



E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; 7(5): 2378-2383
Received: 10-07-2018
Accepted: 12-08-2018

Syeda Sana Fatima
Ramniranjan Jhunjhunwala
College, Ghatkopar west,
Mumbai, Maharashtra, India

Snehal U Govekar
Scientific Research Centre,
KET's V.G. Vaze College
Campus, Mithagar Road,
Mulund East, Mumbai,
Maharashtra, India

Dr. Kshitij V Satardekar
Scientific Research Centre,
KET's V.G. Vaze College
Campus, Mithagar Road,
Mulund East, Mumbai,
Maharashtra, India

Dr. Siddhivinayak S Barve
Scientific Research Centre,
KET's V.G. Vaze College
Campus, Mithagar Road,
Mulund East, Mumbai,
Maharashtra, India

Pranjali P Dhawal
Animal Biotechnology and
Biochemistry Division, Scientific
Research Centre, KET's V.G.
Vaze College Campus, Mithagar
Road, Mulund East, Mumbai,
Maharashtra, India

Correspondence

Pranjali P Dhawal
Animal Biotechnology and
Biochemistry Division, Scientific
Research Centre, KET's V.G.
Vaze College Campus, Mithagar
Road, Mulund East, Mumbai,
Maharashtra, India

In vitro analysis of ethanolic extract of flowers of *Calendula officinalis* for antioxidant, antimicrobial and uv-h₂O₂ induced DNA damage protection activity

Syeda Sana Fatima, Snehal U Govekar, Dr. Kshitij V Satardekar, Dr. Siddhivinayak S Barve and Pranjali P Dhawal

Abstract

The ethanolic extract of *Calendula officinalis* was screened for the presence of antioxidant, antimicrobial, and UV- Hydrogen peroxide induced DNA damage protection activity. Preliminary phytochemical analysis revealed the presence of flavonoids, saponins, and polyphenols. These phytochemicals are known to have antioxidant and antimicrobial properties which were evident from the results obtained for DPPH and FRAP activity of the extracts. The IC₅₀ for radical scavenging activity was 116µg/mL. There was a statistically significant correlation between the ferric reducing antioxidant potential and free radical scavenging activity ($P < 0.05$). The extract also showed significant antimicrobial activity against *Staphylococcus epidermidis* and *Staphylococcus aureus*. Further, DNA damage activity of the extract explained the use of marigold in treating DNA damage caused by oxidative stress.

Keywords: *Calendula officinalis*, DPPH, frap, uv-h₂O₂, phytochemical

Introduction

India, a diverse cultural heritage, having assorted ways of celebrating events and festivals, uses an abundant amount of Marigold flowers for offering to God and decoration purposes [1]. Every day these flowers are offered by the devotees and left unused. About 3.5-4 tonnes of floral waste is left behind in the city of temples [2]. Degradation of floral waste is a very slow process in comparison to kitchen waste degradation [3]. There is, thus a need for proper and eco-friendly processes for the treatment of floral waste.

Plant extracts are also commonly used by cosmetic industries, primarily due to presence of compounds such as saponins, flavanoids, resins and essential oils [4]. Similarly, therapeutic use of marigold is assigned to the various bioactive compounds that it contains. Marigold is known to have abundant poly-phenols such as saponins and phenolic acids [5]. Polyphenols are known to have antioxidant and antimicrobial properties [6].

Plant pharmacological studies have suggested that extracts from *Calendula officinalis* have anti-viral, anti-genotoxic and anti-inflammatory properties. Calendula in suspension is used topically to treat acne, reducing inflammation, controlling bleeding and soothing irritated tissue. There is "limited evidence" that calendula cream or ointment is effective in treating radiation dermatitis and UV induced skin disorders [7].

Throughout the journey of our lives, we tend to accumulate damage generated by UV radiation. This includes inflammation, immune changes, physical changes, impaired wound healing and DNA damage. DNA damage promotes cellular senescence and carcinogenesis [8]. UV radiation is composed of the longest wavelength UVA (315-400nm, 90-95% sunlight), mid-range UVB (280-315nm, 5-10% of sunlight) and shortest wavelength UVC (100-280nm-absorbed by the ozone before reaching earth) [8]. Recently, UVA is identified as a carcinogen likely through its pro-oxidative effects and possibly through other mechanisms such as telomere shortening [9]. In addition, UVA have less ability to induce melanin production compared to UVB, leaving the skin less able to protect itself against further UV insult [10].

Natural antioxidants capable of scavenging reactive oxygen species such plant extracts can be exploited for its use in cosmetic and pharmaceutical industry. *In-vitro* assay such as ability to protect plasmid DNA degradation caused by hydroxyl radical formation (through interactions between hydrogen peroxide and UV light) can be utilized to have preliminary insight into plants ability as UV protective agents. The objective of this study was to evaluate the antioxidant, antimicrobial, phytochemical constituents and UV (UVA, UVB)-H₂O₂ induced DNA damage protection activity of the ethanolic extracts of *Calendula officinalis*.

Materials and Methods

Materials:- α , α -diphenyl- β - picrylhydrazyl (Sigma Aldrich), Tryptic Soy Broth and Columbia media (HiMedia), UV-Vis Spectrophotometer (Cary 50, Varian), UV-A and UV-B light (Philips)

Extraction of Phytochemicals

Collection of Plant Material

For the present work, flowers were collected in the month of Nov'17 from a local market in Mulund, Maharashtra, India. Flowers were shade dried at room temperature and coarsely ground using a mechanical grinder.

Extraction was carried out using Soxhlet apparatus. 15 grams of crushed dried flowers were added to 300mL of ethanol and extraction was conducted for 20 cycles at 70 °C. Followed by extraction, the extract was concentrated in rotary evaporator to recover the solvent and the extract was further dried at room temperature. The dried *Calendula officinalis* extract (COE) was kept at 4 °C throughout the investigations.

Phytochemicals analysis

Tests for flavanoids, saponins, tannins, terpenoids, phenols, cardiac glycosides, ^[10] anthraquinones, alkaloids ^[11] and reducing sugars ^[12] were done as per the standard protocols.

Antioxidant Activity

A, α -diphenyl- β - picrylhydrazyl (DPPH) Free Radical Scavenging Activity

DPPH a free radical on accepting a hydrogen (H) atom from an antioxidant, gets reduced to DPPH₂. The antioxidant reacts by addition of an odd electron to the nitrogen atom in DPPH and is visualized by change in colour from purple to yellow. There is concomitant decrease in absorbance at 517 nm with increasing concentrations of antioxidants. The colour change is monitored by spectrophotometer and utilised for the determination of percent scavenging activity of the plant under study ^[13].

DPPH scavenging potential of crude COE was measured according to the method described by Brand-Williams *et al* with slight modifications (W. Brand-Williams *et al.*, 1995). Different concentrations 50, 75, 100, 200, 300 μ g/ml of COE were prepared in methanol and used. 500 μ L of each concentration of COE were added to 500 μ L of 0.1M DPPH. Colour blank to negate the colour of plant extracts were prepared as above by replacing methanol with DPPH. Positive control was prepared by adding 500 μ L of DPPH to 500 μ L of methanol. Ascorbic acid was used as standard (same concentrations as plant extract) and prepared in the similar manner as the test. All tubes were incubated in dark at room temperature for 30 min and absorbance was taken at 517nm. Experiment was conducted in triplicates. The antioxidant activity was measured by the formula given below,

$$\text{Antioxidant Activity} = \frac{\text{Abs of Positive Control} - (\text{Abs of actual test})}{\text{Abs of Positive control}} \times 100$$

Where,

Abs= Absorbance

Abs of actual test = Abs of test – Abs of color blank

Ferric Reducing Antioxidant Potential assay (FRAP)

Electron-donating antioxidants are described as reductants, and inactivation of oxidants by reductants can be described as redox reactions in which one reactive species is reduced while another is oxidized. In this context, therefore, "total

antioxidant power" may be referred to analogously as total reducing power ^[14].

Different concentrations 50, 75, 100, 200, 300 μ g/ml of COE were prepared in 0.2M Phosphate Buffer (pH 6). 500 μ L of COE of different concentrations were added to 500 μ L of 1% potassium ferricyanide K₃ [Fe (CN)₆] for test and colour blank. Ascorbic acid was used as standard (same concentrations as plant extract) and prepared in the similar manner as the test. All the tubes were placed in water bath at 50 °C for 30 min. 500 μ L of 10% trichloroacetic acid (TCA) were added to these tubes to terminate the reaction. The tubes were centrifuged at 3000rpm for 10 min. 500 μ L of supernatant was taken and added to 500 μ L of distilled water. 100 μ L of 0.1% ferric chloride (FeCl₃) was added to test and standard and 100 μ L of distilled was added to colour blank. Absorbance was calculated at 700nm. Experiment was conducted in triplicates. The results were given as the difference between absorbance of test and absorbance of blank at 700nm.

Antimicrobial Assay

The antimicrobial potential of COE was evaluated using two bacterial strains viz; *Staphylococcus aureus* and *Staphylococcus epidermidis* responsible for causing skin infections. The bacterial strains were provided by the Department of Microbiology at Scientific Research Centre, KET's V.G. Vaze college, Mulund.

Each bacterial strain was sub-cultured and incubated overnight at 37 °C in Tryptic Soy Agar (*S. aureus*) and Columbia Agar (*S. epidermidis*). After 48 hours of bacterial growth, loopful of the cultures from respective plates was inoculated in 5 ml of respective broth medium, to obtain a culture density of 10⁷ CFU/ml which was estimated using a spectrophotometer at 620nm.

Disc diffusion method was used to evaluate antimicrobial activity of the COE. Loopful of each culture was spread across on respective culture media by spread plate technique. The plant extract 1500 μ g/mL made in sterile distilled water was loaded on sterile filter paper discs (6 mm in diameter) and these were placed on agar plates. The plates were incubated at 37 °C for 48 h and zone of inhibitions was observed. ^[15, 16].

UV- H₂O₂ Induced DNA damage protection assay

Comet assay ^[17] was used with modifications. A UV-A tube (Philips, India) was used as a UV-A source with a wavelength range of 315- 400 nm and peaked at 365 nm, whereas a UV – B Bulb (Philips, India) emitting a radiation from 280- 315 nm and peaked at 312 nm served as a UV-B source. For UV –A the intensity was 1.8 mW/cm² and UV – B the tubes were irradiated at an intensity of 5 mW/cm² ^[18].

pBR322 plasmid was used for the assay. 10ng of plasmid was used. 1 μ L of 1500 μ g/mL of COE was prepared in distilled water was added to 1 μ L of 10ng of plasmid. To this 1 μ L of 0.1M H₂O₂ was added and volume was made up to 10 μ L with TE buffer pH (8). Control for complete damage was used by replacing COE with TE buffer pH (8). Control for total protection was made by replacing H₂O₂ and COE with TE buffer. Plant control (no band) was made by adding 1 μ L of 1500 μ g/mL of COE in 9 μ L of TE buffer. All the tubes except control for protection were placed in UV box under UV-A and UV-B light for 15 min. The samples were then loaded in 2% Agarose (70V).

Statistical Analysis

For all the tests the results were reported as Mean \pm SD. Regression analysis was carried out and P value less than 0.05 was considered as statistically significant.

Results and Discussion

Nature and colour of COE

After drying, the extract was weighed and percent yield was calculated as,

$$\text{Percent yield} = \frac{\text{weight of dried extract in grams}}{\text{weight of sample crushed sample}} \times 100$$

Table 1: Nature and colour of COE

Parameters	COE
Yield	6.3 g
% Yield	39.8%
Consistency	Sticky
Colour	Deep yellow

Phytochemical Analysis

Table 2: Results for Phytochemical analysis

Test For	COE
Flavonoids	+
Saponins	+
Tannins	+
Terpenoids	+
Phenols	+
Cardiac glycoside	+
Antraquinones	-
Carbohydrates	+
Alkaloids	-
Reducing sugars	+

KEY:- (+ = PRESENT), (- = ABSENT)

The preliminary phytochemical analysis was conducted on the ethanolic extract of *C. officinalis*. The extract showed positive results for the presence of flavonoids, saponins, terpenoid, phenols, tannins, reducing sugars and carbohydrate. The presence of these phytochemicals confers upon the plant with antioxidant, antimicrobial and anti-inflammatory abilities.

Antioxidant Assay

DPPH radical scavenging activity

For COE and Standard Ascorbic acid, percent scavenging activity was calculated by the formula,

$$\frac{\text{Abs of Positive Control} - (\text{Abs of actual test})}{\text{Abs of Positive control}} \times 100$$

Where, Abs= Absorbance, Actual test= Abs Test - Abs Colour Blank. With increasing concentration, absorbance of COE and ascorbic acid decreased at 517 nm and percent scavenging activity increased.

Table 3: Percent Scavenging Activity of COE and Ascorbic Acid

Conc (µg/ml)	COE PSA \pm SD	Ascorbic Acid PSA \pm SD
50	25.61 \pm 0.0800	48.07 \pm 0.06361
75	33.96 \pm 0.0762	72.28 \pm 0.06369
100	51.67 \pm 0.0545	96.145 \pm 0.0493
200	87.64 \pm 0.0396	97.295 \pm 0.0353
300	91.08 \pm 0.0038	97.415 \pm 0.0282

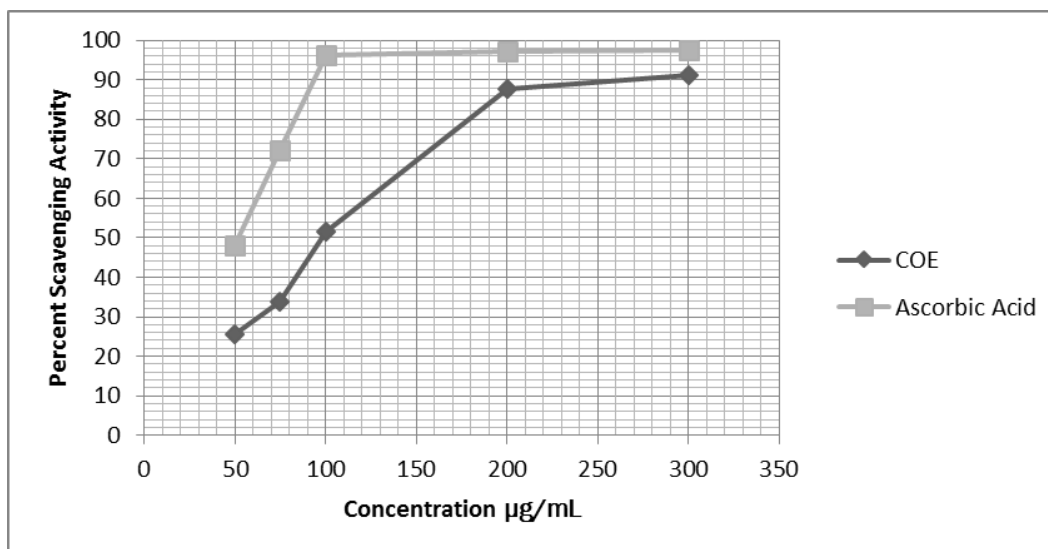


Fig 1: Graph showing Percent Scavenging Activity of COE and Ascorbic Acid

From the above graph [Figure 1], the concentration at which 50% DPPH radicals are scavenged is considered as IC_{50} and it was calculated by regression line analysis that was found to be at 116 μ g/mL. Concentration of COE and percent radical scavenging activity showed positive correlation ($R^2 = 0.89$).

The regression line analysis from which the IC_{50} was calculated is,

$$y = 0.2745x + 18.194$$

Ferric reducing antioxidant potential

Absorbance of all the tubes was taken at 700nm. Absorbance of colour blank without ferric chloride was subtracted from absorbance of test of COE. Similar calculations were made for ascorbic acid. Graph was plotted for absorbance at 700nm on y-axis versus concentration of COE and ascorbic acid on X- axis. With increasing concentration, absorbance increased which indicates concentration and ferric reducing antioxidant potential are directly proportional to each other.

There also existed a strong correlation between ferric reducing antioxidant potential and radical scavenging activity that was statistically significant. ($P < 0.05$)

Table 4: FRAP Activity of COE and Ascorbic Acid

Conc (µg /ml)	COE (T - CB) ± SD	Ascorbic Acid (T - CB) ± SD
50	0.7039±0.143	0.2123±0.031
75	0.9817±0.221	0.2851±0.012
100	1.1416±0.010	0.3217±0.010
200	1.5121±0.089	0.3551±0.013
300	1.8988±0.099	0.3774±0.004

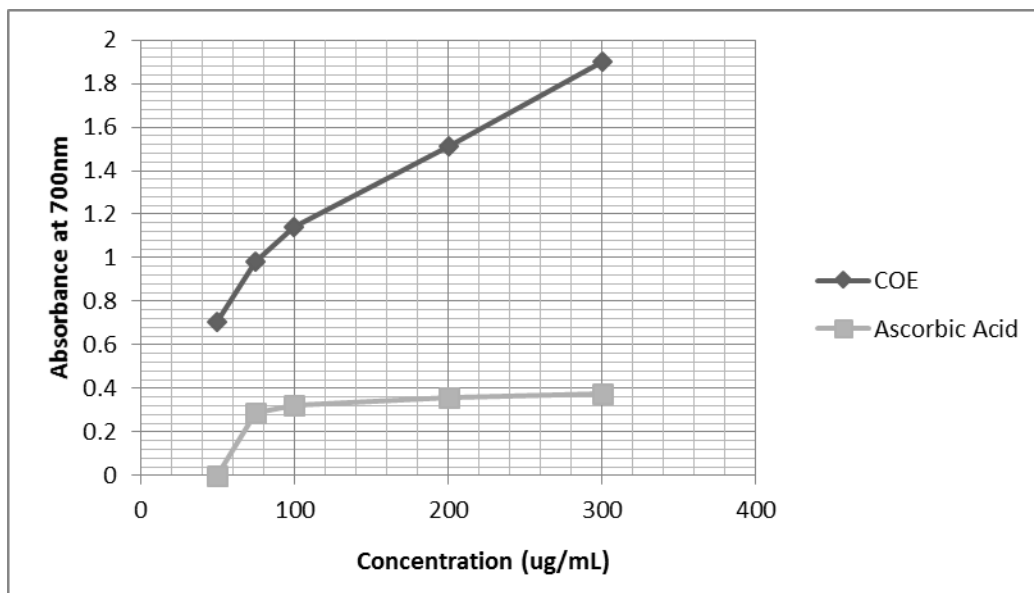


Fig 2: Graph showing Percent Scavenging Activity of COE and Ascorbic Acid

From the above graph [Figure 2], with increasing concentration of COE, absorbance at 700nm increased exponentially and it was evident from the strong positive correlation obtained between them ($R^2 = 0.96$). Results indicated that ethanolic extract of *C officinalis* was found to effectively scavenge DPPH radical and also had good ferric reducing antioxidant potential in a dose dependent manner. Studies show that *C officinalis* possesses antioxidant activity with polyvalent action towards various free radicals such as superoxide anion, hydrogen peroxide, and hydroxyl radicals. This indicates the efficiency of *C. officinalis* extract and its applications in antiaging [19].

Antimicrobial assay

Table 5: Antimicrobial Activity of COE

Organism	Zone of inhibition (mm)*
<i>S. aureus</i>	11
<i>S. epidermidis</i>	13

* Disc diameter 6mm.

From the table 5, it is evident that extract of *Calendula officinalis* showed growth inhibitory effects against gram positive bacteria. This extract can thus be considered as a natural herbal product to have significant antibacterial effect on pathogenic bacteria, especially gram-positive. These microorganisms contribute to the development of skin infections, food poisoning, pneumonia, sepsis, osteomyelitis, and infectious endocarditis [20, 21].

UV-H₂O₂ induced DNA damage protection assay

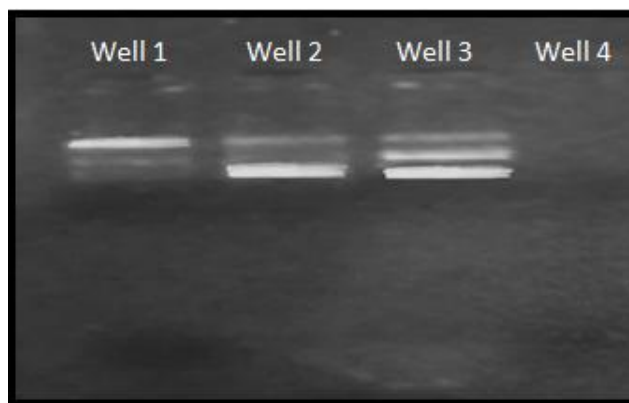


Fig 3: DNA damage protective activity of COE [From right to left of the screen, well 1: complete damage, well 2: treated, well 3: intact plasmid, well 4: COE only]

This assay was based on the ability of extracts to protect the pBR322 plasmid DNA against damage caused by hydroxyl ($\cdot\text{OH}$) radicals generated by hydrogen peroxide when exposed to UV radiations. These reactive species- hydroxyl radicals are known to cause oxidative induction of breaks in DNA strands to yield its open circular or relaxed forms. Hydroxyl radicals react with nitrogenous bases of DNA producing base radicals and sugar radicals. The base radicals in turn react with the sugar moiety causing breakage of sugar phosphate backbone of nucleic acid, resulting in strand break [22].

The scavenging effect of extract was evaluated in plasmid nicking assay. Fig 3 depicts the ability of the extracts to prevent oxidative damage of plasmid DNA. When plasmid DNA was allowed to react with hydrogen peroxide activated by ultraviolet light, it resulted in the formation of single-stranded relaxed nicked DNA (R-Form) and double-stranded nicked and linear DNA (L-Form) [Well 1]. Addition of COE [well 3] resulted native circular (C) plasmid DNA formation and relaxed (R) form, causing disappearance of linear (L) forms of plasmid DNA. The circular form (C) of untreated DNA was converted into nicked forms like relaxed (R) and linear (L) DNA, and upon treatment with extract regained its native form of DNA into circular form (C) and protected DNA from the hydroxyl ($\cdot\text{OH}$) radical induced oxidative DNA damage. More resolution is required to confirm exact protection. Intact plasmid in well 2 showed the presence of circular (C) form of DNA only.

Well 4 is an indication that extract used as test did not contain in DNA contamination and this was an indication that no false positive results were obtained.

Conclusion

Preliminary phytochemical analysis revealed that the ethanolic extract of *Calendula officinalis* contains alkaloids, carbohydrates, proteins, tannins, phenolic compounds, flavonoids, terpenoids, and glycosides. In addition, the COE exhibited significant free radical scavenging activity and ferric reducing antioxidant potential. The extract also showed activity against *S.aureus* and *S. epidermidis*. Moreover, the COE extract showed significant protection activity against UV- H_2O_2 induced DNA damage on pBR322 plasmid. These activities of COE extract may be attributed to the presence of various phytochemical constituents such as polyphenolics, flavonoids, and phenolic acids which were reported earlier to neutralize different free radicals. Although a large number of active compounds are isolated from *C officinalis*, further studies are needed for isolation, structural elucidation, and screening of any of the above-mentioned active principles to proposed activity of drug. This plant material should be extracted in various other solvents to analyze and determine any variation in the current results.

Conflict of Interest

We declare that we have no conflict of interest.

Acknowledgement

Authors would like to thank Dr. B. B. Sharma (Principal), KET's V.G. Vaze College, for letting us use the laboratory space for conducting a few assays.

References

1. Kedare Sagar B, Singh RP. Genesis and development of DPPH method of antioxidant assay. Journal of food science and technology. 2011; 48(4):412-422.
2. Mishra Krishnanand, Himanshu Ojha, Nabo Kumar Chaudhury. Estimation of antiradical properties of antioxidants using DPPH assay. A critical review and results. Food chemistry. 2012; 4(1):036-1043.
3. Jadhav AR, Chitanand MP, Shete HG. Flower Waste Degradation Using Microbial Consortium. Journal of Agriculture. 2013; 3(5):1-4.
4. Moghaddasi Mohammad S, Kashanisup HH. Pot marigold (*Calendula officinalis*) medicinal usage and cultivation. Scientific Research and essays. 2012; 7(14):1468-72.
5. Chakraborty GS. Phytochemical screening of *Calendula officinalis* Linn leaf extract by TLC. Int J Res Ayurveda Pharma. 2010; 1(1):131-134.
6. Raal A, Kirsipuu K. Total flavonoid content in varieties of *Calendula officinalis* L. originating from different countries and cultivated in Estonia. Natural product research. 2011; 25(6):658-662.
7. Golla U. Bhimathati. Evaluation of antioxidant and DNA damage protection activity of the hydroalcoholic extract of *Desmostachya bipinnata* L. Stapf. The Scientific World Journal. 2014, ID 215084, 215084-215092
8. Amaro-Ortiz A, Yan B, D'Orazio JA. Ultraviolet radiation, aging and the skin: prevention of damage by topical cAMP manipulation. Molecules. 2014; 19(5):6202-6219.
9. Grant WB. The effect of solar UVB doses and vitamin D production, skin cancer action spectra, and smoking in explaining links between skin cancers and solid tumours. European Journal Cancer. 2008; 44(1):12-5.
10. Yin B, Jiang X. Telomere shortening in cultured human dermal fibroblasts is associated with acute photodamage induced by UVA irradiation. Postepy Dermatol Alergol. 2013; 30(1):13-8.
11. Dhawal P, Satardekar K, Hariharan S, Barve S. *In-vitro* analysis of cinnamomum verum for formulation of bio-active cosmetic gel. International journal of pharmaceutical sciences and research. 2017; 8(7):2988-2995.
12. Njoku Victor O, Chidi Obi. Phytochemical constituents of some selected medicinal plants. African journal of pure and applied chemistry. 2009; 3(11):228-233.
13. Yadav RN, Munin A. Phytochemical analysis of some medicinal plants. Journal of Phytology. 2011; 3(12):10-14.
14. Benzie Iris FF, Strain JJ. Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods in enzymology. 1999; 299:15-27.
15. Bisignano G, Sanogo R, Marino A, Aquino R, 'angelo VD, Germano MP, *et al.* Antimicrobial activity of *Mitracarpus scaber* extract and isolated constituents. Letters in applied microbiology. 2000; 30(2):105-108.
16. Mostafa AA, Al-Askar AA, Almaary KS, Dawoud TM, Sholkamy EN, Bakri MM. Antimicrobial activity of some plant extracts against bacterial strains causing food poisoning diseases. Saudi Journal of Biological Sciences. 2017; 25(2):361-366.
17. Collins Andrew R. The comet assay for DNA damage and repair. Molecular biotechnology. 2004; 26(3):249.
18. Pranita A Tendulkar, Manjushri A Deodhar. Pretreatment of Zeaxanthin Extracted from Thermotolerant Strain *Synechocystis pevalekii* Protects 3T3 Fibroblasts against Ultraviolet Radiations. International Journal of Scientific and Engineering Research. 2018; 9(1)682-687.
19. Braga Pier Carlo. Antioxidant activity of *Calendula officinalis* extract: inhibitory effects on chemiluminescence of human neutrophil bursts and electron paramagnetic resonance spectroscopy. Pharmacology. 2009; 83(6):348-355.
20. Chessa D, Ganau G, Mazzarello V. An overview of *Staphylococcus epidermidis* and *Staphylococcus aureus* with a focus on developing countries. The Journal of Infection in Developing Countries. 2015; 9(6):547-50.

21. Jafari B, Ahmadizadeh C. The *In Vitro* Study of Antimicrobial Effect of Marigold (*Calendula officinalis*) Extract on Infectious Microorganisms. *Electronic J Biol.* 2017; 13(4):348-352.
22. Buxton GV, Greenstock CL, Helman WP, Ross AB. Critical review of rate constants for reactions of hydrated electrons, hydrogen atoms and hydroxyl radicals ($\cdot\text{OH}/\cdot\text{O}^-$) in aqueous solution. *Journal of physical and chemical reference data.* 1988; 17(2):513-886.