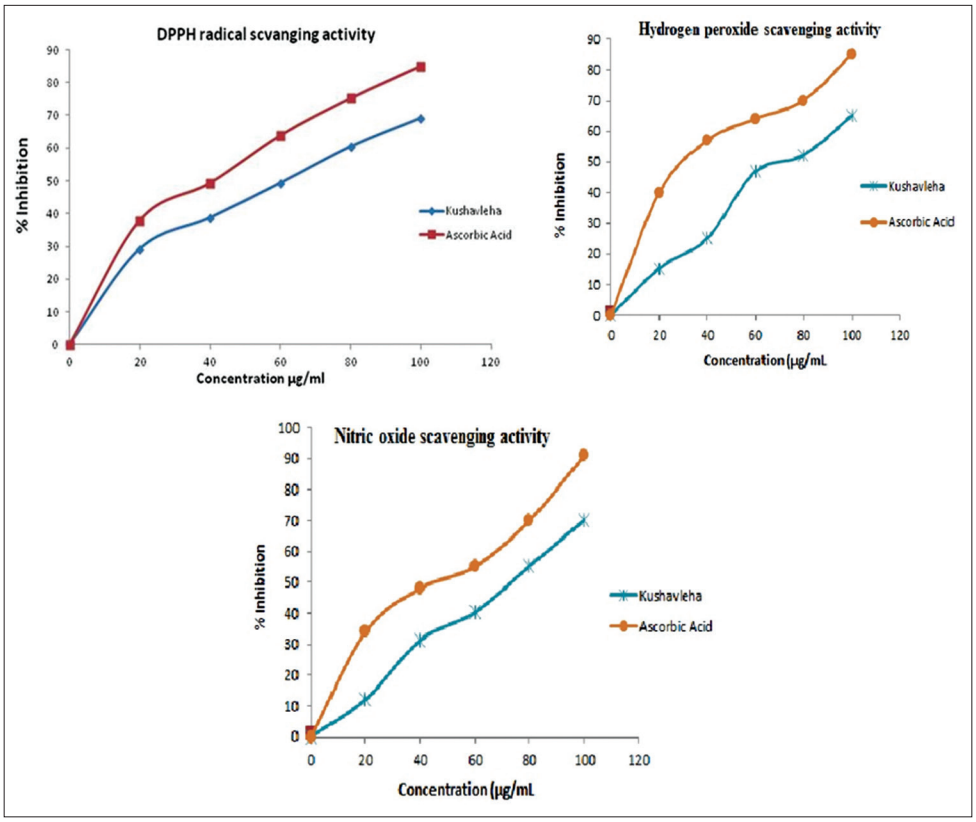

Figure 3: Antioxidant activity

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