In vitro study on anti-oxidant and anti-inflammatory properties of *Varnya Mahakashaya Dashemani* (aqueous extract): A polyherbal formulation

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Abstract

Background: Plants used in *Varnya Mahakashaya Dashemani* (VMD) formulation were investigated individually by many scientists. Most of them have exhibited antioxidant, anti-inflammatory and antimicrobial activities when they have been extracted with the different solvents. Here, an attempt has been made to analyze these activities in aqueous extract of the whole formulation. **Aim:** The aim of this study was to evaluate antioxidant and anti-inflammatory potential of polyherbal formulation VMD. **Material and Methods:** Phytochemical constituents of VMD extract were analyzed using standardized protocols and Fourier transform infrared spectroscopy analysis for functional groups. The amount of total phenolics and flavonoids was determined using the Folin–Ciocalteau and aluminum chloride method, respectively. The *in vitro* antioxidant properties of VMD aqueous extract was screened by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays. Anti-inflammatory potency was evaluated with inhibition of 15-lipoxygenase (15-LOX). **Results:** Phytochemical analysis indicated the presence of alkaloids, flavonoids, tannins, saponin and phenols. The total phenolic content of VMD extract was 50 µg/ml of gallic acid equivalent and the total flavonoids content was 90 µg/ml Quercetin equivalent. It showed higher free radicals quenching capacity with an IC_{50} value of 34.20 ± 3.03 µg/ml for DPPH and ferric reducing ability by FRAP with an equivalent value of 560 µM $(Fe^{+}+)$/g extract. Significant inhibition of 15-LOX enzyme was prominent with increasing concentration of the sample with an IC_{50} of 33.62 ± 5.8 µg/ml. **Conclusion:** VMD has high antioxidant, anti-inflammatory potential and further studies can lead to identification and isolation of more potent therapeutic bioactive compounds from this extract.

Keywords: Anti-inflammatory activity, antioxidant activity, aqueous extract, *Varnya Mahakashaya Dashemani*

Introduction

The existence of herbal medicine worldwide has a long history; they were being used and well documented by ancient Indians, Egyptians, Greeks and Chinese for various therapeutic purposes.[1] It has been recorded from predated history that, knowledge about the use of plants for healing purposes was acquired from either folklore or traditional practice.[2,3] Among the knowledge of ancient medicinal practices, Ayurveda stays unique from a vast variety of healing methods used and discussed in the literature. Increasing interest in the use of natural and traditional medicines worldwide reopened the opportunities to the Ayurvedic practitioners, chemists and pharmaceutical biotechnologists to work together in developing products of herbal formulations authenticated with rational scientific evidence and carefully standardized using authentic botanicals.[2,4]

The concept of *Saundarya* (esthetics) is as old age as origin of mankind. As skin and its complexion is said to be one of the manifest forms of beauty or esthetics, it plays a significant role

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on individual's physical and mental health. Thus, skin and its complexion is one of the ways of expression of beauty as well as health of an individual. In Ayurveda, complexion is referred as *Varṇa.* It is included among the signs of health as well as measurement of the status of health. *Varṇa* (complexion) has various physiological and pathological implications.[5] *Varṇa* includes different parameters of skin. They are color, texture, luster, appearance, nourishment and also dermatological parameters such as skin hydration (dryness-oiliness), skin pigmentation (pigmented-none pigmented), skin sensitivity (sensitive-resistant) and skin wrinkling (wrinkle tight). Hence, disturbance in any of the above components of the skin is considered as *Vaivarnya* (skin discoloration).[6] *Varnya* is a classical term given for the task of restoring and retaining the natural hue, texture and tone of the skin, that is, enhancement of complexion.

Varnya Mahakashaya Dravya are the group of drugs which are used to treat and prevent the *Vaivarnya* related conditions, to maintain and enhance the complexion in healthy. These can be administered both internally and externally. According to *Charaka Samhita, Varnya Mahakashaya* is the eighth group of 50 *Mahakashaya* described in fourth chapter of *Sutra Sthana* of *Charaka Samhita* and includes viz. drugs: *Chandana* (*Santalum Album)*, *Tunga* (*Calophyllum inophyllum)*, *Padmaka* (*Prunus cerasoides)*, *Ushira* (*Vetiveria zizanioides)*, *Madhuka* (*Glycyrrhiza glabra)*, *Manjishtha* (*Rubia cordifolia)*, *Sariva* (*Hemidesmus indicus)*, *Payasya* (*Pueraria tuberosa),* *Sita* (*Cynodon dactylon*) and *Lata* (*Cynodon linearis)*[7] [Table 1].

Thus, in an attempt to understand the mechanism of action of *Varnya Mahakashaya Dashemani* (VMD), the present study deals with the preliminary evaluation of antioxidant potential using *in vitro* antioxidant assays including scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ferric reducing antioxidant power and anti-inflammatory activity through inhibition of 15-lipoxygenase (15-LOX).

Material and Methods

Plant sample extraction

Drugs

The raw drugs were procured from Government Sandalwood Depot, Mysore, Karnataka and Alva's pharmacy, Moodbidari. VMD powder was prepared after cleaning followed by grinding in pulverizer and fine powder of all the ten drugs taken in equal quantity was prepared using a pulverizer. Dried powder of VMD was extracted with 400 ml of cold distilled water with gentle stirring for 3 days and kept in the dark with intermittent shaking.[8] After extraction, the liquid was filtered through Whatmann No. 1 filter paper and combined liquids were clarified by centrifugation at 8000 rpm for 5 mins. The filtrate was collected, lyophilized, transferred to glass vials, and kept at 4°C before use.

Preliminary phytochemical screening of *Varnya Mahakashaya Dashemani* aqueous extract

Preliminary qualitative screening of VMD aqueous extract for the presence of phytochemicals such as phenols, alkaloids, flavonoids and tannins was analyzed according to the standard protocols for phytochemicals present in the sample.[9] [Table 1]

Fourier transform infrared spectroscopy spectroscopic analysis

Fourier transform infrared spectroscopy (FTIR) analysis is used for the identification of the functional groups which attributes to the secondary metabolites present within the sample. It produces combination of characteristic vibrations to each functional group, based upon the absorption of the infrared radiation.[9] Aqueous extract of VMD was subjected to FTIR to record the presence of various functional groups that attributed to the different secondary metabolites present in the plants. This was achieved by directly placing the sample on the probe of the instrument (Perkin Elmer Spectrum Version 10.03.09) and the spectra were collected over the wave number ranged from 4000 cm^{-1} to 400 cm^{-1}.[10,11]

Total phenolic content

Phenolic metabolites in the samples were estimated using a method as described by Ainsworth and Gillespie, (2007) with slight modification.[12] Briefly, 50 µL of each sample and standard were added in a vial. 200 µL of 10% Folin–Ciocalteu reagent (v/v) was pipetted and vortexed to mix thoroughly. To the mixture, 800 µL of 700 mM Na_2CO_3 was added and incubated at room temperature for 2 h. After incubation, the absorbance of each sample was measured at 765 nm. Gallic acid concentrations ranging from 0 to 250 µg/mL

Table 1: Plants used in the preparation of *Varnya Mahakashaya Dashemani*

Drug	Latin name	Family	Parts used
Chandana	*Santalum album* Linn.	Santalaceae	*Kanda Sara* (Heart wood)
Tunga	*Calophyllum inophyllum* Linn.	Guttiferae	*Kanda twak* (Stem bark)
Padmaka	*Prunus cerasoides* D. Don.	Rosaceae	*Kanda twak* (Stem bark)
Ushira	*Vetiveria zizanioides* (Linn.) Nash	Graminae	*Mula* (Root)
Madhuka	*Glycyrrhiza glabra* Linn.	Leguminaceae	*Mula* (Root)
Manjishtha	*Rubia cordifolia* Linn. sensu Hook. f.	Rubiaceae	*Mula* (Root)
Sariva	*Hemidesmus indicus* (L.) R. Br.	Asclepediaceae	*Mula* (Root)
Payasya	*Pueraria tuberosa* (ROXB. EX. WILLD.) DC.	Leguminaceae	*Kanda* (Stem)
Sita	*Cynodon dactylon* (L.) Pers.	Poaceae	*Panchanga* (Whole plant)
Lata	*Cynodon linearis* Wild.	Poaceae	*Panchanga* (Whole plant)

were prepared and the standard calibration curve was used to calculate total phenolic content. The samples were estimated in duplicate.

Total flavonoid content

Total flavonoids were estimated using aluminum chloride method.[13,14] In brief, 50 µL of sample and 300 µL of $NaNO_2$ (1:20 w/v) were pipetted into a 10 ml test tube as the reaction volume was more than 1 ml. The contents were vortexed for 10s and left at room temperature for 5 min. 300 µL of $AlCl_3$ (1:10 w/v), 2 mL of 1M NaOH and 1.9 mL of distilled water were then added to the mixture. After vortexing for 10 s, the absorbance of each sample was measured at 510 nm. Quercetin concentrations ranging from 0 to 500 µg/mL were prepared and the standard calibration curve was obtained using a linear fit. The samples were analyzed in duplicate.

Determination of antioxidant activity

1,1-diphenyl-2-picrylhydrazyl scavenging assay

DPPH scavenging potency of VMD was evaluated by employing the modified protocol.[15,16] DPPH solution (300 µM) was prepared in methanol and 95 µl of DPPH was added to each well of a microtitre plate. Different concentrations of test samples (5 µl) were added to the respective wells. The plate was incubated for 30 min at room temperature and the absorbance was recorded at 517 nm. Ascorbic acid (AA) was used as positive control. The results were expressed as total antioxidant capacity (TAC) and a dose-dependent curve was plotted to calculate the IC_{50} value. The values are represented as mean ± SD of three independent experiments. The percentage radical scavenging was calculated from the following formula.

$$\% \text{ scavenging} = ([A_c\text{-}A_s]/A_c) \times 100$$

Where A_c was the absorbance of the control and A_s was the absorbance of the sample.

Ferric reducing antioxidant power assay

The reactive principle of chemicals in which iron reacts with a colorimetric probe to produce a blue product was used to quantitate antioxidant activity as earlier.[17,18] An aliquot of 30 µL sample was mixed with 90 µL water and 900 µL ferric reducing antioxidant power (FRAP) reagent (2.5 mL of 20 mmol/L of 2,4,6-tri-2-pyridinyl-1,3,5-triazine in 40 mmol/L of HCl, 2.5 mL of 20 mmol/L of ferric chloride, 25 mL of 0.3 mol/L of acetate buffer (pH 3.6) and incubated at 37°C for 30 min. After incubation, the absorbance values were recorded at 593 nm with ultraviolet-visible (UV-vis) spectrophotometer. Known ferrous sulphate contents ranging from 400 to 2000 µmol were used to generate the calibration curve. From the curve, the ferrous ions reduced by the sample were calculated using regression equation. The antioxidant activity was expressed as the amount of extract required to reduce 1 mmol of ferrous ions.

Evaluation of *in vitro* anti-inflammatory activity

Lipoxygenase inhibition assay

Anti-inflammatory efficacy of extracts was assessed by measuring the inhibition of Soybean 15-LOX spectrophotometrically with the minor modifications.[16,19] The substrate 0.2 µM linoleic acid was prepared in 0.2M borate buffer (pH 9). Different concentrations of plant extracts, that is, 1 µg to 60 µg in duplicate were mixed with 15-LOX enzyme and incubated for 2 min at room temperature. The substrate was added to the mixture and the absorbance was measured at 243 nm using UV-Vis spectrophotometer (Beckman Coulter, DU 730 Life Sciences). AA was used as positive control and methanol as a negative control. A dose-dependent curve was plotted to calculate the IC_{50} value.

Results

Qualitative phytochemicals screening

Preliminary qualitative screening of VMD aqueous extract for the presence of phytochemicals showed the presence of alkaloids, flavonoids, tannins, saponin and phenols [Table 2].

Fourier transform infrared spectroscopy spectroscopic analysis

The FTIR spectrum of VMD extracts indicates the presence of differential functional groups which will help in predicting the possible phytochemicals present in the extract with reference to the available spectral data of the previous research based on the peak/frequency value in the region of infrared radiation. Extract analyzed showed the presence of different functional groups such as O-H, C-H, C = C and N-H. The slanted bend at the region of 3271.98 cm^{-1} due to O-H is suggestive of the presence of secondary amines and phenolics in the tested sample. Peaks observed at the frequency of 2967.25 cm^{-1} and 2881.26 cm^{-1} may be due to stretching of both strong and weaker aliphatic C-H group. The bend observed at the frequency of 1566.75 cm^{-1} due to the N-H bending aid in predicting the presence of amides in the extract. Stretching of C = C in the aromatic compounds was noted by observing frequency at 1457.07 cm^{-1}. Although the bends between the frequencies of 1420–990 cm^{-1}, confirm the stretching of S = O due to the presence of sulphoxides or sulphonamides, etc., favors us to predict this as symmetric stretching at frequency of 1390.21 cm^{-1} due to the presence of Nitro "N" groups in the extract [Figure 1].

Total phenolic content

Gallic acid as a standard for phenolic content, the VMD extract was examined for the amount of total phenolic content. With

Table 2: Phytochemicals constituents of *Varnya Mahakashaya Dashemani* extract

Phytoconstituents	Aqueous extract
Alkaloids	+
Flavonoids	+++
Tannins	+
Saponins	+
Quinones	–
Phenols	++
Anthocyanin	–
Proteins	–

+: present, ++: moderately present, +++: strongly Present, - : not present

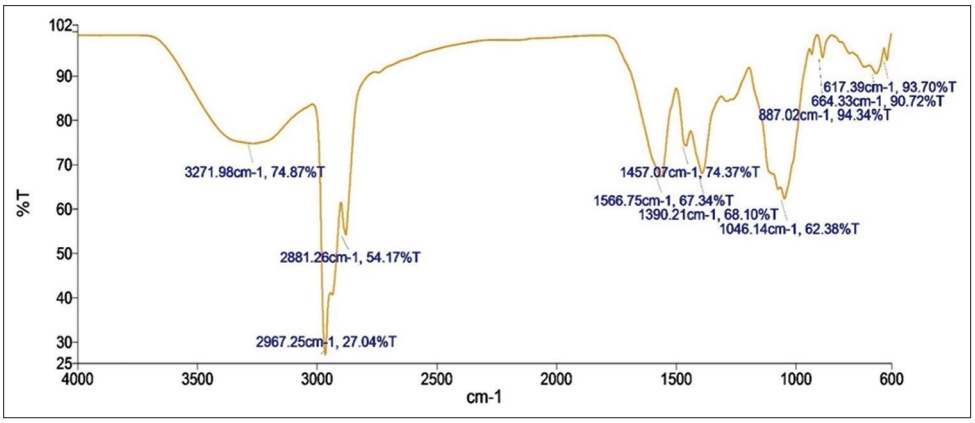

Figure 1: Fourier transform infrared spectroscopy spectra of *Varnya Mahakashaya Dashemani* extract showing different spectral bands

the gallic acid standard curve ($R^2 = 0.9$), the phenolic content was recorded to be 50 µg/ml gallic acid equivalent [Figure 2].

Total flavonoid content

VMD extract was analyzed for the quantitative estimation of total flavonoid content, with quercetin as standard for flavonoid content. Total flavonoid content that was determined from quercetin standard curve ($R^2 = 0.9$) was found to be 90 µg/ml quercetin equivalent [Figure 3].

Antioxidant activity (1,1-diphenyl-2-picrylhydrazyl assay)

Free radicals play a major role in the skin-related problems as it is well indicated by many findings. Hence, the scavenging of free radicals plays an important role in the management of skin-associated complications.

The antioxidant activity of VMD extract was analyzed through DPPH assay, where it has shown the scavenging of the free radicals in a concentration-dependent manner. The results were expressed as IC_{50} value indicating the concentration of the extract required to scavenge 50% of DPPH. The IC_{50} value VMD extract was found to be 34.20 ± 3.03 µg/ml, while the standard AA has higher scavenging activity with an IC_{50} 4.5 µg/ml.

Ferric reducing antioxidant power assay

Ferric reducing ability of VMD extract was determined by FRAP with known ferrous sulfate contents ranging from 400 to 2000 µmol to generate the standard calibration curve ($R^2 = 0.9$). The mechanism of FRAP is based on the transfer of electrons to reduce the Fe^{3+} complex to Fe^{2+}. The extract was prepared in 1 mg/ml concentration and 30 µl of sample was taken for the assay. Based on this, the extract taken was 30 µg (560 µM (Fe^{+}+)/g extract). The antioxidants present in the extract had reduced the Fe^{3+}/ferricyanide complex to the ferrous form and thus proved the reducing power. The reducing ability observed was 560 µM (Fe^{+}+)/g extract [Figure 4].

Anti-inflammatory activity: 15-lipoxygenase inhibition assay

Anti-inflammatory activity of VMD extract was evaluated using a contemporary assay, where the inhibition of lipoxygenase was monitored by the formation of hydroperoxylinoleic acid spectrometrically at 234 nm. The extract has shown

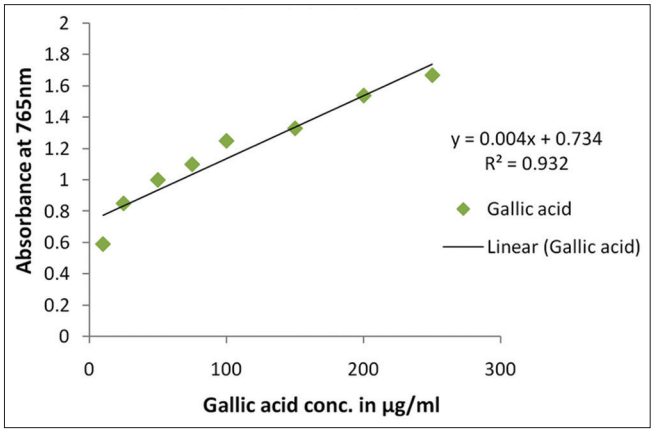

Figure 2: Standard gallic acid curve for total phenolic content

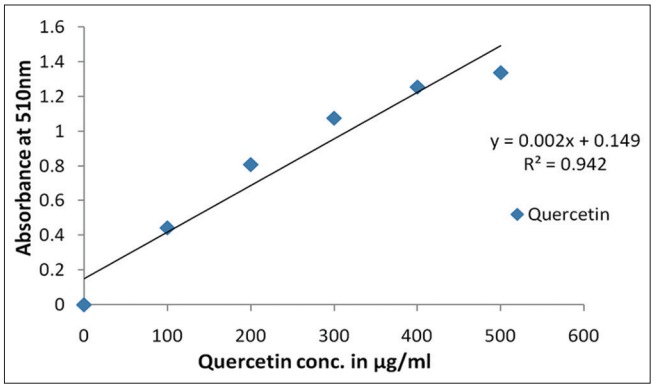

Figure 3: Standard quercetin cure for total flavonoid content

anti-inflammatory activity in a concentration-dependent manner with an IC_{50} value of 33.62 ± 5.8 µg/ml, whereas the reference compound AA exhibited IC_{50} value of 9.60 ± 0.046 µg/ml.

The total phenolic content, flavonoids content, DPPH, FRAP, and 15-LOX values of VMD extracts are shown in Table 3.

Discussion

Due to low cost and high effectiveness, almost 80% of Indian population are found to be dependent on traditional health care system in treating skin diseases.[20] Many studies have been

Table 3: Total phenolic content, total flavonoids content, 1, 1diphenyl 2 picrylhydrazyl, ferric reducing antioxidant power, and 15-lipoxygenase values of *Varnya Mahakashaya Dashemani* extract

Sample	Total phenolic content	Total flavonoids content	DPPH IC_{50}	FRAP (mmol (FeII)/g extract)	15-LOX IC_{50}
VMD extract	50 µg/mg	90 µg/mg	34.20 ± 3.03 µg/ml	560 mM	33.62 ± 5.8 µg/ml

VMD: *Varnya Mahakashaya Dashemani*, DPPH: 1, 1diphenyl2picrylhydrazyl, FRAP: Ferric reducing antioxidant power, 15-LOX: 15-lipoxygenase, IC_{50}: Half maximal inhibitory concentration

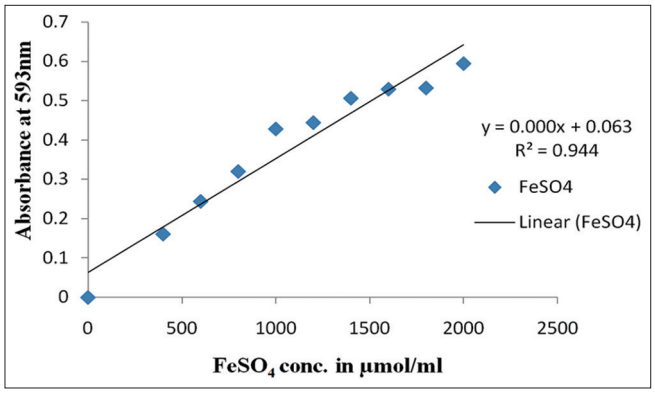

Figure 4: Standard $FeSO_4$ cure for ferric reducing antioxidant power

conducted in evaluating the abilities of herbs/formulations toward treating skin abnormalities.[21] Taken together, use of *Allium cepa* extracts in seborrheic keratosis[22] and the antifungal activity of *Allium sativum and A. cepa* extracts in inhibiting the growth of *Malassezia furfur*, *Candida albicans*, *Candida* sp, in addition to 35 various dermatophyte species highlights the effectiveness of herbs in treating skin infections.[23] The chemopreventive ability of *Azadirachta indica* leaves extracts against murine skin carcinogenesis,[24] antiwarts efficiency of *Ficus carica* latex,[25] *in vivo* evidence of lavender oil in inhibiting immediate-type allergic reactions,[26] and the significant efficiency of sandalwood oil in decreasing papilloma incidence[27] are some of the other examples of efficacy of plant components for treating skin disorders. Even though attempts have been made to unravel the mechanism of action of some plant components,[26] many herbal remedies are still not completely analyzed.[25,28] With newer insights, researchers disagree with "one drug fits all" concept due to renewed understanding in multi-ingredient interaction of traditionally designed polyherbal formulations.[2] With this line of interest, in the present investigation; the experiments were designed with the intention of evaluating *in vitro* bioactive potentials of *Varnya Mahakashaya Dravya*; a polyherbal formulation in an aqueous extract was used to treat *vaivarnya*. Several treatment modalities with varying efficacy for skin health have been developed due to esthetic unfavorability.[29] The importance of active radical scavengers in the protection of skin against both the intrinsic and extrinsic environment has been well documented by many researchers worldwide.[30,31]

Varnya Mahakashaya Dravya as the name of the formulation itself indicates the form of usage, that is, *Kashaya* (decoction), wherein water is the base or vehicle through which different forms of polyherbal formulations can be prepared such as *Kashaya* (decoction),[32] *Ghanavati* (tablets/pills)[33] and *Kalka* (paste).[34] Thus, generally, VMD is prescribed for application with the water (cold or warm). Thereby, in this experiment, cold water extraction was carried out.

The results of phytochemical analysis indicated the presence of alkaloids, flavonoids, tannins, saponins and phenols in VMD aqueous extract. FTIR spectra revealed the presence of different predominant functional groups such as O-H, N-H, C-C and N-H in dissymmetric and symmetric fashions. The total phenolic content present in VMD aqueous extract was 50 µg/mg of gallic acid equivalent and the total flavonoids content was 90 µg/mg quercetin equivalent. VMD extract showed free radicals quenching capacity with an IC_{50} value of 34.20 ± 3.03 µg/ml for DPPH, in comparison with the standard AA (IC_{50} 4.5 µg/ml). The Ferric reducing ability by FRAP of the VMD extract was 560 µM (Fe^{+}+)/g. Significant inhibition of 15-LOX enzyme was observed with the increasing concentration of the sample with an IC_{50} of 33.62 ± 5.8 µg/ml. The reactive oxygen species (ROS) generated in the body may induce DNA damage in melanocytes and also affect its proliferation.[35] Thus, the importance of flavonoids and phenolic components of VMD as active radical scavengers in protection of skin against both the intrinsic and extrinsic environment can be understood.

The results of the present study indicate that VMD has potent antioxidant capabilities which may efficiently scavenge ROS generated by the cells and may also take part in inhibiting secondary messengers that may stimulate melanogenesis, with references to the previous studies.[36-38] The antioxidant potency of VMD may also influence skin pigmentation by interacting with copper at active site to hold up the oxidative polymerization of melanin intermediates.[31,36,37] The result of the present investigation could be a preliminary proof to note, VMD is an effective composition to treat/to prevent skin discoloration by applying to the skin, as it composites the treatment composition containing an effective amount of antioxidants and anti-inflammatory agents with it.

Conclusion

Varnya Mahakashaya Dravya has high antioxidant and anti-inflammatory potential and further studies can lead to identification, isolation of the more potent therapeutic bioactive compound/s from this extract.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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