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Research Article

FORMULATION AND *IN VITRO* EVALUATION OF ANTIOXIDANT ACTIVITY OF HERBAL SUNSCREEN FORMULATION

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ABSTRACT

UV radiations reaching to the surface of the earth are responsible to cause skin damage and condition get worse on long exposure. The chemical and physical sunblock agents are not enough to protect skin, whereas natural products are emerging on large scale for their health benefits. The present investigation is an attempt to formulate herbal sunscreen creams and their evaluation for total phenolic, flavonoid content and free radical scavenging property. Flowers of *Butea monosperma*, leaves of *Neolamarckia cadamba*, peel of *Punica granatum* and leaves of *Cymbopogon citratus* were extracted with methanol. Total five creams with concentration of 2% w/w were formulated in different combinations using 1:1 proportion of extracts. The total phenolic, flavonoid content and antioxidant potential was determined. Diphenyl picrylhydrazyl (DPPH), nitric oxide (NO) radical scavenging assays and reducing power assay were studied by using ascorbic acid as reference standard. The total phenolic and flavonoid content of F-5 was higher and found to be 38.76±0.59 mg GAE/g and 47.89±0.36 mg QE/g respectively. F-1 to F-5 creams exhibited antioxidant potential in concentration dependant manner. Cream F-5 shown better antioxidant property with IC50 value 22.97 µg/ml and 51.49 µg/ml for DPPH and NO respectively and increased reducing ability with higher absorbance. The mixture of all extracts indicating synergetic property of polyphenolic phytoconstituents present in F-5. Further the formulation is required to study for its *in vitro* and *in vivo* sun protective property.

Keywords: Antioxidant, sun protective, phenolic compounds, flavonoids and UV radiations

INTRODUCTION

Skin is the largest living organ that having direct exposure to the environment and protects our body from harmful microbes, chemicals, helps to regulate body temperature, fluid balance and offering protection against sunlight[1,2]. Current research specifies that exposure of UV rays or solar radiation damages the skin in different ways. UV-C radiations get filtered through atmospheric ozone layer known as stratospheric layer and not associated with the harmful effect on the skin. UV-B radiations known as burning rays as they are one thousand times more capable of causing sunburn; DNA absorbs it and initiates carcinogenic processes. UV-B rays causes protein damage, oxidative deterioration of lipids and skin lesions. UV-A radiation produces immediate tanning effect and darkening of melanin in the epidermis. It causes premature photoaging, suppression of immunological functions and necrosis of endothelial cells[3]. Ultraviolet radiation increases oxidative stress in skin and generates reactive oxygen species (ROS), chief factor to initiate cancer and deleterious effects to the skin. Long exposure of UV radiation increases the risk of basal cell carcinoma, squamous cell carcinoma as well as malignant melanoma. UVR also causes phototoxic or photo allergic reactions, autoimmune diseases including lupus erythematosus and idiopathic photo dermatosis [4]. Reactive oxygen species called as free radicals, degrade unsaturated lipids and form malondialdehyde (MDA) which acts as a marker enzyme of lipid peroxidation. In addition, affects the level of non-enzymatic antioxidant includes reduced glutathione (GSH), ascorbic acid level (ASC), total protein level (TP) and antioxidant enzymes as superoxide dismutase (SOD) and catalases (CAT) from the skin tissue. Free radicals generated due to UV radiation also causes significant structural variations like erosion of epidermis, altered thickness of epidermis and dermis, structure of connective tissue, elastin fibres, collagen fibres and oedema [5]. The chemical agents block UV radiations more actively in the UV- B region as compare to the UV-A region while the physical sunscreen reflects the harmful rays away from the skin temporarily. The mostly used sunblock chemical agent is avobenzone and the combination of chemical and physical active ingredients gives better sun protection. However, in the USA combinations of chemical and physical sunscreen agents are not permitted. Avobenzone has been reported to be unstable when contained in formulations with physical sunscreens[6]. Titanium dioxide is stable, provides broad-spectrum protection against solar radiation and not causing any photo-allergy, contact dermatitis or any skin irritation. However, their scattering property causes whitening effect which is not recommended for children and individuals with sensitive skin[7].

Hence, there is a need to search for an alternative sources of effective and safer sun protective agents that can be utilized in sunscreen products as well as in cosmetic preparations. In general, whole plant extracts have shown better potential as photoprotective agents due to their complex chemical composition and broad UV absorption spectra as well as their antioxidant power. Although they have not completely replaced the dominance of synthetic materials, the use of these botanical extracts is becoming more common. Green tea and black tea have been reported to ameliorate adverse skin reactions following UV exposure, while *Aloe vera* gel assists in cell regeneration[8-10].

To avoid the side effects of chemical and physical sunscreen ingredients, naturally occurring compounds are gaining significant attention as skin protective agents. Natural compounds act as catalysts in the light phase of photosynthesis and protects plant cells from reactive oxygen species (ROS) especially the antioxidants like vitamin C, vitamin E, flavonoids, carotenoids and phenolic acids. They fight against free radical causes numerous negative skin changes[11]. Phytoconstituents consist of phenolic acid, flavonoids and high molecular weight polyphenols playing a significant role in skin protection by different mechanisms [12].

The flowers of *Butea monosperma,* leaves of *Neolamarckia cadamba*, peel of *Punica granatum* and leaves of *Cymbopogon citratus*, are studied for their different therapeutic activity, qualitative and quantitative phytochemical analysis. They all as an individual or with other plant extracts are proven for their pharmacological effects. *Butea monosperma* and *Punica granatum* are studied for sun protection activity, which can be used as base to form a formulation for synergetic effect. Each selected plant has proven for antioxidant potential, which provided a good platform to study their phenolic content, flavonoid content and antioxidant potential.

The cosmetics are available in different forms or external applications like lotions, gels, creams, powders etc. The creams are more popular among the consumers and they are looking for herbal remedies. The methanolic extract of *Butea monosperma* is having anti-inflammatory, antioxidant activity and wound healing property[13]. Ethyl acetate and butanol fractions of *Butea monosperma* flowers were studied for free radical scavenging activity[14] and also evaluated for its sunscreen activity by absorption spectroscopy and transmission spectroscopy methods[15]. Ethanolic extract of *Neolamarckia cadamba* was studied for total phenolic content and antioxidant assay[16], methanolic extract of cadama plant shown better DPPH radical scavenging activity[17]. Traditionally *cadamba* leaves were used to cure pimples and wounds, its different extracts are reported for their antimicrobial, anti-inflammatory properties[18], *Punica granatum* fruit and peel extracts were reported for their antioxidant and anti-inflammatory property. It is effective in the prevention and treatment of cancer and other chronic and infectious diseases and helps in regeneration of dermis layer of skin [19]. The lemon grass leaves and oil are used for their antioxidant and antimicrobial activity and highly recommended in cosmetic industries for the skin care products[20].

On the basis of literature, plant extracts were selected and considered to incorporate them in the development of sunscreen creams.

MATERIAL AND METHODS

Chemicals and Reagents

The chemicals used were of analytical grade; 1, 1-diphenyl-2-picrylhydrazyl, Ascorbic Acid, quercetin, gallic acid, Folin Ciocalteu's phenol reagent, Sodium nitroprusside dihydrate, Sulphanilamide, aluminium chloride, sodium chloride, Sodium carbonate, phosphate buffer, Griess Reagent, potassium ferricyanide Ferric chloride, Trichloroacetic acid, Cetomacrogol 1000, Cetostearyl Alcohol, Methylparaben, Propylparaben, Light Liquid Paraffin, White Soft Paraffin, Propylene Glycol, Chlorocresol, Sod. Dihydrogen Phosphate Dihydrate and methanol.

Collection and Authentication of Plants

The flowers of *Butea monosperma* (Lam.) and fresh leaves of *Neolamarckia cadamba* (Roxb.) were collected from Ahmedabad, Gujarat. The fruit pericarp that is peel of *Punica granatum (Linn)* and the fresh leaves of *Cymbopogon citratus*, (Stapf) were collected from the medicinal plant Garden of Alard College of Pharmacy, Pune. The selected parts of the plants were dried under shade at room temperature and herbarium specimen were prepared and authenticated at Botanical Survey of India, Western Regional Centre, Pune.

The identified plants and their Specimen Voucher No. are *Butea monosperma* (Lam.) belonging to family Fabaceae BSI/WRC/IDEN.CER./2016/664, *Neolamarckia cadamba* (Roxb.) family Rubiaceae, *B*SI/WRC/IDEN.CER. /2016/666, *Punica granatum* (Linn), family Punicaceae, BSI/WRC/IDEN.CER. /2016/665, *Cymbopogon citratus* (Stapf) family Gramineae BSI/WRC/IDEN.CER. /2016/662.

Extraction of Plant Material

Coarse powders were passed through a 40-mesh sieve; 100 gm of each powder was refluxed for 2 hours using 250 ml of petroleum ether (60-80^{0}C) to remove non-polar compounds. The marc left after was dried and extracted with 250 ml of methanol for 36 hours by continuous hot extraction method in Soxhlet apparatus[21]. The extracts were concentrated under reduced pressure and at the temperature of 40°C using rotary evaporator[22-23]. The concentrated extracts were cooled and finally placed in the desiccators to remove the traces of solvent left over. The percentage of yield obtained were calculated and recorded. The extracts were named as BM for methanolic extract of *Butea monosperma,* NC for methanolic extract *Neolamarckia cadamba*, PG for methanolic extract *Punica granatum* and CC for methanolic extract *Cymbopogon citratus.*

Formulation of O/W Sunscreen Creams

Before formulating the sunscreen, the cream base was prepared by optimizing the proportion of all ingredients. More than 10 cosmetic bases were developed by varying the proportion of all the excipients and the optimized cream base formula was tested for preliminary stability and used further for incorporation of all methanolic extracts [24]. Five O/W emulsion containing 1:1 proportion of different extracts mixture were used to develop cream formulation[25,26]. Then the required quantity of distilled water (80%) was weighed and heated at 70°C. Chlorocresol and sodium dihydrogen phosphate dihydrate were added and stirred on an electric water bath to make aqueous phase. Light liquid paraffin and white soft paraffin were added to another beaker with continuous heating and stirring added with cetomacrogol 1000 and cetostearyl alcohol simultaneously at 70°C with constant stirring. Oil Phase contents were added to the aqueous phase with using high speed with homogenizer for 10 min. and the mixture of extracts was added to propylene glycol at 70ºC to make a clear solution. This solution was then added to the mixing phase and quantity was adjusted with remaining water (20%) and perfume is added to the final preparation.

Table 1: Proportion of extracts in Creams

Sr. No	Extracts (1:1)	Cream (2 % w/w)
1	PG+BM+NC	F-1
2	NC+CC+PG	F-2
3	BM+ NC +CC	F-3
4	CC+ PG+BM	F-4
5	BM+NC+PG+CC	F-5

Table 2: Composition of Herbal Sunscreen Creams

Sr. No	Ingredients	Quantity(gm)	Uses
1	Methanolic Extracts	2gm	Active Ingredients
2	Cetomacrogol 1000	3 gm	Emulsifier
3	Cetostearyl Alcohol	7 gm	Emulsifier
4	Methylparaben	0.15 gm	Preservatives
5	Propylparaben	0.5 gm	Preservatives
6	Light Liquid Paraffin	6 gm	Emollient
7	White Soft Paraffin	14 gm	Emollient
8	Propylene Glycol	6 gm	Humectant
9	Chlorocresol	0.038 gm	Preservatives
10	Sod. Dihydrogen Phosphate Dihydrate	0.0252 gm	Buffer
11	Purified Water	q.s	Vehicle

Determination of Total Phenolic Content

The Folin-Ciocalteu reagent (FCR) or Gallic Acid Equivalence method (GAE) was used to determine phenolic and polyphenolic antioxidants present in the formulations. It measures the amount of the substance needed to inhibit the oxidation of the reagent. Cream formulations were extracted with methanol and concentration of 1 mg/mL was prepared. 0.5 mL of all test samples was mixed with 2.5 mL of Folin-Ciocalteu reagent (10%) and 2.5 mL Sodium bicarbonate (7.5%). Blank solution was prepared by adding 0.5 mL of methanol, 2.5 mL of Folin-Ciocalteu reagent (10%) and 2.5 mL Sodium Bicarbonate (7.5%). All resulting solutions were subjected to incubation at 45°C for 45 min. Blue colour was developed and the absorbance was measured at 765 nm spectrophotometrically [27]. The standard calibration graph was plotted for Gallic acid solution (10-80µg/ mL). Phenolics content of cream formulations were expressed in mg of GAE/g of extract.

Determination of Total Flavonoid Content

The aluminium chloride colorimetric method used to determine total flavonoid content. Cream formulations were extracted with methanol and concentration of 1 mg/ mL was prepared. 50 µL of test samples were diluted up to 1 mL using methanol. Resulting solutions were mixed with 4 mL of distilled water and stood for 5 min. Resulting mixtures were then added with 0.3 mL of $AlCl_3.6H_2O$ (10%) solution followed by 2 mL of NaOH (1.0 M) for the next 5 min. Final volume of mixtures was made up to 10 mL with distilled water and kept aside for 15 min. Distilled water was kept as blank against test. The absorbance's were measured spectrophotometrically at 510 nm[28]. Standard calibration graph was plotted for quercetin solution (10-80 µg/ mL). Flavonoid content of herbal cream was expressed in mg of QE/g of extract.

***In Vitro* Antioxidant Activity of Sunscreen Creams**

DPPH Free Radical Scavenging Activity

Test samples of creams and standard solutions (10-80 µg/ mL) were prepared in methanol. 3mL of each test sample extracts mixed with 5mL DPPH solution and the resulting mixture was shaken properly and allowed to stand for 30 mins at 37^{0}C. A blank test solution was prepared in a similar way omitting cream extract. Absorbance of all samples was measured at spectrophotometrically at 517 nm [29]. The assay was carried out in triplicates and % inhibition was calculated using the formula,

$$\% \text{ Inhibition} = \{(Abs_{std} - Abs_{test})\} / (Abs_{std}) \times 100$$

Where, Abs – The absorbance of test and standard solution

Nitric Oxide Free Radical Scavenging Activity

Test sample of creams and standard solutions (10-80 µg/ mL) were prepared in methanol. 3 mL each test sample extracts were added to Sodium nitroprusside (5 mM) in phosphate– buffered saline (PBS) and shaken. Mixtures were incubated at 25°C for 150 min. Then resulting mixtures then allowed reacting with 1 mL of Griess reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% napthylethylenediamine dihydrochloride). The absorbances of mixtures were measured spectrophotometrically at 546 nm[30] and blank was prepared same way except extract. The assay was carried out in triplicates and % inhibition was calculated using the formula,

$$\% \text{ Inhibition} = \{(Abs_{std} - Abs_{test})\} / (Abs_{std}) \times 100$$

Where, A – The absorbance of test and standard solution

Reducing Power Assay

Test samples of cream and standard solutions (10-80 µg/ mL) were prepared in methanol. 2.5 mL of test samples of extracts was added to 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide (1% w/v) and mixed well. These mixtures were incubated at 50 ^{0}C for 20 min. and cooled. 2.5 mL of Trichloroacetic acid (10% w/v) added to the mixture. Resulting mixtures were subjected to centrifugation at 3000 rpm for 10 min. The mixture was allowed to stand for some time and 2.5 mL of supernatant liquid was withdrawn and mixed with 2.5 mL of distilled water and 0.5 mL of ferric chloride (0.1 % w/v) solution. The blank was prepared same way except the extract. Absorbances were measured spectrophotometrically at 700 nm [31]. Absorbances measured in triplicate and calculated as Mean ± SD.

RESULTS AND DISCUSSION

Extraction of Plant Material

After completion of extraction, extracts were collected, subjected to the rotatory evaporator for removal of methanol. Percentage of

yield was calculated and found to be 4.54 % w/v, 3.79 % w/v, 11.35 % w/v, 3.26 % w/v for BM, NC, PG and CC extract respectively.

Table 3: Percentage (% W/V) Yield of Plant material

Sr. No	Plant	Plant Part	% Yield (W/V)
1	*Butea monosperma*	Flowers	4.54
2	*Neolamarckia cadamba*	Leaves	3.79
3	*Punica granatum*	Peel	11.35
4	*Cymbopogon citratus*	Leaves	3.26

Evaluation of O/W Sunscreen Creams

The optimized herbal creams with different compositions were prepared and further investigated for the determination of Total phenolic, Total flavonoid content and free radical scavenging property.

Determination of Total Phenolic Content

Phenolic compounds are primary antioxidants of natural products composed of phenolic acids and flavonoids, the hydrogen donor, having a good correlation with the antioxidant activity [32]. It was calculated in terms of gallic acid equivalents (mg of GA/g) of formulations by using the standard curve. The equation was y = 0.0022x + 0.2217 and R = 0.9966. The Total phenolic content of the formulations was found to be 37.03 ± 3.73, 27.95 ± 3.82, 31.31 ± 2.62, 33.71 ± 2.06 and 38.76 ± 0.59 mg GAE/g for F-1, F-2, F-3, F-4 and F-5 respectively. F-5 shown the total phenolic content slightly higher compared to F-1 to F-4.

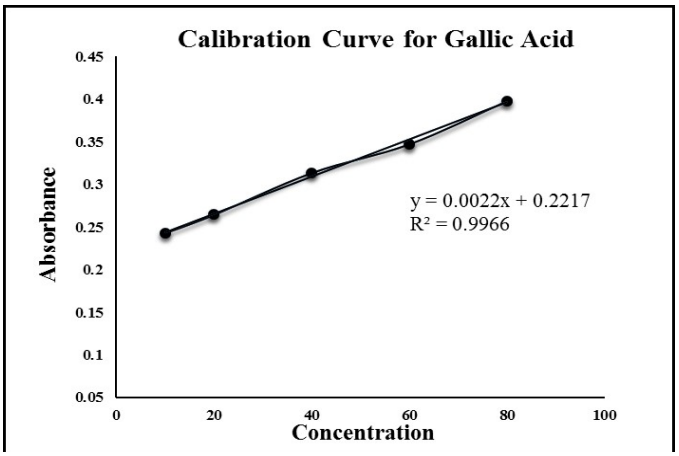

Figure 1: Calibration Curve for Gallic Acid

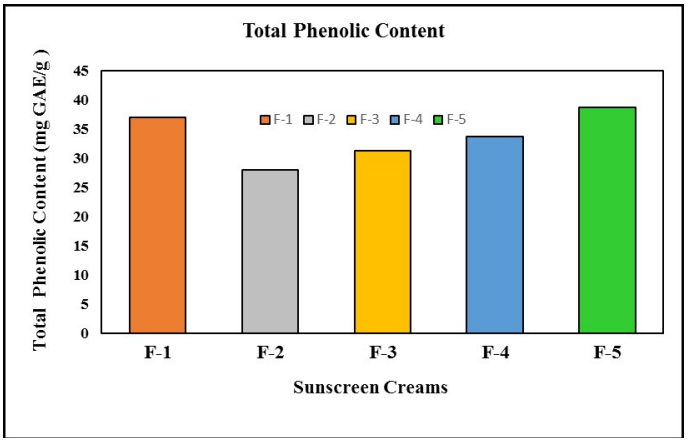

Figure 2: Graph of Total Phenolic Content of Sunscreen Creams

Determination of Total Flavonoid Content

Flavonoids are having hydrogen donating ability and chelator of divalent cations. Flavonoids are having their radical scavenging property[33]. The calibration core was plotted and content total flavonoid content was determined from the calibration curve and expressed as milligram of quercetin equivalent (mg of QE/g of extract). The equation is y = 00026x + .0.1800 and R^2 = 0.9936. The total flavonoid content of the formulations was found to be 42.07 ± 0.09, 41.31 ± 0.58, 38.04 ± 0.62, 46.16 ± 0.24 and 47.89

± 0.36 for F-1, F-2, F-3, F-4 and F- 5. F-5 is having higher flavonoid content compared to F-1 to F-4.

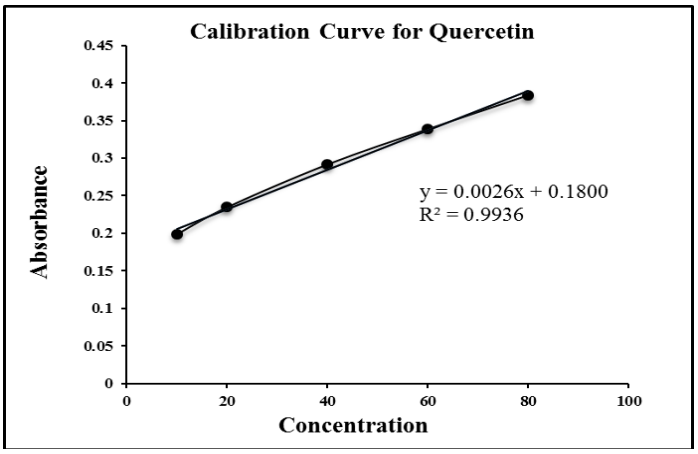

Figure 3: Calibration Curve for Quercetin

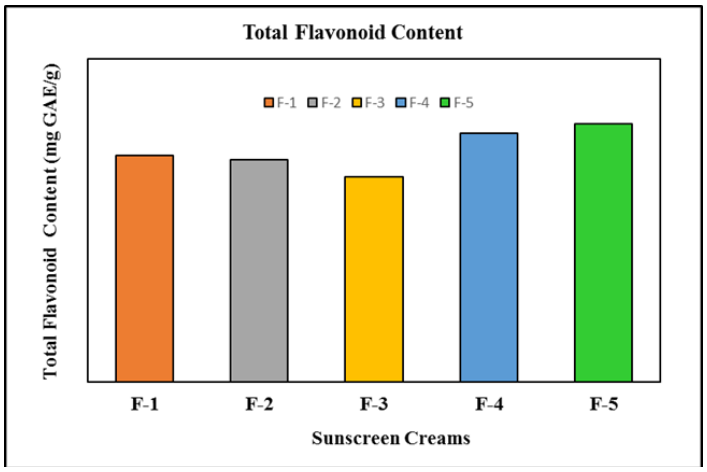

Figure 4: Graph of Total Flavonoid Content of Sunscreen Creams

Table 4: Total Phenolic and Flavonoid Content

Sr. No	Sunscreen Creams	Total Phenolic Content (mg GAE/g)	Total Flavonoid Content (mg QE/g)
1	F-1	37.03±3.73	42.07±0.09
2	F-2	27.95±3.82	41.31±0.58
3	F-3	31.31±2.62	38.04±0.62
4	F-4	33.71±2.06	46.16±0.24
5	F-5	38.76±0.59	47.89±0.36

Determination of *In Vitro* Antioxidant Activity of Creams

DPPH Free Radical Scavenging Activity

The % inhibition found was 74.70 % for Ascorbic acid, 64.10 % for Cream F-1, 58.29 % for cream F-2, 50.77 % for F-3, 64.62 % for F-4 and 66.32 % for F-5. There was a dose–dependent increase in antioxidant activity for all concentrations. The higher % inhibition was found in F-5 and lower was in F-3. The IC_{50} values obtained were 10.73 µg/ml, 40.73 µg/ml, 53.61 µg/ml, 74.73 µg/ml, 31.01 µg/ml, 22.97 µg/ml for Ascorbic acid, F1, F2, F3, F4 and F5 respectively.

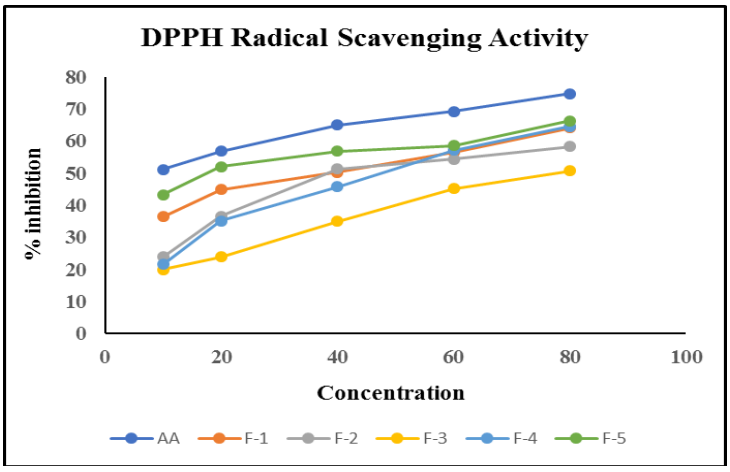

Figure 5: Graph of DPPH Free Radical Scavenging Activity of Sunscreen Creams

Nitric Oxide Radical Scavenging Activity

Test and sample with 10 µg/ml to 80 µg/ml concentration were prepared and ascorbic acid was used as control. The% inhibitions found was 72.23 % for Ascorbic acid, 49.12% for F-1, 40.66% for F-2, 40.26% for F-3, 46.34% for F-4, 60.42 % F-5. F-5 is shown higher % of inhibition and lower for F-3. The IC_{50} values obtained were 41.04 µg/ml, 82.21 µg/ml, 163.70 µg/ml, 188.03 µg/ml, 104.25 µg/ml and 51.49 µg/ml for Ascorbic acid, F1, F2, F3, F4 and F5 respectively.

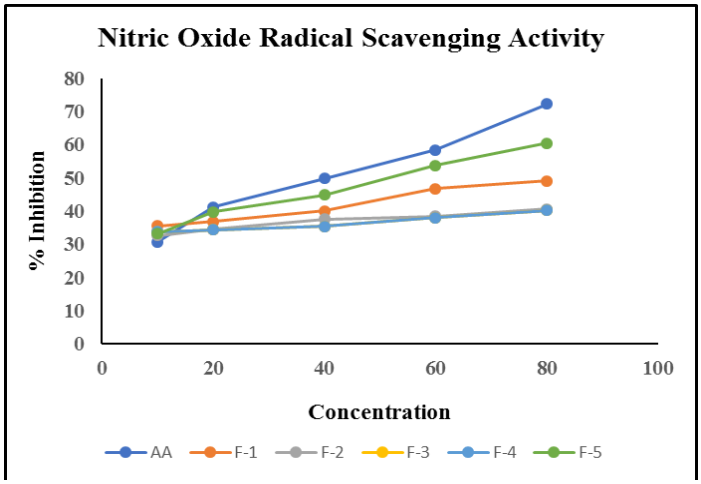

Figure 6: Graph of Nitric Oxide Free Radical Scavenging Activity of Sunscreen Creams

Table 5: IC_{50} values for DPPH and No_2 free radical scavenging activity

	IC_{50} (µg/ml)					
***In vitro* Antioxidant Assay**	**Ascorbic acid**	**F-1**	**F-2**	**F-3**	**F-4**	**F-5**
DPPH	10.73	40.73	53.61	74.73	31.01	22.97
NO_2	41.04	82.21	163.70	188.03	104.25	51.49

Reducing Power Assay

The absorbance of each concentration of samples increased with the increase in their concertation. The absorbance was measured in triplicates and reported as Mean ± SD. The absorbances found were in the range of 0.1650 ± 0.0030 to 0.5820 ± 0.0040 for ascorbic acid served as control. 0.147 ± 0.1473 to 0.324 ± 0.3213 F-1, 0.1117 ± 0.0025 to 0.1870 ± 0.0020 for F-2, 0.1220 ± 0.0030 to 0.2220 ± 0.0026 for F-3, 0.1793 ± 0.0021 to 0.3730 ± 0.0130 for F-4 and 0.1913 ± 0.0035 to 0.4247 ± 0.0067 for F-5. The absorbance shown by F-5 was higher and the low was found in F-2. The increase in absorbance with the increase in concentration indicates the reducing ability for the components present in the creams.

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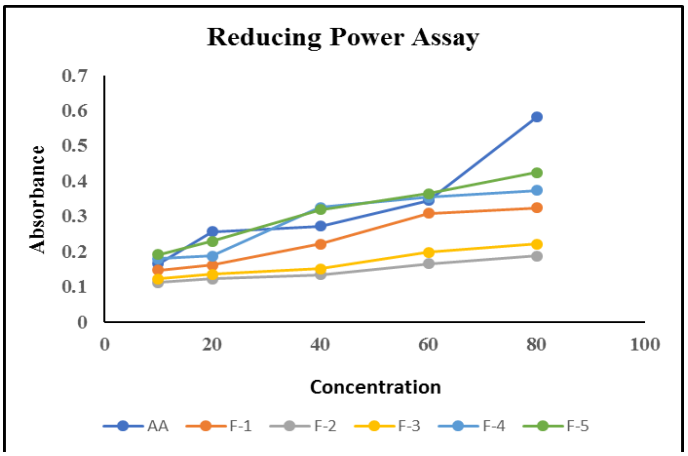

Figure 7: Graph of Reducing Power Assay of Sunscreen Creams

CONCLUSION

Natural antioxidants contributed to the photoaging pathway by inhibiting the oxidative stress conditions and stabilized it by scavenging the reactive oxygen species generated by the UV rays. Many research studies reported that the plant extract or extracts which shows potential antioxidant property and UV absorption ability can help to prevent photo-aging and skin cancer. The present research all the cream formulations are shown ample amount of phenolic and flavonoid compounds and also the free radical scavenging property. The F-5 was better in phenolic and flavonoid content and also shown higher antioxidant properties compared to other creams. The number of phenolic compounds, flavonoids present and antioxidant potential could be responsible for their sun protective effect. The findings obtained from the study indicated the efficacy of the formulation as a prominent source of antioxidants, which will be useful to reduce lipid peroxidation or oxidative stress to protect the skin externally. Further, the prepared creams are required to evaluate for their *in vitro* and *in vivo* sun protection property.

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