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Screening for Antioxidant and Antibacterial potential of common medicinal plants in the treatment of Acne

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Abstract

In the present study, sequential extracts of *Camellia sinensis*, *Glycyrrhiza glabra*, *Calendula officinalis* and *Linum usitatissimum* were analyzed for their antioxidant activity in different test systems. Phytochemical analysis indicated that amongst twelve test extracts, methanolic extract of *Camellia sinensis* was found to have the highest total phenolic content (104.93 ± 1.630 mg GAE /g) and FRAP value (1046.330±1.948 mg TE/g). Flavonoid content (115.503±2.984 mg RuE /g dry extract) of methanolic extract of *Glycyrrhiza glabra* was found to be superior among all the extracts. Highest DPPH scavenging (IC_{50}=44.03 ±1.784 µg/ml) effect was also observed in methanolic extracts of *Camellia sinensis*. *In vitro* anti-microbial screening indicated that methanolic extracts showed promising antimicrobial activity against *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Propionibacterium acnes*.

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Acne, Antioxidant assay, Free radicals, Medicinal plants.

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Introduction

Acne vulgaris is the most common skin disorder of pilosebaceous unit caused by hormonal, microbiological and immunological factors. It affects all age groups i.e. teenagers (85%), 25-34 year (8%) and 35-44 year (3%) and is characterized by the presence of bacteria namely *Staphylococcus aureus, Staphylococcus epidermidis* and *Propionibacterium acnes* in the follicular canal [1]. The severity of this skin disorder generally increases with age and time. *Propionibacterium acnes* evokes mild local inflammation by producing neutrophil chemotactic factors. Consequently, neutrophils get attracted to

the acne lesions and constantly release inflammatory mediators such as reactive oxygen species (ROS) [2]. Reactive oxygen species (ROS) namely hydroxyl (·OH) and super oxide ($O_2^{\cdot-}$) and reactive nitrogen species (RNS) like nitrous oxide (N_2O) nitroxyl anion (NO^-) are the most common free radicals. These toxic ROS can also act as a messenger in the induction of several biological responses such as NF-kB and AP-1. These radicals are formed with the reduction of oxygen to water. Normally, the production of these radicals is slow and they are removed naturally by the antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT) and glucose-6-phosphate dehydrogenase (G6PD) existing in the cell, but due to depletion of immune system and natural antioxidants in different ailments, it becomes necessary to use antioxidants as free radical scavengers for removal of ROS to reduce cell damage that occurs during acne inflammation [3]. Antioxidant compounds have the ability to capture, deactivate and repair the damage caused by free radicals (Alonso et al., 2002) [4]. Hence, the objective of the present study was to investigate antioxidant potential of the following four medicinal plants viz: *Camellia sinensis* [5] *Glycyrrhiza glabra* [6] *Calendula officinalis* [7,8] and *Linum usitatissimum* possessing antimicrobial and anti-inflammatory potential also.

Materials and Methods

Plant materials and chemicals

Fresh and dried plant materials were collected from medicinal gardens and authorized herbal stores in Delhi. Their botanical identities were determined and authenticated at the National Institute of Science Communication and Information Resources (NISCAIR), New Delhi, India vide voucher specimen NISCAIR/RHM/consult/2007-08/936/120 and NISCAIR/RHM/consult/2008-09/978/09.

Clindamycin phosphate was procured from Sri Ram Institute of Industrial Research, New Delhi. All other chemicals used were of analytical grade and obtained from either Sigma-Aldrich or Merck.

Preparation of Extracts and Phytochemical Screening

The air dried leaves, flowers, stolons, roots and seeds were pulverized and used for extraction in soxhlet apparatus at room temperature. Sequential extraction of 200 g was done with solvents of increasing polarity i.e. petroleum ether (PE), dichloromethane (DCM) and methanol (ME) and the extracts were abbreviated as: *Camellia sinensis* (CSPE, CSDCM, CSME), *Glycyrrhiza glabra* (GGPE, GGDCM, GGME), *Calendula officinalis* (COPE, CODCM, COME) and *Linum usitatissimum* (LUPE, LUDCM, LUME). The extracts were evaporated under vacuum conditions using a rotary evaporator and stored at 4˚C in air tight containers for further studies. Preliminary phytochemical screening and percentage yield of the twelve extracts was recorded [9].

Determination of total phenolic content

The total phenolic content of the thirteen extracts was determined using McDonald method with modifications [10]. 100 µl of the diluted extracts containing 500 µg and standard phenolic compound gallic acid (10-50µg/ml) were mixed separately with (62.5µl) Folin-Ciocalteu reagent and diluted with 0.287 ml distilled water and 0.375 ml of 20% aqueous Na_2CO_3. The mixtures were then allowed to stand for 2 hrs and the total phenolic content was determined using spectrophotometer at 765 nm. The concentration of the total phenolic compounds was calculated using the equation ($y = 0.02769x + 0.0103$: $r^2 = 0.9984$) and the total phenolic content was expressed as mg of Gallic acid equivalents (GAE) / g of dried extract (Table 1).

Table 1: Total phenolic and flavonoid content of test extracts

Scientific name	(GAE) PhenolicContent (mg/g)			(RuE) Flavonoid content (mg/g)		
	PE	DCM	ME	PE	DCM	ME
Camellia sinensis (le)	14.16 ± 0.91	17.88 ± 1.85	104.93 ± 1.63	19.29 ± 2.56	24.29 ± 0.90	28.87 ±0.51
Glycyrrhiza Glabra (ro& st)	60.52 ± 1.94	73.51 ± 1.45	95.38 ± 2.49	41.14 ± 0.56	115.48 ± 3.69	115.5 ±2.98
Calendula officinalis(fl)	ND	52.26 ± 1.28	91.00 ± 1.66	2.49 ± 1.66	62.35 ± 2.40	73.42 ±2.68
Linum Usitatissimum (se)	1.62 ± 0.69	2.69 ± 0.25	15.14 ±0.12	14.39 ± 1.03	27.25 ± 0.19	7.59 ±0.21

le (leaves) ; ro& st (roots and stolons); fl (flowers); se (seeds);PE (petroleum ether extract); DCM (dichloromethane extract); ME (methanolic extract); ND (not detected).
Results were mean ± standard deviation

Determination of total flavonoid

The total flavonoid content of all the extracts and TTO was determined using existing Chang et al. method with some modifications [11]. 100µl of the extract containing 500 µg was mixed with 300 µl of distilled water and 30 µl of 5% $NaNO_2$. The mixture was kept at room temperature for 5 min followed by addition of 30µl of 10% $AlCl_3$, 0.2 ml of 1mM NaOH and 1ml distilled water. The absorbance of the reaction mixture was measured at 415 nm with UV spectrophotometer. The concentration of the flavonoid compounds was calculated using the equation ($y = 0.01083x - 0.00476$: $r^2 = 0.9945$) obtained from the rutin (20-100 µg/ml) calibration curve and the flavonoid content was expressed as mg of rutin equivalents (RuE) / g of dried extract (Table 1).

Scavenging effect on 1, 1-diphenyl-2-picrylhydrazyl (DPPH)

The free radical scavenging activity was estimated by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay using Blois method with some modifications [12]**.** The reaction mixture contained 100µl of test extracts (100-500 µg/ml) and 1 ml of methanolic solution of 0.1mM DPPH radical. The mixture was then shaken vigorously and incubated at 37°C for 30 min. The absorbance was measured at 517 nm using ascorbic acid (100-500 µg/ml) as positive control. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity which was calculated using the following equation:

DPPH scavenging effects (%) = 100 x $(A_o - A_1)/(A_o)$

where A_o is the absorbance of the control reaction and A_1 is the absorbance of reaction mixture containing DPPH and extract at 517 nm. The antioxidant activity of the extract was expressed as IC_{50} value which is defined as the concentration (µg/ml) of extract that inhibits the formation of DPPH radicals by 50%. It was obtained from linear regression analysis (Figure 1).

Ferric reducing antioxidant power (FRAP) assay

Freshly prepared FRAP reagent contained 5 mL of a 10mM TPTZ (2, 4, 6 -tripyridyl- 2- triazine) solution in 40 mM HCl, 5 mL of 20mM $FeCl_3.6H_2O$ and 50ml of 300 mM acetate buffer (pH 3.6) and was heated at 37°C. 100 µl of various extracts (10mg/ml) was mixed with 900 µl of FRAP reagent and the mixture was then incubated at 37°C for 6 min. Ferric reducing antioxidant power of the extracts was determined by modified Benzie and Strain method [13]. The absorbance of the coloured reaction mixture (ferrous tripyridyltriazine complex) was measured at 595 nm using standard trolox (1 mg/ml) to estimate the percentage of iron reduced. The results were calculated as mg of trolox equivalent (TE) per g of dried extract using the

equation: (y = 0.0749 + 0.003X, r^2 = 0.9888) (Figure 2).

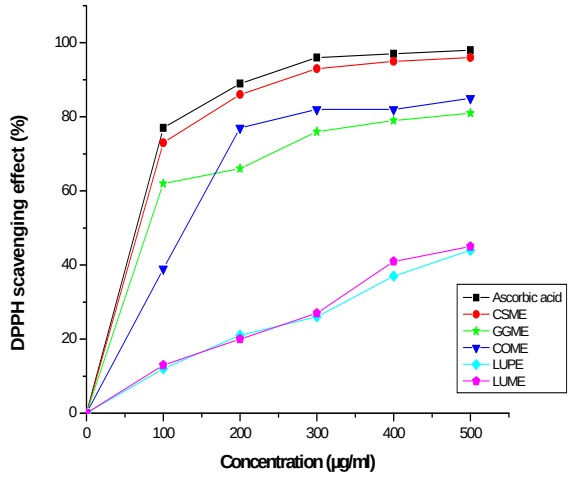

Figure 1: DPPH scavenging effect (%) of active plant extracts and ascorbic acid

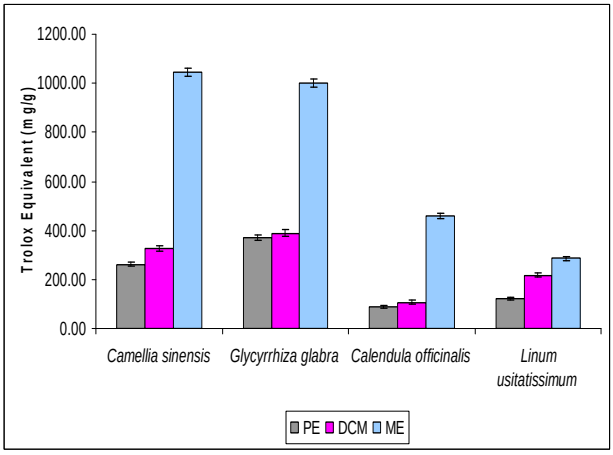

Figure 2: Ferric reducing antioxidant power of test extracts

Antimicrobial screening

Microorganism and media

Aerobic bacteria: *Staphylococcus aureus* (MTCC 96), *Staphylococcus epidermidis* (MTCC 2639) and anaerobic bacteria: *Propionibacterium acnes* (MTCC *1951) were obtained from the Microbial Type Culture Collection Centre, Institute of Microbial Technology, Chandigarh. Fresh cultures of the isolates of aerobic and anaerobic bacteria were suspended in nutrient broth and reinforced clostridium medium respectively. *S. aureus* and *S. epidermidis* cultures were incubated for 24 h at 37°C and 30°C, respectively. *P. acnes* culture was incubated in an anaerobic chamber at 37°C consisting of 10% CO_2, 10% H_2 and 80% N_2 for 48 h.

Antimicrobial screening using disc diffusion method

Antibacterial activity of the extracts was tested using agar disc diffusion method [14]. 100 µl of fresh culture suspension of the test bacteria was evenly spread on nutrient agar and reinforced clostridial agar plates. The concentration of cultures was $5x10^5$ CFU/ml. For screening, 6 mm diameter filter paper disc, impregnated with 20 µl of extract solution equivalent to 0.2 mg of extract was placed on the surface of inoculated media agar plates. Incubation was done at 37°C or 30°C for 24 h and 48 h depending upon the type of bacteria under optimum conditions. Clear zones of inhibition were measured in mm, including the diameter of the disc. Zone measuring 10 mm or more was considered as effective against test organisms. Clindamycin (10 µg/disc) was used as positive control and the respective solvents, which were used for extraction, served as negative control.

Statistical analysis

All the samples were run in triplicate and mean values were used for the result analysis. The statistical significance between the antioxidant activity of extracts and standards was evaluated using SPSS version 10.0.1 and the comparison was done using Mann-Whitney U test.

Results and Discussion

In the present study, the percentage yield of test extracts indicated that GGME showed the highest percentage yield (20.11) followed by COME (11.22) and CSME (9.55). Preliminary phytochemical screening indicated the presence of the following phytoconstituents in selected medicinal plants: *C.*

sinensis (alkaloids, flavonoids, terpenoids and tannins), *G. glabra* (carbohydrate, glycosides, flavonoids, saponins, terpenes and sterol), *C. officinalis* (flavonoids, saponins and terpenoids) and *Linum usitatissimum* (terpenes, proteins and fatty acids). In recent years, attention has been focused on the oxidative stress while treating acne vulgaris because the rate of generation of ROS is more than the rate of its removal. Though the biological system tries to protect with the help of enzymes like SOD and CAT in case the activity of CAT gets reduced, yet it leads to the accumulation of superoxide radicals and hydrogen peroxide [15]. In the present investigation, results clearly indicated (Table 1) that amongst all test extracts, CSME showed the highest amount of GAE of phenolic compounds (104.934 ± 1.630 mg/g) and the lowest was observed in LUPE (1.620 ± 0.692 mg/g). COPE didn't show any phenolic content. Total phenolic content of the extracts was found to be significant ($P< 0.05$). *Camellia sinensis is* well known to be rich in polyphenolic content and possesses antioxidant activity. The constituents of *C. sinensis* include large amounts of (-)-epigallocatechin, (-)-epicatechin, (+)-gallocatechin, (+)-catechin, their derivatives [16], rutin and myricetin which have been shown to possess high antioxidant and free radical scavenging activity and have a positive effect on the human health. Phenols are the simplest bioactive phytochemicals having free radical scavenging ability due to the presence of hydroxyl groups. The site and the number of hydroxyl groups present are related to their relative toxicity to microorganisms, showing that increased hydroxylation results in increased toxicity [17]. Flavonoids are polyphenolic compounds which play an important role in stabilizing lipid oxidation and are also associated with antioxidative action. Flavonoid content of the extracts in terms of (mg/g) rutin equivalents was recorded (Table 1). Highest and lowest flavonoid content was observed in GGME (115.503 ± 2.984 mg/g) and COPE (2.488 ± 1.659 mg/g) respectively. This could be due to the licorice flavonoid constituents possessing free radical scavenging effect and antioxidant potential. Licorice flavonoids contain mainly flavones, flavonols, isoflavones, chalcones, dihydroflavones and dihydrochalcones [18].

The in-vitro antioxidant activity of test extracts was estimated using DPPH assay. All methanolic extracts except LSME exhibited potent antioxidant activity when DPPH radical was used as a substrate to evaluate the free radical scavenging activity. The antioxidants reacted with DPPH, a purple coloured stable free radical which accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The amount of DPPH reduced was estimated by measuring the decrease in absorbance at 517 nm. A lower IC_{50} value indicated a greater antioxidant activity [19]. Our experimental data indicated that though all the test extracts demonstrate H-donor activity, still the highest DPPH radical scavenging activity was observed in CSME (IC_{50} = 44.03 ± 1.784 µg/ml) followed by GGME (IC_{50} = 51.07 ± 3.050 µg/ml) and COME (IC_{50} = 111.96 ± 1.129 µg/ml) presented in (Fig. 1) whereas LUME (IC_{50} = 687.79 µg/ml) didn't show much radical scavenging activity. Mann-Whitney U test showed comparison of ascorbic acid and active methanolic extracts. P value < 0.05 was observed in COME indicating data to be significantly different whereas CSME and GGME showed P value > 0.05. FRAP on the other hand gives a direct measure of antioxidants or reductants in a sample which react with ferric tripyridyltriazine (Fe^{3+} TPTZ) complex and produce a coloured product, ferrous tripyridyltriazine (Fe^{2+} TPTZ) [20]. All the plant extracts showed a dose-dependent reducing activity (Fig. 2). None of the plant extracts exhibited absorbance higher than the standard Trolox. Reducing activity of the extracts in terms of mg/g of

TE (y = 0.003x + 0.0749: r^2= 0.98824) was between (1046.330 ± 1.948 and 86.248 ± 2.329). The highest FRAP activity was observed in GGME (946.330 ± 1.948) followed by CSME (901.506 ± 2.044) and COME (460.089 ± 1.943) whereas the lowest value was recorded in COPE (86.248 ± 2.091).

Screening for *In vitro* antimicrobial activity using clindamycin phosphate as a positive control clearly indicated that CSME, GGME and COME showed promising antimicrobial activity against all the three organisms. Furthermore, LUPE was found to be more effective than LUME against aerobic and anaerobic test strains. Highest zone of inhibition, 17.8 ± 0.016 mm, was observed for CSME against *S. epidermidis*. Literature revealed strong relationship between the total phenolic content and antioxidant activity in many plant species because phenolic compounds not only attack cell walls and cell membranes by affecting their permeability but also interfere with membrane functions like electron transport, protein synthesis and enzyme activity. Hence, active phenolic compounds could lead to the destruction of pathogens [21].

Conclusion

The results of this study indicated that a higher concentration of phenolic compounds in methanolic extracts makes them a strong free radical scavenger, which further indicates that these plants can be a good source of natural antioxidants to prevent free radical mediated oxidative stress in case of acne. Therefore, further investigation is needed to explore the parameters essential for formulation so that the antioxidant and antibacterial potential of these medicinal plants can be utilized to provide safe and effective topical herbal formulation for the treatment of acne.

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