Formulation and *In-vitro* Evaluation for Sun Protection Factor of *Crinum asiaticum* Linn flower (Family-Amaryllidaceae) Extract Sunscreen Creams

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ABSTRACT

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Crinum asiaticum Linn. flowers commonly known as white lily flowers were explored to find flavonoids, so, thin layer chromatography was performed and some of the flavonoids were identified as flavon (Apigenine), flavonol (Quercetin), flavan-3-ols (Catechin), isoflavones (Daidzein). flavonol (Fisetin, Myricetin and Kaempferol). The reported flavonoids were known to have sunscreen activity and hence SPF factor of the dried flower extract (aqueous) was incorporated in topical formulation and evaluated in vitro with the help of Optometric Model-290 SPF. Extraction of flowers was done in two steps; maceration in MeOH: Water (9:1) followed by maceration in MeOH: Water (1:1) respectively. The two extracts were combined and methanol was then evaporated with the help of rotary evaporator, the resultant aqueous extract was used for Thin Layer Chromatography and then into topical formulation. In vitro determination of SPF was done with the help of method specified by The Comité de Liaison de la Parfumerie in Europe (COLIPA). The SPF was found to be 1.17 and Boots Star rating as 3, indicating that Crinum asiaticum Linn flower extract (aqueous) can be considered as good candidate for Sunscreen or as an additive in any other sunscreen formulation.

Keywords: Sun Protection Factor, in vitro method, flavonoids, Crinum asiaticum L. (Amaryllidaceae) flowers.

INTRODUCTION

As a barrier and immunological organ in the human, the skin especially epidermis, is particularly subjected to external effects. Light is the major environmental component to which skin is exposed daily and this light comprises of UV radiations which have been reported for damaging effects to the skin.[1] There are three types of UV rays; UV-A (320-400 nm), UV-B (280-320 nm), UV-C (200-280 nm).[2] Exposure to UV-A radiation results in damage to the elastic and collagenic fibers of connective tissue of skin, which causes premature ageing (photo-ageing), while UV-B radiation bring about acute inflammation (sun burn) and intensification of photo-ageing.[3] In addition to these changes, UV-B radiations are also reported to induce immune-suppression which reduces normal immunological defense mechanisms of the skin, therefore chances of development of malignant tumor increases.[1,3,4] The application of sunscreens is an efficient method of protecting skin against UV radiations. Thus, it has become a necessity to develop a validated topical sunscreen product which will provide protection against both UV-radiations and hence, topical formulations like sunscreen cream, lotion, spray, gel are prepared.

The efficacy of sunscreens is characterized by the sun protection factor (SPF). The SPF is a numerical rating system to indicate the degree of protection provided by a sun care products like sunscreen.[5] SPF is defined as the ratio of the minimal erythema dose (MED) of solar radiation measured in the presence and in the absence of a sunscreen agent.[6]

Regulatory agencies like the US-FDA and COLIPA (*The Comité de Liaison de la Parfumerie in Europe*) has made *in vivo* testing on human volunteers using an erythemal endpoint to determine the SPF of topical cream mandatory.[7] Although it is a recommended and recognized method by COLIPA, it has several disadvantages like being expensive, time-consuming and is potentially hazardous to human clinical subjects. Having said this, there are still many questions left unanswered about both the scientific accuracy and reproducibility of *in vivo* measurements of SPF, whereas, an *in vitro* measurement has the advantage of not exposing human subjects to harmful UV radiation, is cost-effective and provides us with statistically significant data which helps us to develop an effective sunscreen product. Thus, for economical, practical and ethical considerations a suitable method for *in vitro* determination of SPF is used more often.[8]

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Sunscreen creams incorporate a wide variety of chemicals like derivatives of 3-benzylidenecamphor, 4-aminobenzoic acid, cinnamic acid, salicylic acid, benzophenone and 2-phenylbenzimidazole, Avobenzone and Zinc oxide[1] which have particular absorbance and are effective over various areas of UV spectrum. In order to get a broad spectrum UV protection, more than one active sunscreen ingredients are added in the sunscreen product.[8] The EU has regularly listed 27 different organic and inorganic sunscreen ingredients since two decades, which are approved by Australian Government- Department of Health and Ageing, Therapeutic Goods Administration (TGA) for use in Australia whereas only 16 ingredients are listed in US-FDA monograph, out of which Avobenzone and Zinc oxide are used frequently since 1978.[9] The inorganic materials like Titanium dioxide incorporated in formulation as sunscreen reflect and scatter ultraviolet and visible radiation from a film of inert metal particle which forms an opaque barrier, they are photo stable, do not react with organic sunscreens and due to their light scattering properties there is less variability in the photo-protective effect of inorganic agents as compared to organic agents. However, inorganic sunscreens are cosmetically unacceptable because of their opaque quality and occlusiveness. The higher refractive index of Titanium dioxide explains its whiter appearance and thus lower cosmetic acceptability.[10] Also, these sunscreen ingredients have been increasingly reported for allergic and contact dermatitis, phototoxic and photo-allergic reactions, contact urticaria and even solitary cases of severe anaphylactic reactions. [11] Therefore, the researchers have turned their attention towards developing herbal sunscreen agents which are effective with less or no side effects.

Flavonoids are widely distributed plant pigments. They are water soluble and commonly occur in vacuoles, membrane-enclosed structures within cells. Chemists have identified more than 3,000 naturally occurring flavonoids. Flavonoids are placed into 12 different classes, the best known of which are the anthocyanins, flavonols, and flavones. All flavonoids have 15 carbon atoms and consist of two 6-carbon rings connected to one another by a carbon ring which contains an oxygen atom. Most naturally occurring flavonoids are bound to one or more sugar molecules. Small changes in a flavonoid's structure can cause large changes in its color.[12]

Flavonoids often occur in fruits, where they attract animals which eat the fruits and disperse the seeds. They also occur in flowers, where they attract insect pollinators. Many flavones and flavonols absorb radiation most strongly in the ultraviolet (UV) region and form special UV patterns on flowers which are visible to bees but not humans. Bees use these patterns, called nectar guides, to find the flower's nectar which they consume in recompense for pollinating the flower. UV-absorbing flavones and flavonols are also present in the leaves of many species, where they protect plants by screening out harmful ultraviolet radiation from the Sun.[13]

Literature survey has reported that flavonoids like flavonols (quercetin, rutin, kaempferol, myricetin, fisetin, vitexin)[14, 15, 16] flavones (apigenin, luteolin) flavanones (naringenin, hesperitn, naringin, and hesperidin), isoflavones (genistein)[17,1] and anthocyanins [18] have shown the potential as sunscreen agents. Flowers and fruits often consist of few or more flavonoids, therefore flowers of *Crinum asiaticum* L. (Amaryllidaceae) were considered as project topic wherein isolation of flavonoids and its characterization was done firstly by determination of total flavonoid content, then characterized by Thin Layer Chromatography technique and subsequently doing its SPF evaluation in a topical sunscreen cream.

Flowers of *Crinum asiaticum* L. (Amaryllidaceae) is commonly known as white lily flower and can be found in the local cultivars of Thane, Dahanu, Nashik and Konkan, it is originally found at the seashores.[19] Flowers are white in color, fragrant at night and have reddish anthers.[20] On drying, the flower color changes to yellow indicating that flavonoids might be present. Literature survey has reported alkaloids but very little investigation has been done in exploring other constituents, although one flavonoid has been reported in literature like 4′-hydroxy-7-methoxy flavan.[21] As not much of a work is reported in exploring flavonoids, thus, the study was aimed at isolating and identifying the flavonoids followed by developing a validated and effective topical dosage form.

MATERIALS AND METHODS

2.1 Plant material:

Flowers of *Crinum asiaticum* L. (Amaryllidaceae) was collected from Tapovan Nursery, Panchvati, and District-Nashik, Maharashtra state, India. It was authenticated taxonomically with the courtesy from Botanical Survey of India, Koregaon Park, Pune. The herbarium was deposited with Reference No. CRAEK4 on 2/2011.

2.2 Extraction of dried flowers of *Crinum asiaticum* Linn (Family-Amaryllidaceae):

Dried flowers were ground and extracted in two steps, firstly with MeOH: H_20 (9:1) and secondly with MeOH: H_20 (1:1). At each step, sufficient solvent was added to make slurry and the mixture was left for 6-12 hours, followed by vacuum filtration with Whatmann No.1.The two extracts were then combined and rotary evaporated (Equitron Rotary Evaporator, Medica Instrument Mfg, Co, Mumbai) to 10ml its volume at 40°Cunder vacuum. The resultant aqueous extract was cleared of low polarity contaminants by extraction with chloroform several times. The solvent-extracted aqueous

layer, containing the bulk of flavonoids, was then evaporated to dryness.[22]

The UV spectra of the naturally occurring flavonoids, like flavones and flavonols have been determined as a function of concentration in aqueous solutions. These spectra indicate that the extent of keto–enol phototautomerism in both flavonoids is greatest at high concentrations: a situation which favours molecules aggregation/dimerization. Such behaviors is consistent with phototautomerism being facilitated by a concerted, intermolecular transfer of protons between the partners in the flavonoid dimer. This excited state tautomerism dissipates absorbed energy harmlessly and as such provides a possible mechanism by which these molecules may function in the protection of plants from damaging UV radiation. Along with UV spectroscopy the fluorescence excitation spectra of both flavones and flavonols at high concentrations in aqueous solutions indicate the presence of significant amounts of the enolic tautomeric form in the ground-state. The absorption of the enolic tautomer is at longer wavelengths (510 nm) than that of the keto tautomer (440 nm) and as it extends into the blue spectral region, would account for the yellow appearance of these flavonols in flower petal extracts. Therefore, the wavelengths for flavonoid and flavonol content determination are different.[23]

2.3. Determination of total flavonoid content: [24]

Aluminium chloride colorimetric method was used for flavonoids determination. 1ml of aqueous extract of flowers of *Crinum asiaticum* L. (Amaryllidaceae) or 500*µ*g/ml standard solution of rutin (provided by Yucca enterprises, Wadala, Mumbai) was added to 10ml volumetric flask containing 4ml of water. To the above mixture, 0.3ml of 5% $NaNO_2$ was added. After 5min, 0.3ml 10% $AlCl_3$ was added. At 6th min, 2ml 1M NaOH was added and the total volume was made up to 10ml with water. The solution was mixed well and the absorbance was measured against reagent blank at 510nm. Total flavonoid content of aqueous extract was expressed as mg rutin/g of extract.

Formula:

$$X = A.\ mo.10/A_0.m$$

Where,

X=flavonoid content, mg/g of plant extract,

A=the absorption of plant extract solution,

A_0=the absorption of standard rutin solution,

m=the weight of plant extract in g

mo=the weight of rutin in solution in mg

The total flavonoid content was found to be 0.375 mg/g. (Table1)

Table 1: Observations for Aluminium chloride assay of white lily flowers

Sr. No.	Solution	Concentration (µg/ml)	Absorbance at 510nm
1.	Standard (Rutin)	500	0.2027
2.	*Crinum asiaticum L.* flower extract	1000	0.1405

2.4 Determination of total flavonol content:

The content of flavonol was determined by Yermakov, *et al* (1987). The rutin calibration curve was prepared by mixing 2ml of 0.5, 0.4, 0.3, 0.2, 0.116, 0.1, 0.05, 0.025 and 0.0166mg/ml rutin ethanolic solution with 2ml (2%w/w) aluminum trichloride and 6ml (5%w/w) sodium acetate. The absorption at 440nm was read at 2.5hr at 20°C. The sample procedure was carried out with 2ml of plant extract (1%w/w) instead of rutin solutions. The absorbances of standard rutin and aqueous extract of flowers of *Crinum asiaticum* L. (Amaryllidaceae) are mentioned in Table 2

The content of flavonols, in rutin equivalents (RE) was calculated by the following formula:

$$X = C.V/M$$

Where,

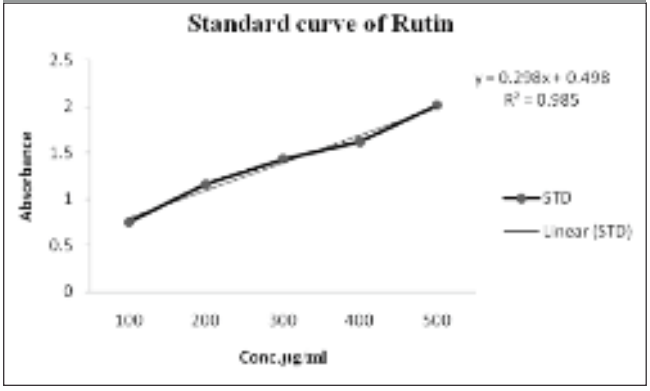
X= flavonol contents, mg/g plant extract in rutin extract

Table 2: Absorbance of standard Rutin for flavonol content determination

Conc. µg/ml	Absorbance
100	0.750
200	1.156
300	1.432
400	1.618
500	2.010
CAAE	0.6872

*CAAE: Crinum asiaticum aqueous extract

Fig.1: Standard curve of Rutin



C= the concentration of rutin solution established from calibration curve, mg/ml

V, M= volume and weight of plant extract ml, g

The flavonol content was found to be 0.33mg/g.

2.5 Thin Layer Chromatography:[22]

The fact that a spot is visible at all under conditions is a likely indication that it represents phenolic compound. Flavonoids often account for the majority of visible (in UV) spots although blue fluorescent, pink, whitish, orange and brownish spots must be considered as unlikely to represent flavonoids until further investigated (by UV-vis spectroscopy). The typical flavone and flavonol glycoside spot will appear dark purple in the UV. TLC was performed in five different mobile phases like Butanol: Acetic acid: Water (BAW 4:1:5), Forestal (Acetic acid: Water: Hydrochloric acid - 30:10:3), Chloroform: Acetic acid: Water (CAW 3:1.5:0.2), Ethyl acetate: Pyridine: Acetic acid: Water (EPAW 36:36:7:2), Butanol: Benzene: Pyridine: Water (BuBzPW 5:1:3:3). (Table 3) Each of these mobile phases showed dark purple spots under UV at 366nm, the interpretations of probable flavonoid type are as follows; Apigenin, Quercetin, Catechin, Epicatechin, Fisetin, Myricetin, Kaempferol and Daidzein, Pinocembrin and Anthocyanins.

2.6 Formulation of Sunscreen cream:

The Sunscreen cream was prepared by following procedure, the formulation of the cream is specified in (Table 4).

Table 3: TLC Profile of white lily flowers ' extract

Sr. no.	Solvent system	No. of Spots	Detection	Rf value	Compounds
1	BAW (4:1:5)	5	UV-light	0.90, 0.79, 0.69, 0.60, 0.44	Glycosides, aglycones, sugars, glucose and galactose not separated
2	Forestal (HOAc:H_2 0:HCl) (30:10:3)	1	UV-light	0.84	Flavones, Flavonol, Anthocyanin aglycones
3	CAW (3:1.5:0.2)	4	UV-light	0.93, 0.88, 0.63, 0.54	Iso- rhamnetin, kaempferol, syringitin-laricitrin mixtures
4	EPAW (36:36:7:2)	3	UV-light	0.93, 0.89, 0.45	Sugars
5	BBzPW (5:1:3:3)	1	UV-light	0.82	Anthocyanin

*B-n-butanol, A-acetic acid, W-water, OHAc-Acetic acid, H 2 0-water, HCl-hydrochloric acid (concentrated), C-Chloroform; E-ethyl acetate, P-pyridine, Bz- Benzene.

Table 4: Formulation of white lily flowers' extract sunscreen cream

Sr. No.	Ingredients	Components
1.	Cetostearyl alcohol	5 %w/w
2.	Stearic acid	4 %w/w
3.	Petroleum Jelly	1 %w/w
4.	Glycerin	5 %w/w
5.	Potassium hydroxide	1 %w/w
6.	Water	85 %w/w
7.	Methyl paraben sodium	0.20 %w/w
8.	Propyl paraben sodium	0.05 %w/w
9.	Aqueous extract of white lily flower	2 %w/w

Step 1- Aqueous phase preparation: Potassium hydroxide (1%w/w) was dissolved in deionised water (85%w/w), followed by addition of glycerin (5%w/w), sodium methyl paraben (0.2%w/w) and aqueous extract of dried lily flowers (1%w/w). The resulting mixture was then heated up to 80°C.

Step 2- Oil phase preparation: Sodium propyl paraben (0.05%w/w), stearic acid (4%w/w), cetostearyl alcohol (5%w/w) petroleum jelly (1%w/w) was added and heated at 80°C.

Step 3- Mixing phase: Oil phase was added to aqueous phase at 80°C with continuous stirring for 20-25 mins and then it was homogenized at 8000 rpm till uniform emulsion was obtained. The emulsion was then poured into wide mouthed container and stored at temperature not exceeding 37°C.

2.7 Determination of physical parameters of cream:

Preparation of herbal cream has always been a challenging task and the cream is accepted only if it is tested appropriately for various physical parameters like ease of spreadability, appearance, pH, viscosity and pleasant feeling as specified in (Table 5)

Table 5: Physical Parameters of white lily flowers' extract sunscreen cream

Sr. No.	Parameters	Observations
1.	Color	Brown
2.	Odor	Aromatic
3.	Spreadability	Good and uniform
4.	pH	6.7
5.	Specific gravity	0.93
6.	Viscosity	1690 cp

2.8 Determination of *in vitro* SPF:

This study was performed by Transmittance measurement of the flowers of *Crinum asiaticum* L. (Amaryllidaceae) cream. The Optometrics Model SPF-290 Analyzer measures the sun protection factor of the cream over a wavelength range from 290nm-400nm. Approximately 110mg of sample was applied and spread on 56cm^2 area of Transpore tape to obtain a sample film thickness of 2μl/cm^2 (to get an even film) as suggested in the operational manual of Optometrics LLC for the sample application technique. The samples thus prepared were exposed to Xenon arc lamp for determining the SPF and Boots Star Rating.

WIN SPF has used the following equation for calculating SPF value.

$$SPF_{scan} = \frac{\sum_{290}^{400} E\lambda\, B\lambda}{\sum_{290}^{400} \frac{E\lambda\, B\lambda}{MPF\lambda}}$$

Where,

MPFλ= scan MPF value

Eλ = spectral irradiance of terrestrial sunlight under controlled conditions

Bλ= erythemal effectiveness

RESULT

The aqueous extract of dried flowers of *Crinum asiaticum* Linn. was subjected to Thin Layer Chromatography using different solvents like Butanol: Acetic acid: Water (4:1:5), Forestal (Acetic acid: Water: Hydrochloric acid - 30:10:3), Chloroform: Acetic acid: Water (3:1.5:0.2), Ethyl acetate: Pyridine: Acetic acid: Water (36:36:7:2), Butanol: Benzene: Pyridine: Water (5:1:3:3). Different number of spots were obtained in different solvents like 5, 1, 4, 3, 1 respectively (Table No.3), all of them appeared as Dark Purple and fluorescent yellow color spots under UV light at 366nm. The compounds were quercetin, kaempferol, fisetin, myricetin, pinocembrin and anthocyanins (yellow color spot). Some of these compounds have reported to have sunscreen activity, therefore, topical formulation was made and evaluated for *in vitro* SPF determination using Optometrics – 290 SPF model.

The topical cream prepared was brown in color, showed good and uniform spreadability, pH was found to be 6, viscosity and specific gravity was found to be 1690cp and 0.93 respectively (Table No.5), the parameters of cream complies with official acceptance criteria. SPF of this cream is found to be 1.17±0.02 with Boots Star Rating 3 at critical wavelength of 375.1 nm, generally, if the Boots Star rating is more than 2 and when critical wavelength is more than 375 nm (Table No.6), then, the product developed provides good UV-A and UV-B protection, hence, flowers of *Crinum asiaticum* L. (Amaryllidaceae) topical cream can be considered as good candidate for sunscreen cream or as an additive to any other sunscreen formulation.

Table 6: Results of SPF and other Parameters of white lily flowers' extract sunscreen cream

Sr.no	Parameter	Scan I	Scan II	Scan III	Average
1	SPF	1.24	1.15	1.13	1.17
2	Standard deviation	0.03	0.01	0.02	0.02
3	UVA/UVB ratio	0.499	0.625	0.635	0.586
4	Critical wavelength	373.8	376.2	375.4	375.1
5	Boots star rating	2	3	3	3

Fig. 2: SPF-290 Graph Report of white lily flowers' extract Sunscreen Cream (Scan 1)

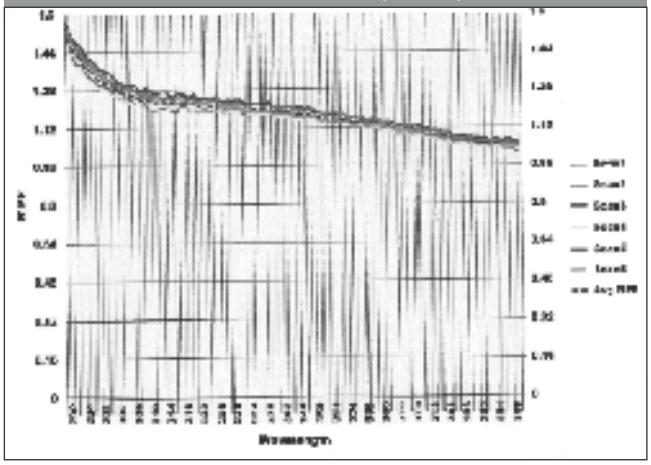
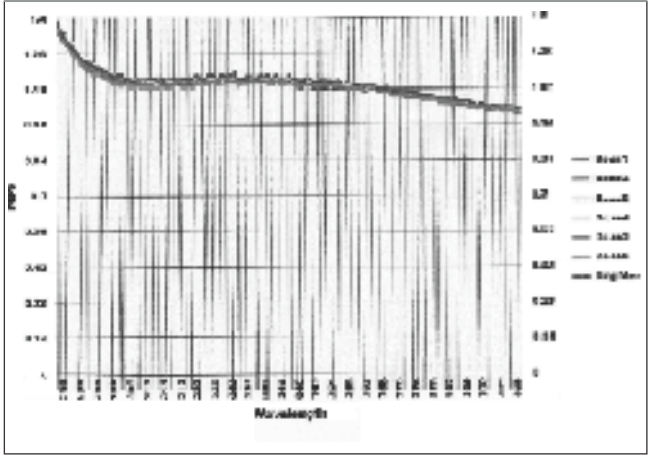


Fig. 3: SPF-290 Graph Report of white lily flowers' Sunscreen Cream (Scan 2)

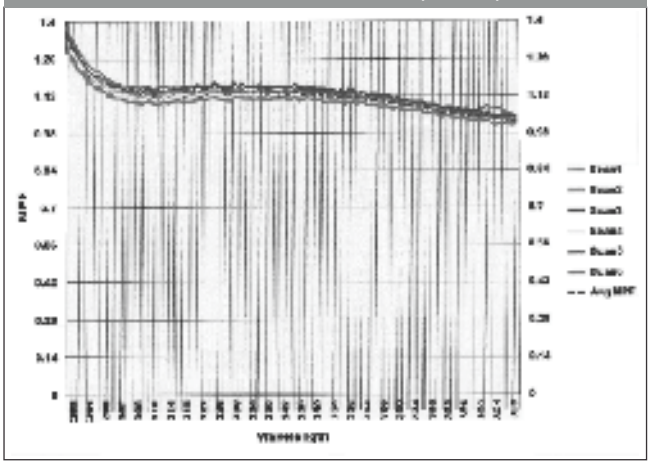


DISCUSSION

The Optometrics Model SPF-290 Analyzer is a computer controlled instrument that is designed to measure the sun protection factor of sunscreen preparations. For US-FDA standards the protection factor is calculated over the wavelength range from 290-400nm. To initiate an analysis a reference scan was done with the blank substrate (which consists of data from 23 wavelengths) in the incident beam. The sample was then applied to the substrate and the first sample scan was made. Data was collected in the same manner as the reference data, ratioed to the reference and plotted as a MPF (Monochromatic protection factor). Ratioing the sample signal to the reference signal negates any effect of wavelength dependent variables in the optical system (source, monochromator and detector). Up to 6 sample scans were made to compensate for variables in the substrate and sample application.

The SPF-290 software uses Trapezoidal Approx calculation technique to approximate the integral for SPF and Erythemal UVA protection factor. These include UVA/UVB ratio, critical wavelength and cumulative absorbance. The Average Absorbance method is used for calculating average protection factor; this method averages and computes the standard deviation based on the absorbance scan data. This method of calculation gives a better average value assuming that sample thickness is the largest variable in performing a protection factor measurement.

Fig. 4: SPF-290 Graph Report of white lily flowers' extract Sunscreen Cream (Scan 3)



For the calculation of standard deviation, Diffey's method is used, based on B. L. Diffey's paper [25] on using Transpore Tape® as the substrate for SPF measurements. Diffey's equation applies weighing by recognizing that the MPF measurements for a set of scans have some distribution. Therefore, the standard deviations of the MPF measurements at each wavelength are factored in to the Diffey SPF standard deviation calculation.

Physical Parameters include color, pH, odor, spreadability, specific gravity and viscosity. The color of cream was found to be brown and odor was aromatic, this was done by sensory evaluation, while pH was determined with the help of electronic pH meter by preparing buffer solutions of 4 and 7 pH, calibrating the instrument and followed by measurement of pH, which was found to be 6.7. Specific gravity was determined on the basis of procedure specified in USP, wherein a tared and dry pycnometer was filled first with water boiled till 25°C and calibrated, and then the sample cream was boiled till 25°C and filled in pycnometer. The weight was done only after the temperature was equal to that of balance and then weighed. The tared weight was then subtracted from the filled weight and specific gravity was measured, which was found to be 0.93. The viscosity of White lily cream was determined as per the procedure mentioned in the manual of Brookfield viscometer. The software of Brookfield viscometer (Model no. LV-II + Pro LV) was switched on and

the programming of parameters was done. The selection of spindle number (spindle No. LV-IV) for determination of viscosity was done on trial and error basis. The behavior of viscosity of the dosage form was studied at variable RPM (Shear rate) at constant temperature. The viscosity was therefore found to be 1690cp.[26]

CONCLUSION

The described *in vitro* method, though, presents some limits; it has spared the exposure of human subjects to harmful ultraviolet radiations that can pose potential health risks and ethical issues connected with it hence, it is still preferred and is undoubtedly beneficial as it gives accurate and reproducible results. This method has helped to determine the SPF value of herbal alternatives like flowers of *Crinum asiaticum* L. (Amaryllidaceae) and stating that it has good sunscreen activity and can be considered as active sunscreen agent or can be incorporated into other sunscreen formulations as an additive to enhance the activity.

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