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ARTICLE

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EXPLORATION OF PHARMACEUTICO-ANALYTICAL PROFILES AND IN –VITRO ANTIOXIDANT ACTIVITY OF *VARNAKGHRITA*

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Introduction

Ayurveda drug compendium comprises thousands of formulations for different ailment. Herbs, metals, minerals and animal bi-products are the sources of them. These natural substances are converted into medicaments through different pharmaceutical procedures called Ayurvedic pharmaceutics (*Kalpana*). Juice (*Swarasa*), paste (*Kalka*) and aqueous extracts (*Kashaya, Hima, Phanta*) of herbs are considered as the basic pharmaceutical dosage forms[1]. Based on them, various secondary dosage forms viz. powders (*Churna*), tablets-pills (*Guti-Vati*), wicks (*Varti*), ointment (*Malahar*) confectioneries (Avaleha-*Khanda*), lipid-based drugs (*Sneha Kalpa*), distillates (*Arka*), hydro-alcoholic fermentation (*Sandhana)* are being designed by the seers[2]. These dosage forms are meant to be use for internal (oral, nasal & ano-rectal) and as well as external application. A major portion of the formulation used for external application are lipid based drugs which are advocated in different dermatological conditions (*Kustha*) and minor disorders (*Kshudra Roga*). Preparation of medicated ghee or oils expedite lipid soluble phyto-constituents of herbs and their extracts of different organic media (water, milk, buttermilk, cow urine etc.). Lipid-based drug delivery systems (LBDDS) are being preferred for topical application for enhancing the potency of drug substance, convenient for lipophilic and hydrophilic drug, greater absorption, bioavailability, control drug release and pharmaceutical stability[3]. *Varnak Ghrita* (VG) is a lipid base classical ointment formulation mentioned in *Kshudrarogadhikara* of *Chakradatta* to improve skin complexion. It comprises ingredients like *Madhuka* (*Glycyrrhiza glabra* L), *Chandana* (*Santalum album* L), *Padmaka* (*Prunus cerasoides* D. Don*)* of group of ten complexion promoting *dravya*(*Varnya Mahakashaya)*[4], *Sarshapa* (*Brassica juncea* (L.) Czern), *Priyangu* (*Callicarpa macrophylla* Vahl.), *Kaliyaka (Coscinium fenestratum* (Gaertn.) Coleb.*), Haridra (Curcuma longa Linn.), Lodhra (Symplocos racemose Roxb.), Kesar (Crocus sativus L.)* having complexion enhancing (*Varnaprasdana)*[5] and blood detoxifying (*Raktaprasadana)*[6] properties. In contemporary research, these herbs are reported to have wide variety of pharmacological activities viz. anti-inflammatory[7], analgesic[8], antioxidant[9], chemo-preventive[10], anti-tumor, melanogenesis inhibitor[11] and anti-microbial activities[12] etc. Despite of these, VG is one of the under-explored drug in current treatment modalities. Therefore, present study has been conceptualized to explore its pharmaceutical, analytical and pharmacological profiles.

Material and Methods

Preparation of Test Drugs : *Varnak Ghrita* (VG) was prepared as per the classical method (with modification) mentioned in *Chakradutta*[13]. All the ingredients (Table 1) were purchased from the Kharibauli market, New Delhi and authenticated by the experts of *Drayaguna* (Ayurvedic pharmacogonosy & pharmacology) before use.

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Table 1: Ingredients and part used of VG

Sl. No.	Content	Part Use	Botanical name	Ratio
1.	*Madhuka* (Licorice)	Root	*Glycyrrhiza glabra* L.	1 part
2.	*Chandana* (Sandalwood)	Hart wood	*Santalum album* L	1 part
3.	*Priyangu* (Beauty berry)	Seeds	*Callicarpa macrophylla* Vahl.	1 part
4.	*Sarshapa* (Indian Mustard)	Seeds	*Brassica juncea* (L.) Czern	1 part
5.	*Padmaka* (Bird Cherry)	Hart wood	*Prunus cerasoides* D.Don	1 part
6.	*Kaliyaka* (Yellow vine)	Stem	*Coscinium fenestratum* (Gaertn.) Coleb.	1 part
7.	*Haridra* (Turmeric)	Tuber	*Curcuma longa Linn.*	1 part
8.	*Lodhra* (Symplocos tree)	Stem	*Symplocos racemose Roxb.*	1 part
9.	*Kesar* (Saffron)	Stigma	*Crocus sativus* L.	8 part
10.	*Ghrita* (Cow ghee)	---		32 part
11.	*Siktha* (Bee wax)	---		8 part

Preparation of Paste (Kalkadravya): All the herbal drugs were powdered separately with the help of grinderfollowed by sieving (# 40). Then a little quantity of water was mixed to prepare boluses out of it.

Preparation of Medicated Cow Ghee (Ghrita): Ghee preparation was done as per the general principle of lipid-based drugs (*Snehakalpana)* mentioned in *SarangadharSamhita*[1]. Cow ghee was taken in a stainless steel vessel and heated on low flame to obtain the moisture free state. Then the paste in bolus form were added into it and fried till it become light brown in colour. Further, water was added into the ghee and processed on low flame for two days and after achieving the chief desire characteristici.e firmness of the paste (*Ishatkathinkalka)*of 3rd stage of processed lipid (*Kharapaka)*, heating was stopped. After that, the mixture was filtered through four-fold cotton cloth and stored in air tight glass container. Temperature at different stage viz. frying, boiling, 1st stage (*Mridu*), 2nd stage (*Madhyam)* and 3rd stage (*Kharapaka)* were measured with the help of thermometer and recorded.

Preparation of Ointment (Malahar): Stigma of *kesara* was triturated with the prepared gheein a mortar and pestle for 20 minutes. After complete trituration, bee wax was added at 70^{0}C and triturated well to get a smooth homogenous ointment like consistency. Finished product was stored in air tight glass container and packaging was done as per standard guidelines[2].

Physico-chemical Analysis: Various quality control parameters like Organoleptic test (colour, odour, touch, consistency etc.), physico-chemical parameters (pH, specific gravity, moisture content, extractive values, acid value, peroxide value, iodine value, saponification value, mineral oil content and refractive index) were performed as per standard guidelines[3]. Heavy metals (Hg, As, Lead and cadmium) were also analyzed through ICP-MS instrument for detection of possible contamination.

***Qualitative Screening of Phyto-chemicals*:** Organic solvents of different polarities were used to extract phyto-chemicals present in the formulation. Methanol, ethyl acetate and petroleum ether extracts of VG have been tested forthe presence of Alkaloid[4], flavonoid[5], saponins[6], steroid[7] and glycosides[8] by following standard method and guidelines.

Quantitative Estimation of Phyto-chemicals: Total phenolic content (TPC) of Methanol extract was quantified as per Folin ciocalteu methodthrough UV-Vis spectroscopy[9]. The sample was analyzed in triplicate at 517 nm wavelength while the standard curve was created by using Gallic acid at various concentrations ranging from 5-100 µg/ml in methanol.

Estimation of Targeted Metabolite: The test drug consists numerous phyto-chemicals i.e. secondary metabolites. Curcumin is one among them which was quantified through High performance thin layer chromatography (HPTLC) with reference standard in the present study. Test samples were prepared at a concentration of 100mg/ml in methanol followed by sonication for 20 minutes, while the curcumin (98% pure Sigma-Aldrich) reference standard was prepared in same solvent at a concentration of 1mg/ml. Then sample in

triplicate (6, 8 and 10 **µl)** and standard in 1, 2, 4, and 6 µl were applied for generation of calibration curve on the TLC plates silica gel 60 F 254 (stationary phase) by automatic sample Applicator (CAMAG Linomat, Spray gas-Inert gas, Sample solvent type-Methanol, Dosage speed-150 nl/s, Syringe size- 100 ml, Band width- 8.00 mm). Mixture of n-Hexane: ethyl acetate: Glacial acetic acid: Methanol (10:1.1:1.1:2.5) was used as mobile phase. Scanningwas performed at (λ = 254nm, 366 nm) and quantification (λ = 404 nm) were done by CAMAG TLC scanner having specifications of (Scanner_230698(2.01.02), Slit dimensions- 6.00×3.00 mm, micro, Scanning speed-20nm/s, Wavelenth-254 nm, Lamp-D2, Measurement type-Remission, Measurement mode-Absorption).

FTIR (Fourier Transform Infrared Spectroscopy): The test drug was analyzedfor presence of functional groups by FTIR (Bruker, 3000 Hyperion Microscope with Vertex 80 FTIR System, Germany) and the spectra were taken in the region of 4000-400 cm^{-1} to determine the organic bonds[10].

In-vitro Anti-oxidant Activity: DPPH scavenging activity of methanol extract of the test drug was analyzed through UV-VIS spectroscopy. 3 ml of a 0.5 mM DPPH methanol solution was added to 1 ml of sample solutions of different concentrations, and allowed to react at room temperature. After 30 min the absorbance values were measured at 518 nm. Ascorbic acid (2, 4, 6,8,10,12 µg/ml) were used to generate a standard curve[11].

Result

Preparation of Test Drugs: The standard manufacturing procedure was developed by preparing three batches of VG. An average yield of 93.66 % and loss of 2.64 % was observed in the finished product. (Table 2). The temperature pattern and duration of cooking were depicted in fig. 1, 2& 3.

Table 2: Observation of different batches of VG

Observations	Batch 1	Batch 2	Batch 3	Mean value
Initial quantity of ghee (gm)	500	500	500	**500**
After heating(moisture free)	481.5	490	489	**486.8**
Yield of Varnak ghrita (gm)	465	470	470	**468.3**
Yield of Varnak ghrita (%)	93	94	94	**93.66**
Mixing of *Kesar* and *Siktha*	560	568	570	**566**

Physico-chemical Parameters: Observed organoleptic, Primary and specific physico-chemical parameters were specified in table 3. Mercury and lead were found below the detection limit while Arsenic and cadmium were detected as 0.471 & 0.239 ppm respectively which are within the limit prescribed by Ayurvedic Pharmacopoeia of India.

Table 3: Organoleptic and Physico-chemical parameters of VG

Organoleptic parameter	
Colour	Yellow
Odour	Characteristic
Touch	Soft
Consistency	Greasy

Sl	Parametern = 3	Varnak ghrita (Mean ± SD)
1.	Refractive index	1.4539 ± 0.002
2.	Specific gravity	0.9079 ± 0.003
3.	Moisture content /LOD	0.3 ± 0.002 %
4.	Saponification value	220.62 ± 2.62
5.	Iodine value	23.0 ± 3.05
6.	Acid value	3.5571 ± 0.05
7.	Peroxide value	17.5513 ± 1.89
8.	Mineral oil	-ve

Qualitative Screening of Phyto-chemicals**:** Steroid was not found in any of the tested extracts. Methanol extract have shown the presence of alkaloid and flavonoid while glycoside and saponnin were found in ethyl acetate and petroleum ether extracts along with them.

Detection and quantification of Curcumin: Separation of curcumin and VG were well observed in white light, 254 & 366 nm respectively. Initially scanning was performed at 254 & 366 nm andcurcumin was identified at R_f value of 0.121±0.047 (254 nm). After assigning the reference substance spectra was taken between 254 to 450 nm. The observed λ max was noted as 404 nm and on quantitation, curcumin of 287µg/g was quantified in the test drug with CV of 4.52%. (Fig. no 4)

FTIR: Scanning in IR light (4000-400 cm^{-1} wavelength) detected 16 peaks in the test drug in the region of 583.87, 721.59, 869.44, 966.09, 1099.03, 1112.94, 1160.63, 1235.75, 1377.38, 1417.73, 1464.37, 1613.37, 1743.29, 2852.68, 2921.94, 3474.10 cm^{-1}respectively. Various functional groups viz.

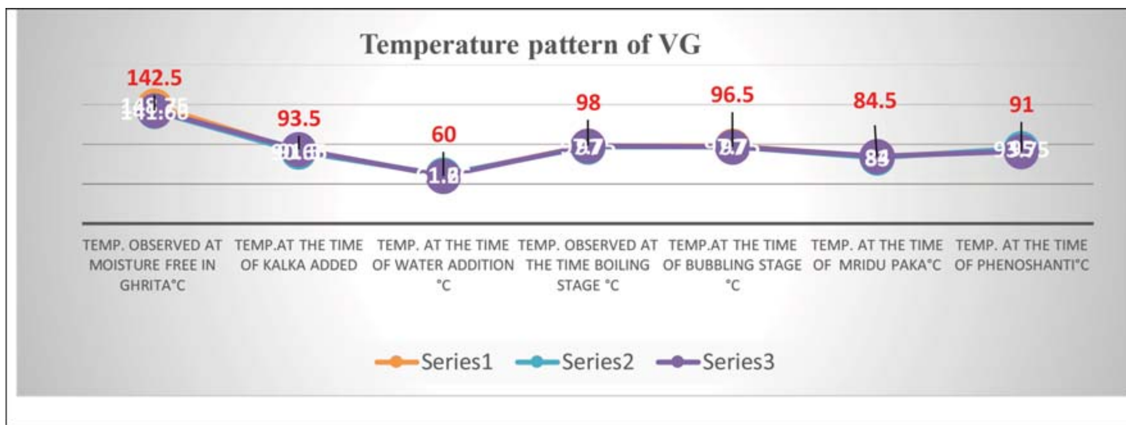

Fig 1: Temperature pattern of VG in different stages

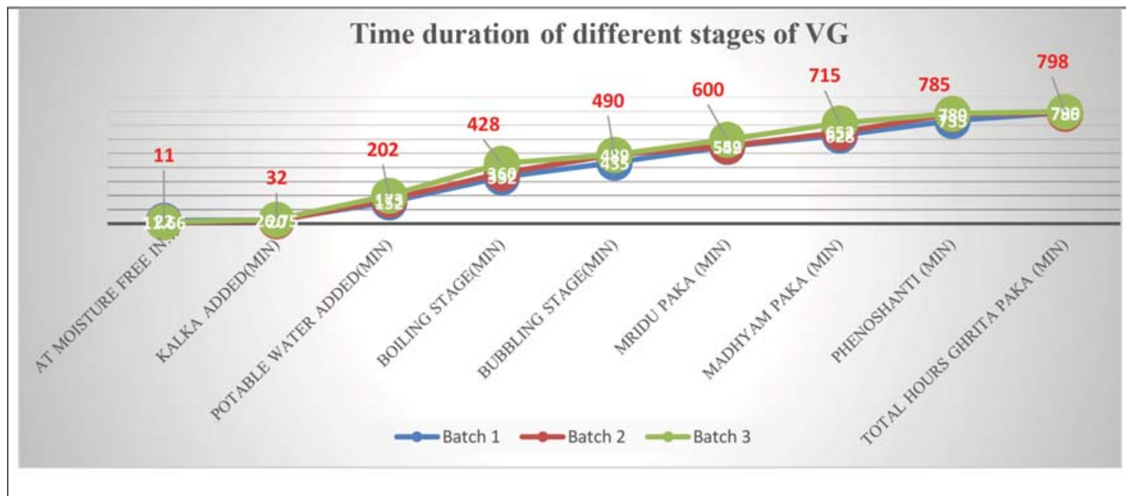

Fig. 2: Time duration of different stages of VG

Preparation of paste | Moisture free of ghee | Frying of paste

Processing of ghee | Mixing of Kesar and bee wax | Packaging of VG

Fig. 3: Preparation of Varanak Ghrita

Alcohol (O-H), Alkyl (C-H), Alkene (C-C), Alkylhalide (C-H wag) having Strong/medium, broad and stretching vibration were found. (Fig no. 5)

Total Phenolic Content (TPC) and In-vitro Antioxidant Activity (DPPH scavenging): TPC was calculated as 10.64± 0.44µg/g/GA while standard curve was developed with different concentration of Gallic acid (R^2= 0.9962).In DPPH scavenging activity IC 50 was calculatedas 14.10 ± 0.80 µg/ml when standard curve was developed with different concentration of Ascorbic acid (R^2=0.9843).(Fig No. 6 and 7).

Discussion

Present research work is the exploration of ancient wisdom. "*Varnakghrita* (VG)" derived its name from its pharmacological action i.e. enhance the glow, texture and contour of the skin. The ingredients present in this formulation having vivid pharmacological action related to the pathology of various skin diseases.

Preparation of the ointment and the ghee are the two major steps involved in theprocessing of present formulation. As the classics have not mentioned about liquid media (*Dravadravya)*, water was used as liquid media in accordance with general guidelines of lipid base drug (*Snehapaka)*. Coarse powder (#40) of the ingredients was used to make bolus of paste (*Kalka)*, as it is suitable for a media to extract active pharmaceutical componentsand prevents loss and charring as fine or very fine particles are susceptible to burn and trappingof lipid media. Making raw ghee moisture free is necessary to increase stability and solubility of lipid soluble components[25]. The paste was added at low temperature to avoid burning and loss of thermo-labilebioactive components. Processing of ghee at mild temperature restrict the degradation of API (Active

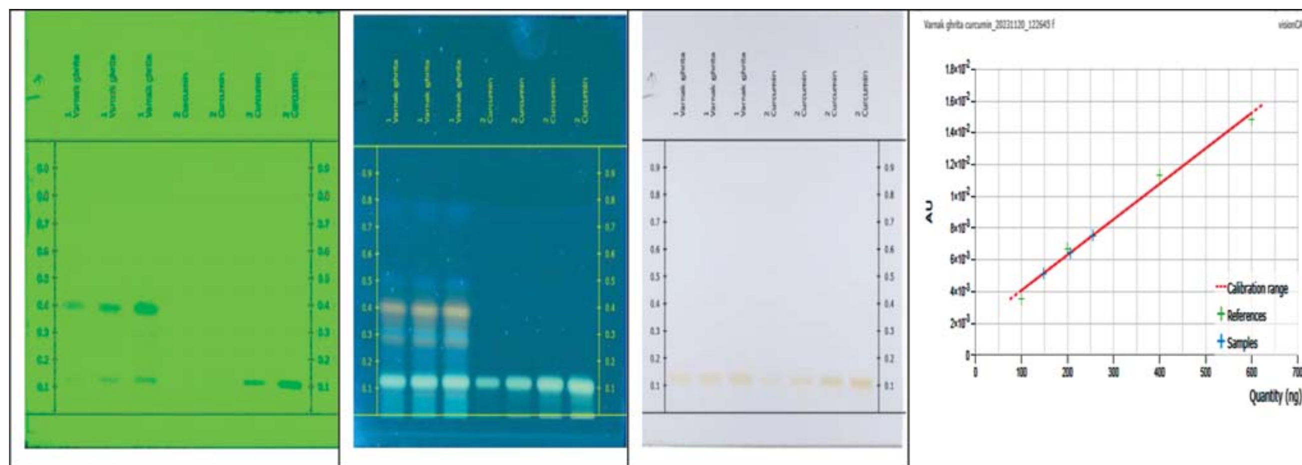

Fig. 4: HPTLC fingerprinting for quantitation of curcumin

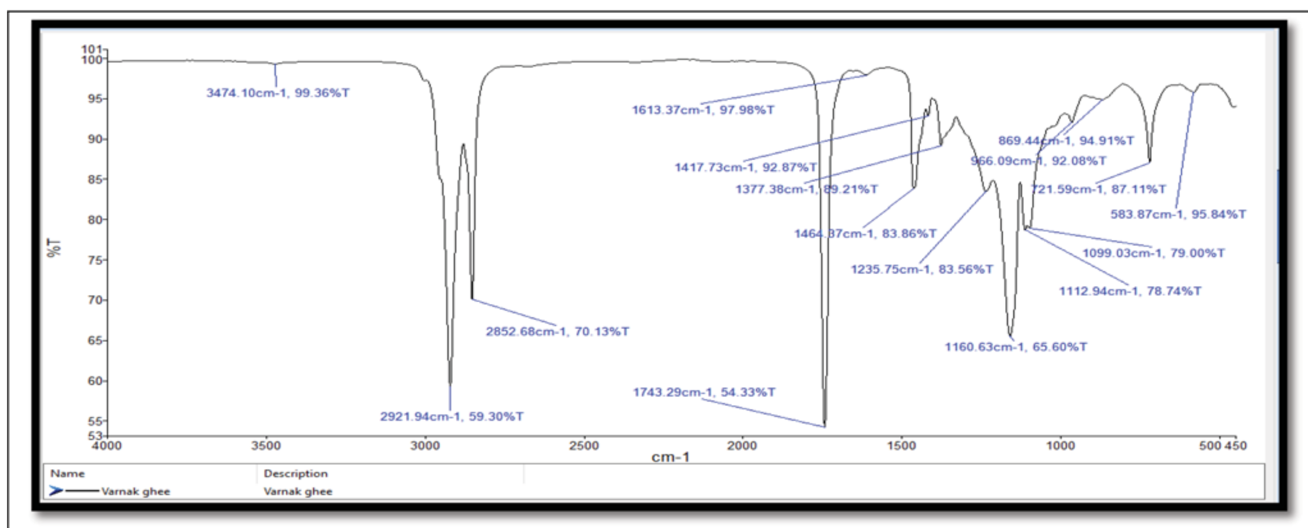

Fig. 5: FTIR spectra denoting different functional group

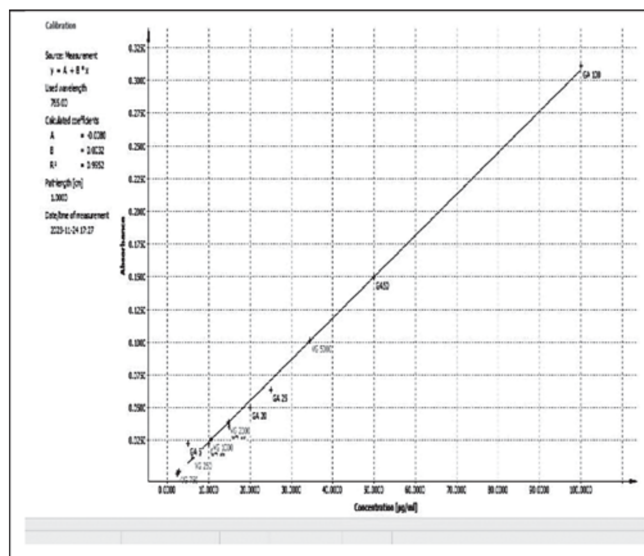

Fig.6: Standard curve for TPC estimation

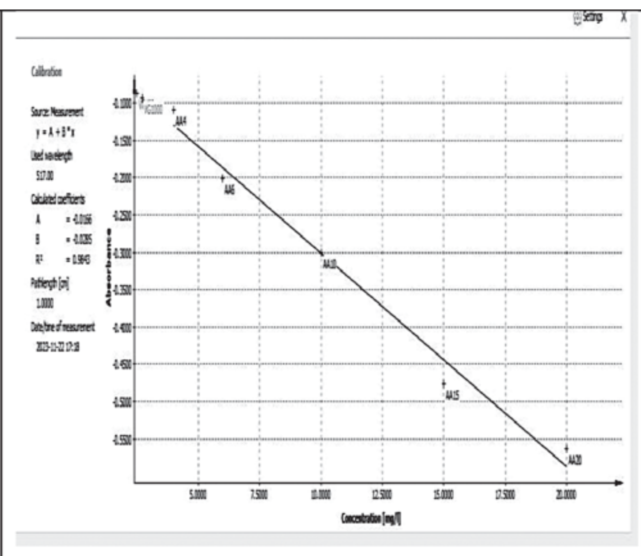

Fig.7: Standard curve for DPPH estimation

Pharmaceutical Ingredients). It is evident that when ghee is heated above 140^0C, produce primary oxidation product which decompose later to make secondary oxidation product.[26] In classics, $1/4^{th}$ quantity of bee wax has been mentioned. However, in this ratio, very hard consistency of the ointment was observed in pilot batch which is not suitable for dispense. Pilot batches with different ratio ($1/4^{th}$, $1/6^{th}$, $1/8^{th}$ and $1/10^{th}$) of bee wax were prepared. It has been found that, $1/10^{th}$ ratio of bee wax is suitable to achieve desired consistency. Modification in the ratio of *Kesar*to $1/10^{th}$, instead of $1/4^{th}$ was also done due to its high cost value.

Organoleptic characters facilitate yellow color which is due to ingredients viz. *Haridra*, *Priyangu*, *Yastimadhu* and *Keshar*. The base used i.e bee wax in a suitable proportion facilitates soft and greasiness consistency. Refractive index (RI) of a substance determines the capability to bend the light compared to vacuum. It is used to evaluate the purity or concentration of a substance. In a medium like ghee, high RI indicate more concentration of light which infers rancidification thus lower stability period[27]. In present work, 1.4539 RI of VG has been found which is indicative of good shelf life. Specific gravity of VG was found to be 0.9079 which indicate relatively less dense than the water which provide better spreadibility. Moisture play a pivotal role on drug's quality, safety and efficacy. It can affect a drug through water-solid interactions, water-amorphous interaction and molecular disorders of crystalline structures interaction.[28] Low moisture content (0.3%) revealed that the test drug is very less susceptible to degradation or microbial/fungal contamination. Saponification value indicate average molecular weight of triglycerides which is inversely proportionate to the molecular weight of fatty acid in a sample[29]. Study reported that, high saponification value (220.62) indicate a higher proportion of short carbon chain in fatty acid present in sample[30]. Iodine value express notion of unsaturation in a fatty substance. The unsaturation of a fat or oil is directly co-related to the amount of C=C bond present which reflects the vulnerability of a sample to oxidation[31]. The lesser degree of unsaturation (23.0) indicate great stability and high quality. Acid value reveled the amount of free fatty acid (FFA) by measuring carboxylic acid group (-COOH) in a sample. FFA is produced by the hydrolysis of fat due to various factors viz. storage, processing, heating etc. High FFA value indicate less stability and poor quality[32]. Peroxide value indicate the primary oxidation of fat, current data suggest that VG has a lower primary oxidation value which directly correlated with the quality of the product. Mineral oil is derived from the mineral sources which is a mixture of higher alkanes. It is foundto be mixed with many edible fat or fat base used for cosmetics. Various toxic effect of mineral oil viz. carcinoma, erythema, allergy, diarrhea etc. has been reported[33]. Absent of it suggests it is safe to be use. Heavy metals (Hg, As, Pb, Cd)as contaminants become the major concern for the safety of herbal medicines. Its presence above the threshold level can be hazardous for human being through its bioaccumulation[34]. All the heavy metals in VG were detected below the permissible limit mentioned by API[35].

Phytochemicals of different groups viz. alkaloid, flavonoid, glycoside and saponin were detected in different organic media. Each one have their own pharmacological actions through different pathway to mitigate various patho-physiology.Curcumin, structurally [1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-Dione], is a natural polyphenol having unsaturated aliphatic group. In one gram of VG, 287 µg curcumin is found to be present through HPTLC. The aliphatic heptene linker act as a very potent proton donor when it is in the bis-keto state in weak acidic media. Mostly, it facilitates the scavenging activity of Reactive Oxygen Species (ROS) thus acts as a potent anti-oxidant[36]. It exhibits anti-inflammatory and immunomodulatory activities through suppression of TNF-α, vascular cell adhesion molecule-1 (VCAM-1)[37], NF-κB, IL-2, forkhead box P3 (Foxp3), and CD25 expression[38]. It is reported to homeostat dermal pathological condition by increasing fibroblastic cells, collagen content and alter the expression of vascular endothelial growth factor (VEGF) and plasminogen activator inhibitor-1[39]. Furthermore, it is reported to have anti-atherosclerotic, thrombolytic, anti-lipidimic, anti-carcinogenic, chemo-preventive and neuroprotective activities[40]. Despite of enormous potency, it has disadvantages of auto-degradation, poor absorbance, poor bio-availability, poor skin penetration and low aqueous solubility. Curcumin in lipid base drug delivery system resolve all issues, provide stability, high drug loading and gradual release in targeted organs[41].

FTIR revealed VG having organic substances of different functional groups viz. Alcohol (O-H), Alkyl (C-H), Alkene (C-C), Alkylhalide (C-H wag). The detected O-H groups indicate the presence of phenols in the test drug[42]. Alkyl are the hydrocarbons found in various organic materials viz. Fatty acids, organic acids, terpenes etc. Alkylhalide groups are found in the aromatic substances[43]. It indicates presence of volatile or essential oils.

Total phenolic content is the cumulative of simple phenolics (resorcinol and phloroglucinol), phenolic acids, aldehydes, coumarins, flavonoids, chalcones, aurones, benzophenones, xanthones, stilbenes, benzoquinones, naphtha-quinones, anthraquinones, betacyanins, lignans, and polyphenols[44]. TPC of VG is found to be 10.64± 0.44µg/g/GA. They mainly impart anti-oxidant property[45]. Besides that, these are known to have anti-inflammatory, anti-microbial, anti-viral, anti-fungal activities which can be key factors to mitigate different dermatological pathologies.[46]

Free radicals are the product of oxidation process that is continuously going on in biological system. They are found to have key role in the pathogenesis of various diseases including skin diseases, cancer, cardiovascular disease neural disorders, Alzheimer's disease cognitive impairment Parkinson's disease, alcohol induced liver disease, ulcerative colitis, aging and atherosclerosis etc[47]. Drugs having anti-oxidant property are chosen for breaking of their pathogenesis. In dermatology, melasma, contact dermatitis, urticaria, aging, acne vulgaris, atopic dermatitis, Skin cancer, wound like skin conditionsare reported to be related with abnormal metabolism of ROS (Reactive Oxygen Spices)[24]. The DPPH scavenging activity of VG is found to be 14.10 ± 0.80 µg/ml, which ensure it can be a potent topical formulation for these diseases.

Kesar is an important ingredient of VG. It is reported to have more than 150 volatile aromatic substances and crocin, safranal, pirocrocin, crocetin, zeaxanthin, lycopene, α- and β-carotenes, glycosides, monoterpenes, aldehydes, flavonoids, anthocyanins, vitamins (especially riboflavin and thiamine) and amino acids like non-volatile bioactive compounds[49]. These are responsible for anti-oxidant, anti-inflammatory, anti-cancer, chemo protective, anti-lipidimic activities[50]. In topical application, it imparts anti-tyrosinase potential which helps to cease production and accumulation of excessive melatonin, thus resolve dermatological condition caused by hyperpigmentation[51]. Bee wax is used as potential base for preparation of traditional ointments. It consists of free fatty alcohols (Triacontanol, Octacosanol, Hexacosanol, and Tetracosanol), phenolic compounds, terpenes and hydrocarbon which facilitate antioxidative, anti-inflammatory, anti-microbial and cell regenerative activities to resolve dermatological pathologies.[52]

Cumulatively, the product imparts suitable quality and safety which ensure its stability and efficacy. Present functional groups and phytochemicals are the indicative of wide range of pharmacological activities. In-vitro DPPH scavenging activity suggests its anti-oxidant potency.

Conclusion

Present research is the exploration of a classical formulation. Developed pharmaceutico-analytical profiles of VG can be used as a preliminary data for its standardization. Phytochemical analysis and anti-oxidant activity suggest its use in various dermatological conditions. However, evaluation of its efficacy through preclinical and clinical studies are required to establish its potency. ❒

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