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Pharmacognostic evaluation of *Vasa Ghrita*

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Justicia adhtoda L. is a well-known plant in India traditionally used for treatment of cough and cold. *Vasa ghrita* formulation is prescribed in treatment of bronchial asthma. The main aim was to develop standardization protocol for the *Vasa ghrita* formulation and to establish the standard limits range by considering another two marketed formulations (RVG and KKVG). Preliminary morphological screening was performed to describe the leaf and the formulations, in which a range was observed especially in terms of their colour from orange-yellow (RVG), yellow (KKVG) to green-yellow (INVG). Microscopical, phytochemical and physicochemical analysis of the leaf was in support of the literature data of *Justicia adhtoda* L. A physicochemical study was performed for the formulations with pure ghee as the blank for comparison. The parameters included, specific gravity, rancidity, melting point, saponification value, iodine value, acid value, peroxide value, unsaponifiable matter, TLC and HPTLC, FTIR spectroscopy, total Phenolic Content and Total Flavonoid Content. All the three formulations showed values of similar range, however, KKVG formulation was found to be more rancid with high peroxide value (6.667±0.067) than RVG (3.4±0.116) and INVG (0.933±0.067). INVG formulation was found to contain the maximum TPC (72.521±0.113 mg GAE/g) and TFC (14.877±0.247 mg QUE/g) values. In vitro biological activity was carried out by studying the antioxidant potential using DPPH radical scavenging estimation; RVG was found to have the least IC50 value (348.000±0.01 µg/mL). Microbiological load and toxicity profile of INVG formulation were also determined to establish the safety profile of the formulation of *Vasa Ghrita*.

Keywords: Phytochemical analysis, Physicochemical analysis, Total phenolic content, Total flavonoid content, *Vasa gritha*

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The philosophy of well-being is the central locus of the realm of Ayurveda. Its ideology focuses on the wholesome treatment of the patient, taking into its purview all the several aspects of illness *viz.,* physical, emotional, social, as well as spiritual. This health care system has regained importance in the recent times because of its ability to combat chronic ailments. One such group of chronic ailments which have become highly prevalent in the current scenario are upper respiratory tract disorders ranging from simple cough to severe asthma. This can be widely attributed to both life style imbalance as well as increase in the pollutants in the atmosphere. Improper daily routine and unbalanced diet had led to loss of immunity towards the weakest of the microorganisms, lowering the threshold of response. On the other hand, effluents of different varieties have triggered biological changes whose repercussions are felt much later than they are exposed to. At this conjuncture, mankind has resorted to Ayurvedic system of healing for these group of diseases. Also, presently the increase in demand has led to large scale production of formulations. However, the ability of the formulation in improving the condition of the patient is called its efficiency. In the case of herbal and ayurvedic formulations, this efficiency is found to be variable which is dependent on other variables like the source of raw material (that affects the content of phytoconstituents which are altered by the seasonal, ecological and economical changes); the process employed in handling of the raw materials right from the cultivation and collection to packing; and lastly the standard operating procedures followed during the process of manufacture. Many guidelines have been framed nationally and internationally to limit the possibility of errors at every step of the process. However, a great deal of research needs to be

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undertaken to develop standard data with which the processed formulations can be compared with[1].

One of the medicinal plants which is highly resorted to in the field of respiratory ailments is Vasaka. Its common name is Adulsa and scientifically called as *Justicia adhatoda* L. (Acanthaceae), It is commonly known as Vasaka and is mainly found in India and Sri Lanka, Pakistan, Nepal. It is a sub-herbaceous bush, found throughout the year in plains and sub-Himalayan tracts in India, ascending up to 1200 m. It contains not less than 0.6% of vasicine on dried bases. The leaves of this plant are rich in quinazoline alkaloids, vasicine and vasicinone. Leaves are also rich in vitamin C, carotene and essential oil. The roots contain vasicinolone, vasicol, peganine, sitosterol, β-glucoside-galactose and deoxyvasicine and 2'-hydroxy-4-glucosyl-oxychalcone. The flowers show presence of b-sitosterol-D-glucoside, kaempferol, Adhatonine, Vasinol[2,3].

There are multiple ways in which the vasa formulations can be made *viz., Ghana, Sneha, Sandhana Avaleha kalpanas*. The choice of formulation is dependent upon the patient's prakruti and disease pathology. One of the potent formulations for the treatment prescribed in Ayurveda for the ailments of upper respiratory tract disorders is *Vasa Gritha*. This formulation is prepared using the leaves of *J. adhatoda* L. by incorporating the concentrated extract along with the powder into the ghee[2,3].

Materials & Methods

Collection of plant material

Authenticated *J. adhatoda* L leaves were obtained from the herbal garden of Gomantak Ayurveda Mahavidyala and Research Centre, Shiroda, Goa, India and the same have been authenticated by the department of *Dravyaguna Vignan*. *Adhatoda vasica* is synonymous to *J. adhatoda* L. *Vasa ghrita* was prepared as per the Ayurvedic procedure. Two marketed preparations were procured from an Ayurvedic Pharmacy, Mumbai and labeled as Brand RVG and Brand KKVG.

Preparation of *Vasa ghrita*

Vasa ghrita was prepared in three steps[2-4]

1) Preparation of *Kashaya* (*Swaras*), 2) Preparation of *Kalka* (paste), 3) Preparation of *Vasa ghrita*

Step 1 - Preparation of *Kashaya* (*Swaras*):- The vasaka leaves were cleaned and the dry weight was noted. The leaves were washed & cut into small pieces. Then 4 liters of distilled water was taken into a vessel, to it 1 kg of dried vasaka leaves were added and water level was marked using a marker. The vessel contents were then boiled until it was reduced to half. It was stirred intermittently (Fig. 1a,b).

Step 2 - Preparation of *Kalka* (paste):-250 g of Vasaka powder (*Churna*) was added to a vessel and then required amount of water was added in order to make it into dough like consistency (Fig. 1c).

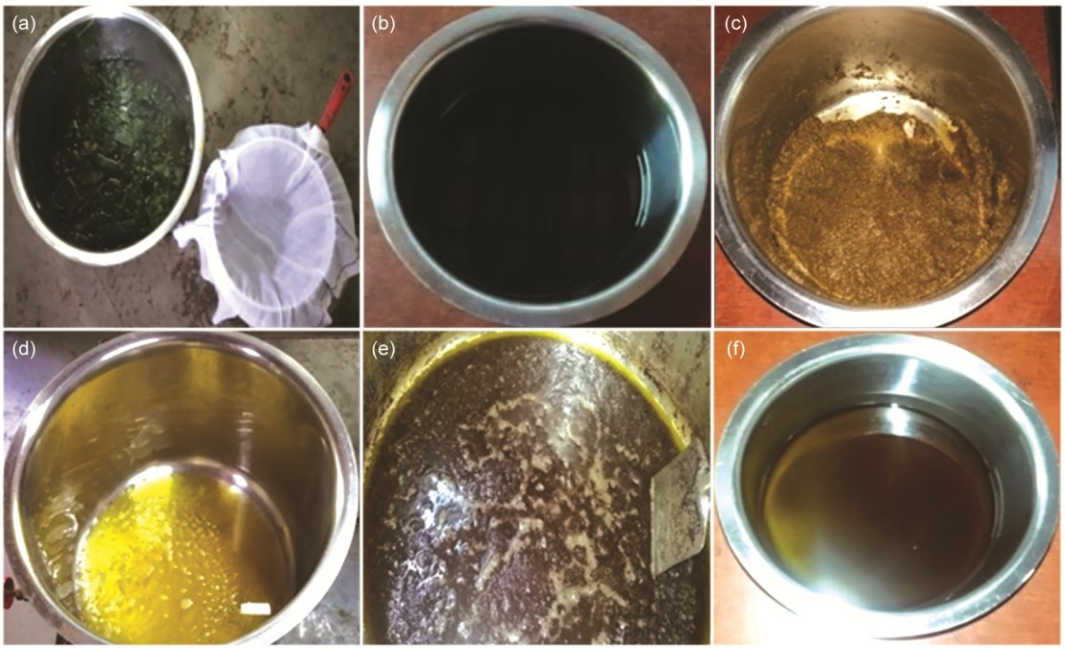

Fig. 1 — Steps for *Vasa ghrita* formulation; (a) Filtration of *Vasa Kashaya*, (b) *Vasa Kashaya*, (c) *Vasa Kalka* (Paste), (d) Heating of pure ghee, (e) Boiling the mixture of *Kalka*, *Kashaya* and *Ghrita*, (f) *Vasa ghrita* (INVG)

Step 3 - Preparation of *Vasa ghrita*:-Empty clean vessel was taken and heated to remove any traces of water then 1 kg pure cow ghee was slowly added (Fig. 1d). The vasa *kalka* (paste) was added and allowed it to form a uniform mixture. Finally, *kashaya* (*Swaras*) was added to the above mixture. The mixture was stirred continuously to prevent charring (Fig. 1e), then *sneha siddhi lakshnas* (confirmatory tests) were checked during the preparation in order to conclude whether the final stage of *ghrita* was reached. The *Vasa ghrita* was filtered using muslin cloth while warm, then transferred into the bottles and packed. This in-house preparation has been labeled as INVG (Fig. 1f).

Sneha siddhi lakshanas (confirmatory tests)[2,3]

Lakshana of *ghrita* preparation was identified by testing for *Varti* formation (wick like shape), *Phenasanti* (disappearance of the froth), crackling sound and *Gandhavarnarasotpatti*.

Morphological evaluation

Morphological studies of the vasaka leaves and the *Vasa ghrita* formulation (INVG, RVG, and KVG) were done including color, odor, size, taste, surface characteristic, etc. using subjective evaluation[5].

Microscopical evaluation[5]

Powder microscopy and leaf constants were determined as per the standard procedures. Vasaka leaf was kept in contact with chloral hydrate for a long time till it turned transparent. The drug powder was boiled in chloral hydrate solution and then the decanted solution was taken and to that, equal proportion of phloroglucinol and hydrochloric acid were added. Leaf constant values were also determined using the standard protocols.

Phytochemical evaluation

Preliminary phytochemical screening was performed for the aqueous extract of the powder of *Adhatoda vasica* leaves and for all formulations for the presence of saponins, alkaloids, tannins, flavonoids, steroids, carbohydrates, etc as per the standard procedures[5].

Physicochemical evaluation

Physicochemical studies like moisture content, total ash, acid insoluble ash, water soluble ash, alcohol soluble extractive, water soluble extractive, ether soluble extractive, foaming index, swelling index, specific gravity, rancidity, melting point, acid value, saponification value, iodine value, peroxide value, unsaponifiable matter, TLC and HPTLC, FTIR and other spectrophotometric analysis were carried out as per the WHO guidelines[5].

TLC and HPTLC analysis

Standard Preparation: 1 mg of standard vasicine drug powder was dissolved in 1 mL of methanol.

Sample Preparation: Partitioning using separating funnel: The molten 10 g of the *ghrita* formulation was taken in a separating funnel and 50 mL of 30% methanol was added. It was shaken for around 4-5 times and then it was allowed to separate. The methanol-water fraction was collected in the conical flask. Methanolic fractions were concentrated and used for the TLC and HPTLC analysis. The procedure was repeated for all the three *ghrita* formulations (INVG, KKVG and RVG). Vasa lyophilized powder labeled as (VLP) sample was prepared with 10 mg of powder in 1 mL methanol. *Vasaka* powder decoction sample labeled as (VPD) was prepared by boiling 0.5 g powder in 5 mL water. All the above samples were subjected to TLC and HPTLC analysis. After trials, the final mobile phase developed was Ethyl acetate: Methanol: Formic acid – (8:2:0.5). Total 6 samples were analyzed which were VLP, VPD, Standard Vasicine, INVG, KKVG and RVG[6,7].

Fourier transform infra-red spectroscopy (FTIR)

FTIR was carried out for all the samples including raw material (Vasa powder, Pure Ghee) and for the *Vasa ghrita* formulations (INVG, KKVG and RVG) in order to obtain spectra for the same[5].

Estimation of total phenolic content[8]

Preparation of standard solution of gallic acid

Stock solution (I) was prepared by dissolving 50 mg of gallic acid in 50 mL of methanol. From this stock solution (I) 10 mL solution was pipette out in 100 mL volumetric flask and volume was made up to 100 mL with methanol (stock solution II). Further dilutions were made by taking 1 mL from the stock solution (II) in 10 mL volumetric flask and volume was made up to with 10 mL methanol so as to obtain 5 µg/mL concentrations. Similar dilutions were done to obtain 10, 20,30,40,50,60,70,80, 90 and 100 µg/mL.

Preparation of sample and procedure to determine total phenolic content of lyophilized powder and ghrita formulations

The total phenolic content of methanolic extract of *A. vasica* lyophilized powder was determined using the

Folin-Ciocalteu reagent method. Stock solution was prepared using 100 mg of crude *A. vasica* lyophilized powder which was weighed in 100 mL volumetric flask and the volume was made up with methanol (stock solution I). Using these stock solutions serial dilution were done to obtain different concentrations (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 µg/mL). 1 mL of each extract was mixed with 2.5 mL of Folin-Ciocalteau reagent and 2.5 mL of 7.5% Na_2CO_3. Similarly, for total phenolic content was estimated for all the brands of *Vasa ghrita*. The stock sample solutions were prepared by partitioning 20 g *ghrita* with 100 mL of methanol. Final Sample solutions were made by diluting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 mL of stock in 10 mL methanol (serial dilution). The mixture was incubated for 45 min. The absorbance was measured at 760 nm using a UV-Vis spectrophotometer. Standard curve for gallic acid was obtained and the results were expressed as milligram of gallic acid equivalence per gram. Each experiment was performed in triplicates at each concentration.

Estimation of total flavonoid content[8]

Preparation of standard solution of quercetin

Stock solution (I) was prepared by dissolving 50 mg of quercetin in 50 mL of methanol. From this stock solution (I) 10 mL was pipette out in 100 mL volumetric flask and volume was made up to 100 mL with methanol (stock solution II). Further dilution was made by taking 0.5 mL from the stock solution (II) in 10 mL volumetric flask and volume was made up to 10 mL with methanol so as to obtain 5 µg/mL concentrations. Similar dilutions were done to obtain 10, 15, 20, 25, 30, 40, 45 and 50 µg/mL.

Preparation of sample and procedure to determine total flavonoid content of lyophilized powder and ghrita formulations

The total flavonoid content of *A. vasica* lyophilized powder was estimated by aluminum chloride method. Stock solution was prepared, 100 mg crude *A. vasica* lyophilized powder was weighed in 100 mL volumetric flask and volume was made up with methanol (stock solution I). Using this stock solution, serial dilutions were done to obtain different concentrations (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 µg/mL). 2 mL of above each sample were mixed with 2 mL of 2% $AlCl_3$ and the mixture was incubated for 1 h. Similarly, total flavonoid content was estimated for all the brands of *Vasa ghrita*. The stock sample solutions were prepared by partitioning 20 g *ghrita* with 100 mL of methanol. Final sample solutions were made by diluting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 mL of stock in 10 mL methanol and then incubated for 1 h. Absorbance was noted at 415 nm using UV-Visible spectrophotometer. Standard curve for quercetin was obtained and the results were expressed as milligram of quercetin equivalence per gram. Each experiment was performed in triplicates at each concentration.

DPPH radical scavenging activity[8]

Preparation of standard solution of ascorbic acid

The standard graph for ascorbic acid with ethyl acetate was obtained by making the concentration of 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 µg/mL. 1 mg of gallic acid was weighed and dissolved 100 mL of methanol (stock solution I). Using this stock solution, serial dilutions were done to obtain different concentrations. Final sample solutions were made by diluting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 mL of stock in 10 mL methanol. Absorbance was measured at 507 nm using Autoanalyzer with 485 µL of DPPH in ethyl acetate as blank.

Preparation of sample of lyophilized powder and ghrita formulations

The DPPH Radical scavenging activity of methanolic extract of *A. vasica* lyophilized powder was determined by using standard protocols. The stock solution of 50 µg/mL was prepared in ethyl acetate. Concentrations of 5, 10, 15, 20, 25 µg/mL were made. 15 µL of sample from each were taken and 485 µL of DPPH solution was added to it. The mixture was incubated in dark for 30 min. For *Vasa ghrita* Stock of 1 g/mL concentration was prepared in for all *vasa ghrita* formulations. Dilutions were made with ethyl acetate to obtain the concentration of 100, 200, 300, 400 µg/µL. From these final dilutions, 15 µL samples were taken and 485 µL of DPPH in ethyl acetate was added to it. Absorbance was measured at 507 nm using Auto analyzer with 485 µL of DPPH in methanol as blank. This was compared with the standard graph for ascorbic acid with ethyl acetate by making the concentration of 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 µg/mL. Each experiment was performed in triplicates at each concentration. The percentage of DPPH radical scavenging was calculated according to the following formula.

% DPPH radical scavenging = [(Ac-At)/Ac] x100

Where Ac: Absorbance of control and At: Absorbance of test

Biological toxicity studies[9]

***Determination of microbial load* (*Plate count for bacteria and fungi*)**

Standard protocol was followed for the determination of microbial load. Polysorbate 80 was used as an surfactant to emulsify the *gritha* formulations. For the analysis of bacteria *casein-soybean digest agar* was used as the medium and *Sabouraud glucose agar* was utilized for the fungi.

Animal toxicity studies[10]

***Acute oral toxicity study* (*OECD guideline 423*)**

6 male wistar rats (200-250 g) were obtained from Raghavendra enterprises, Bangalore, India. Allocated in to a dose range groups and each group consist of 6 animals. Acute oral toxicity for formulations had been carried out following OECD guideline 423. Food but not water, was withheld for overnight before the experiment. And further 2 h after administration of test drug. Animals were observed individually after dosing at least once during the first 30 min, periodically during the first 24 h, with special attention given during the first 4 h and daily thereafter, for a total of 14 days. Furthermore, all the animals were observed for mortality during the entire period of the study. The body weight of each animal was recorded just prior to dosing on day one and 14th day. On 14th day, blood was collected by puncturing supra-orbital plexus by capillary tubes under ether anesthesia for estimation of hematological and biochemical parameters. The parameter measured was as follows: RBC, WBC, hemoglobin, packed cell volume. For estimation of biochemical parameters, serum was separated from collected blood and requisite quantity of serum was fed to the auto analyzer. Biochemical parameters measured were Glucose, total proteins, SGPT, SGOT, Creatinine, Blood urea and Uric acid.

Statistical analysis

The analysis of data was done by using one-way analysis of variance (ANOVA) followed by multiple comparison tests by using Graph pad prism 5.0. $p<0.05$ was considered to be significant.

Results and Discussion

Morphological evaluation is the primary aspect of any standardization protocols. The organoleptic features of the collected sample was matching with that of the standard description of the Adulsa leaves. The leaves were dark green in color with characteristic odour and bitter taste. They had the typical shape of lanceolate to ovate-lanceolate along with an acuminate apex and a tapering base. The length of the leaf was in the range of 28-30 cm with long petioles justifying in all aspects the literature elaboration about the leaf morphology[11,12].

The organoleptic features of the final formulation of *Vasa gritha* however, showed some variation among the three formulations. The colors ranged from Yellowish-orange for RVG, Yellow for KKVG and Greenish-yellow for INVG, as can be seen in Figure 2. This slight variation in color can be attributed to the variation in the ghee that is incorporated in the preparation. Also some formulations can be prepared with the addition of jaggery which can impact the color of the final formulation. However, the odour and taste were consistent as all the three formulations had the characteristic odour and bitter taste[2].

The next in line for evaluation is microscopy. The dried drug powder was subjected to powder microscopy. The leaf showed most prominent identifying features of miscroscopy *viz.,* knee bent trichome along with multicellular trichome, calcium oxalate crystals, diacytic stomata, pitted vessels, spiral vessels and tetrad cells[11,12]. The leaf constants determined were within the limits found in the reference literature[11,12].

Preliminary phytochemical screening was performed both for the powder of Adulsa leaves as well as the formulations showed the presence of alkaloids, flavonoids, tannins, carbohydrates, saponins, amino acids as reflected in the literature[11,12].

Physicochemical parameters for Vasaka powder is summarized in Table 1, all these values were in limits as per the Ayurvedic Pharmacopoeia. The presence of carbohydrate can be corroborated with the swelling index (1.000±0.100 mL) and that of the saponins present in small quantity with the foaming index which is <100.

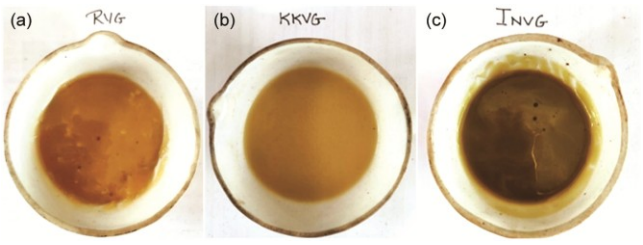

Fig. 2 — Organoleptic characters of *ghrita*

All the comparative physicochemical parameters for *Vasa ghrita* is summarized in Table 2. The values for all the parameters were in the proximity range of the pure ghee with some variations. Moisture content of INVG (0.316±0.001% w/w) was more as compared to RVG (0.133±0.003% w/w) and KKVG (0.090±0.003% w/w); pune ghee (0.237±0.001%w/w)[3,4,6,7].

Fat is lighter than the water. So specific gravity of fat/ ghrita is less. The observed values for specific gravity are RVG (0.939±0.001w/v), KKVG (0.942±0.001 w/v), INVG (0.941±0.002 w/v). Specific Gravity is more for RVG than KKVG and INVG having the least. This indicates that INVG is more lighter weight fat than KKVG and RVG respectively. Pure Ghee has the specific gravity of (0.237±0.009 w/v)[3,4,6,7].

Melting point shows the purity of the compounds. Every compound has its own melting and boiling point. If the substance melt over wide range, then the substance might be impure. Melting point of ghee changes depending on the type of breed of animal and season. In winter melting point might change. Melting point of RVG was 35.333±0.333°C, KKVG (28.333±0.333°C), INVG (31±0.577°C), Pure Ghee (30.333±0.333°C)[3,4,6,7].

Saponification number is the number of milligrams KOH required to neutralize fatty acids resulting from complete hydrolyses of 1g of fat. KKVG (226.27±0.468) showed high saponification value then INVG (224.868±0.468) and RVG (234.857±0.083) respectively. Therefore, molecular weight of the fat content of RVG is more as compared to INVG and KKVG respectively. Pure ghee (234.857±0.083) showed highest saponification value depicting pure ghee has least molecular weight than the *Vasa ghrita* formulations[3,4,6,7].

Iodine value shows amount of unsaturation of the fats and oils. Iodine value is the number of grams of iodine consumed by 100 g of fat. A high iodine value showed indicates high degree of unsaturation. Iodine value for INVG is slightly more (37.055±0.073) than RVG (35.870±0.042) and less for KKVG (34.475±0.042), while pure ghee showed the highest iodine value of 37.055±0.073. This showed INVG is more unsaturated then RVG and KKVG[3,4,6,7].

Epihydrin aldehyde is the compound in rancid fats responsible for the color reaction. Rancidity test was carried out and found that KKVG showed positive test by giving red color, whereas INVG, RVG and pure ghee did not show any color change[3,4,6,7].

The number of mg potassium hydroxide required to neutralize the free acid in 1 g of the fat or oil is defined as Acid value. More the free acid more will be the potassium hydroxide required to neutralize it and hence acid value also increases. Higher acid value gives higher chances if *ghrita* getting oxidized and rancid. Acid value for RVG was found to be 1.141±0.019, KKVG was 5.80±0.019, Inhouse was 1.608±0.019 and that of Pure ghee was 0.3787±0.0193. Since acid value for KKVG is high among all the *ghrita* formulation, it has chances of getting rancid very fast. This was also proved by rancidity test in which KKVG showed red coloration. Pure ghee showed very less acid value as compared with *Vasa ghrita* formulation because the *ghrita* formulation is prepared by boiling and might contains

Table 1 — Physicochemical Parameters for Vasaka Powder

Sr. No.	Physicochemical parameters	Mean ± SEM
1.	Moisture content (%w/w)	0.161±0.004
2.	Ash value (%w/w)	14.143±0.160
3.	Acid insoluble ash (%w/w)	0.268± 0.040
4.	Water soluble Ash (%w/w)	4.335±0.290
5.	Alcohol soluble extractive (%w/v)	4.933 ±0.250
6.	Water soluble extractive (%w/v)	29.803±0.280
7.	Ether soluble extractive (%w/v)	7.560±0.075
8.	Swelling index (mL)	1.000±0.100
9.	Foaming index (%)	< 100

All the values are expressed as Mean±SEM, n=3

Table 2 — Physicochemical parameters for *Vasa ghrita*

Sr. No.	Physicochemical parameter	Brand RVG	Brand KKVG	Brand INVG	Pure Ghee
1.	Moisture content (%w/w)	0.133± 0.003	0.090±0.003	0.316±0.001	0.237±0.001
2.	Rancidity	Not rancid	Rancid	Not rancid	Not rancid
3.	Specific gravity (w/v)	0.939±0.011	0.942±0.001	0.941±0.001	0.942±0.000
4.	Melting point (°C)	35.333±0.333	28.333±0.333	31.000±0.577	30.300±0.333
5.	Acid value (%)	1.141±0.019	5.8300±0.019	1.608±0.019	0.379±0.019
6.	Peroxide value (%)	3.400±0.115	6.667±0.067	0.933±0.067	3.800±0.000
7.	Saponification value	223.932±0.468	226.270±0.468	224.868±0.460	234.857±0.080
8.	Iodine value (%w/w)	35.870±0.0423	34.475±0.042	36.547±0.000	37.055±0.073
9.	Unsaponifiable matter (%w/w)	0.614±0.002	0.807±0.035	0.080±0.023	0.925±0.001

All values are expressed as Mean±SEM, n = 3

moisture which results in oxidation. Acid value KKVG>INVG>RVG>Pure Ghee[3,4,6,7].

The peroxide value gives idea about of the extent of oxidative rancidity, which is similar to the acid value. It is a measurement of the amount of oxygen absorbed at double bonds in unsaturated fatty acids. Peroxide value for RVG was 3.4±0.115, KKVG was 6.667±0.067, INVG was 0.933±0.067 and Pure ghee was found to be 3.800±0.000. KKVG showed High peroxide value than other ghrita formulation which indicate that high extent of rancidity[3,4,6,7].

The unsaponifiable matter consists of substances present in oils and fats, which are not saponifiable by alkali hydroxides and fails to form soap. The unsaponifiable matter for RVG was obtained as 0.614±0.002, KKVG was found to be 0.807±0.035, INVG was 0.080±0.023 and that of Pure ghee showed value of 0.925±0.001[3,4,6,7].

HPTLC was performed after developing a suitable mobile phase wherein different phytoconstituents present can be identified as shown in Figure 3(a). Qualitative analysis indicated the presence of higher vasicine content in INVG. Pure vasicine standard showed Rf value of 0.13. VLP showed Rf value 0.14 which was similar to standard. VPD Rf value was 0.14, INVG, KKVG, RVG showed Rf value of 0.12, 0.13, 0.13 respectively, refer to Figure 3(b). FTIR fingerprinting was carried out. The INVG spectra were matching with that of the other *vasa ghrita* formulations, as seen in Figure 4 spectra[3,4,6,7]. Table 3 gives the quantitative values.

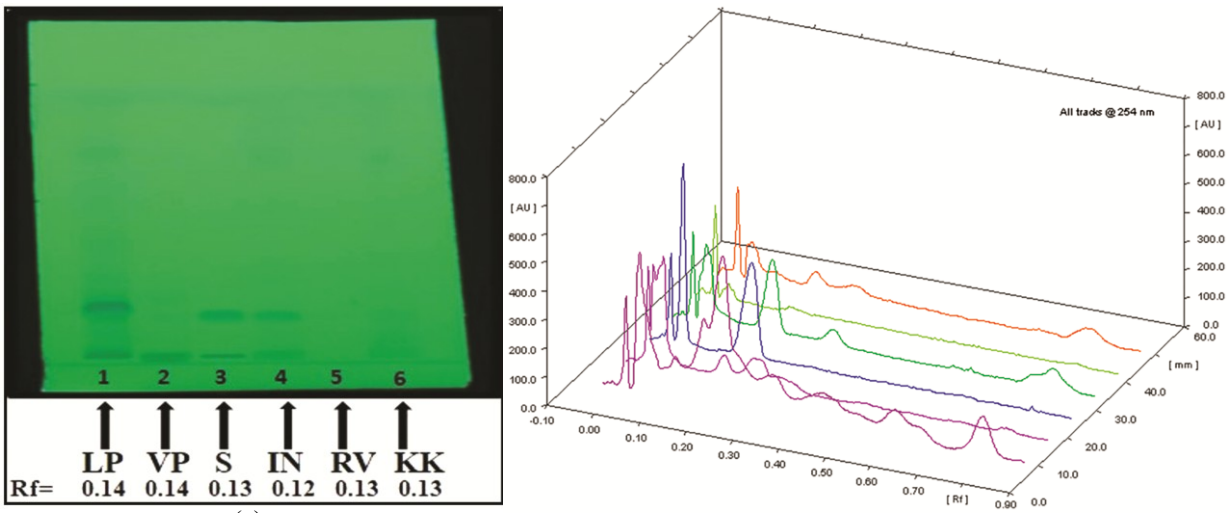

Fig. 3 — (a) HPTLC analysis of VLP, VPD, Standard vasicin, INVG, RVG and KKVG samples were spotted respectively under λ=254 nm (Plate), (b) HPTLC analysis of VLP, VPD, Standard vasicin, INVG, RVG and KKVG samples were spotted respectively under λ=254 nm (3D overlay)

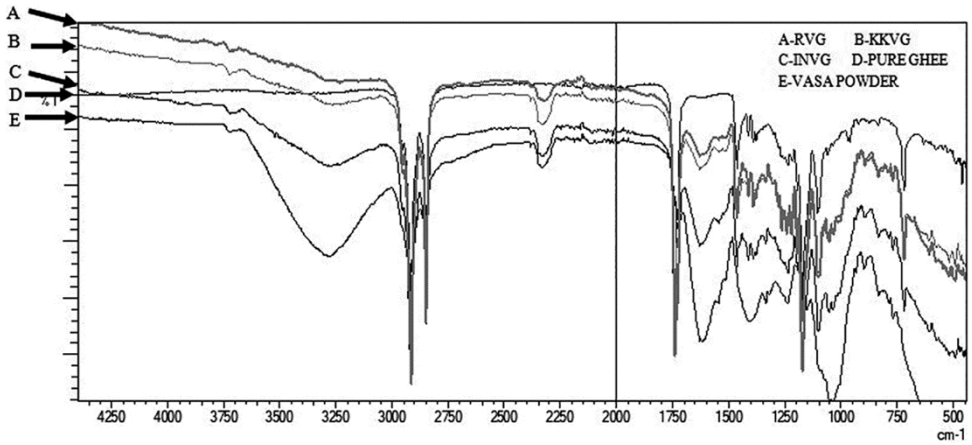

Fig. 4 — Fourier Transform Infra-Red Spectroscopy overlay of Brand RVG, Brand KKVG, INVG, Pure Ghee and Vasa Powder (VLP)

Table 3 — Qualitative identification of Vasicine in vasaka powder and formulations

Peak	Start Position	Start Height	Max Position	Max Height	Max	End Position	End Height	Area
VLP	0.14 Rf	10.8 AU	0.18 Rf	187.5 AU	29.04%	0.19 Rf	157.1 AU	3367.1 AU
VPD	0.14 Rf	2.3 AU	0.17 Rf	63.6 AU	51.54%	0.20 Rf	5.4 AU	1174.9 AU
Vasicine	0.13 Rf	8.8 AU	0.18 Rf	330.3 AU	100.00%	0.23 Rf	2.0 AU	8657.8 AU
INM	0.12 Rf	2.2 AU	0.17 Rf	248.2 AU	100.00%	0.21 Rf	0.3 AU	5553.0 AU
KKM	0.13 Rf	2.4 AU	0.16 Rf	17.4 AU	100.00%	0.19 Rf	7.1 AU	446.2 AU
RVM	0.13 Rf	0.1 AU	0.17 Rf	54.0 AU	67.61%	0.20 Rf	10.2 AU	1232.1 AU

Table 4 — Estimation of Total Phenolic Content, Total Flavonoid Content and IC_{50} values (DPPH method)

Sr. No.	Parameter	*Vasa ghrita*			VLP
		RVG	KKVG	INVG	
1.	Total Phenolic Content (mg GAE/g)	231.913±10.199	258.653±8.405	339.756±12.908	48.677±2.049
2.	Total Flavonoid Content (mg QUE/g)	75.392±8.817	79.904±11.786	138.029±13.284	26.869±2.559
3.	DPPH radical scavenging activity: IC_{50} values (µg/mL)	348.000±0.01	1612.000±0.197	1216.000±0.151	18.725±1.062

All values are expressed as Mean±SEM, n= 3

Table 5 — Hematological parameters and Biochemical changes of INVG treated rat

Sr. No.	Parameters	Control	INVG (2000 mg/kg oral)
1.	RBC ($x10^6/mm^3$)	6.55±0.237	7.35±0.238
2.	WBC ($x10^6/mm^3$)	7.35±0.146	7.45±0.146
3.	Hb (g/dL)	13.0±0.123	13.0±0.125
4.	Packed cell volume ($x10^6/mm^3$)	35.3±0.432	37.4±0.23
5.	Blood glucose (mg/dL)	30.55±5.321	26.67±5.494
6.	SGOT (IU/L)	113±5.92	126±3.58
7.	SGPT (IU/L)	25.2± 1.74	29.0±3.83
8.	Total protein (g/dL)	5.37±0.504	5.42±0.704
9.	Uric acid (g/dL)	1.65±0.208	1.74±0.186
10.	Creatinine (mg/dL)	2.815±0.1007	2.668±0.2817

All values are expressed as Mean±SEM, n = 3

The DPPH radical scavenging activity was determined and compared with the standard ascorbic acid. IC_{50} for ascorbic acid was found to be 8.923±0.019 µg/mL and for other samples as shown in Table 3.

The total phenolic content for RVG was found to be 231.913±10.199 mg GAE per gram. The value was higher for KKVG having 258.653±8.405 mg GAE per gram and highest was for INVG having 339.756±12.908 mg GAE per gram. Vasa lyophilized powder showed 48.677±2.049 mg GAE per gram. Aluminum chloride also forms acid labile complexes with the ortho-dihydroxyl groups in the A-or B-ring of flavonoids. The total flavonoid content for RVG was 75.392±8.817 mg QUE per gram, KKVG was 79.904±11.786 mg QUE per gram, INVG was 138.029±13.284 mg QUE per gram and Vasa Lyophilized powder showed 26.869±2.559 mg QUE per gram. Table 4[3,4,6,7].

Total bacterial growth at the end of 72 h was seen more in KKVG followed by RVG and INVG. The

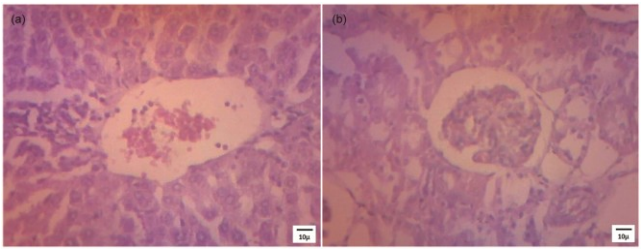

Fig. 5 — Histopathology of Liver and Kidneys, (a) Vasaka treated liver-liver with central vein dilatation and peri sinusoidal inflammation, (b) Vasaka treated Kidney-Normal cytoarchitecture

bacterial growth should not be more than 300 CFU/mL. Fungal growth was not seen for the all the three brands except for INVG which showed only one colony at the end of 120 h. The fungal growth should not be more than 100 CFU/mL. The fungal and bacterial growth was found to be within the limits[5].

For toxicity evaluation of *Vasa ghrita* in rats, weight variation was observed in control and *Vasa ghrita* treated rats. There was significant increase in body weight in all treatment groups at the end of the study in comparison to day 1 body weights. The control group showed difference of 33.5±7.68 g whereas INVG showed difference of 56.7±6.47 g. The hematological results showed that there was no significant change in RBC, WBC, Hemoglobin and packed cell volume in *Vasa ghrita* treated rats in comparison to control rats during toxicity study as given in Table 5. It was also observed that there was no significant change in biochemical parameters like blood glucose, SGOT, SGPT, Total Proteins, Uric acid, Creatinine of *Vasa ghrita* treated rats in comparison to control rats during toxicity study as shown in Table 5. Histopathological examination of organs observed that in *vasa ghrita* treated liver there was perisinusoidal inflammation and Central vein dilatation Figure 5 (a)

whereas in kidney, normal cytoarchitecture is observed; Figure 5 (b)[12].

Conclusion

In the last few decades the faster effects of allopathic medicines have tempted people to take up these as a remedy for the quick disappearance of the symptoms produced by the diseases. But now after the prolonged use of these for a period of time have brought to the general awareness of the public that allopathy is more of a discrete treatment than a holistic one. Also, the advent of various side effects, drug interactions and withdrawal symptoms encountered with many allopathic medicines are increasing by the day[13,14]. Hence now, slowly the focus is again turning towards the traditional systems of medicines be it Ayurveda, Unani, Siddha, Homeopathy etc[15]. In this process, more and more interest is being shown by the pharma companies to manufacture the traditional medicines to meet their increasing demand. This offers a great potential in the form of contributing to the national economy as these products are also preferred in the international market.

However, there is a big challenge facing these products in the international scenario as standardized protocol for the qualitative and quantitative evaluation of many of the formulations has not yet been well established[16]. A lot of work needs to be performed in this area of research to develop dependendable techniques which can take into consideration the various factors that affect the final herbal/ayurvedic formulation (time of collection, processing, manufacturing, packaging and storage).

In the present study one such important formulation "*Vasa ghrita*" has being considered to develop the protocol for its evaluation. An inhouse formulation was prepared following the standard protocol of Ayurveda and this was compared with two other formulations already available in the market to see the variations in different parameters of study. The study was conducted on morphology, microscopy, physicochemical, phytochemical and biological parameters of the individual plant materials and the formulations.

It has been observed that there are differences existing in the data obtained from the three different formulations which in turn indicates that there is a great scope of variability. There is a greater need to emphasis the exact procedures, which need to be followed right from the stage of collection, all through the processing and final package and storing of the formulations. More such data needs to be generated making multiple assessments of the same formulations from various companies. This data bank once generated can serve as a guide for better production of the standardized formulations.

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Animal Ethical Committee Approval

IAEC/XIII/01/RIPER/2019

Conflict of Interest

There are no conflicts of interest.

Author Contributions

N M S has carried out all experimental part and writing of this manuscript. P L K has done the formatting of manuscript. M K J has supervised the entire experiment and manuscript writing. Y N S has supervised in carrying in out the microbial studies. A S has helped in the concept of the experiment. S R has helped in carrying out the animal studies.

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