# APPENDIX A: IDENTIFICATION REPORTS

<table>
<thead>
<tr>
<th>Name Of Working Standard</th>
<th>Clindamycin HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratory Reference For Working Standard</td>
<td>APL/WRS/185</td>
</tr>
<tr>
<td>Assay</td>
<td>860 µg/mg (On As Such Basis) (Clindamycin)</td>
</tr>
<tr>
<td>Water</td>
<td>4.70 % w/w</td>
</tr>
<tr>
<td>Quantity Of Working Standard</td>
<td>1 gm</td>
</tr>
<tr>
<td>Date Of Preparation</td>
<td>21/01/2008</td>
</tr>
<tr>
<td>Date of Validity</td>
<td>20/01/2009</td>
</tr>
<tr>
<td>Storage Conditions</td>
<td>preserve in tight container.</td>
</tr>
</tbody>
</table>

Authorized Signatory
For Arbro Pharmaceuticals Ltd
Dr. H.B. Singh  
Head  
Raw Materials Herbarium & Museum  
Phone: 25841143  
E-mail: hbs@niscair.res.in;

Dear Mrs. Nand,

Kindly refer to your letter No. nil dated 1 January 2008 regarding identification of five crude drug samples. The samples have been identified as given below:

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>Sample received as</th>
<th>Sample identified as</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Azadirachta indica leaves (Market sample)</td>
<td>Melia azadirach Linn.</td>
<td>Wrong</td>
</tr>
<tr>
<td>2</td>
<td>Azadirachta indica leaves (Self collected)</td>
<td>Azadirachta indica A. Juss.</td>
<td>O.K.</td>
</tr>
<tr>
<td>3</td>
<td>Azadirachta indica Bark (Market sample)</td>
<td>Azadirachta indica A. Juss.</td>
<td>O.K.</td>
</tr>
<tr>
<td>4</td>
<td>Glycyrrhiza glabra stolens</td>
<td>Glycyrrhiza glabra Linn.</td>
<td>O.K.</td>
</tr>
<tr>
<td>5</td>
<td>Calendula officinalis flowers</td>
<td>Calendula officinalis Linn.</td>
<td>O.K.</td>
</tr>
</tbody>
</table>

With regards

Yours sincerely,

Mrs. Pratibha Nand  
E-1024, Ramjas Vihar  
Delhi-110034
Ref. NISCAIR/RHMD/Consult/-2008-09/978/09

Dr. H.B. Singh
Scientist F. & Head
Raw Materials Herbarium & Museum
Phone: 25841143
E-mail: hbs@niscain.res.in; hbsbhati@yahoo.com

25 April, 2008

Dear Mrs. Pratibha,

Kindly refer to your letter No. 11 dated 22 April 2008 regarding identification of one crude drug sample. The sample has been identified as given below:

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Sample received as</th>
<th>Part</th>
<th>Sample identified as</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Linseed / Flaxseeds</td>
<td>Seeds</td>
<td>Linum usitatissimum Linum</td>
<td>O.K</td>
</tr>
</tbody>
</table>

With regards

Yours sincerely

(Dr. H.B. Singh)

Mrs. Pratibha Nand
E-1024, Saraswati Vihar
DELHI-34
Dr. H.B. Singh  
Chief Scientist & Head  
Raw Materials Herbarium & Museum (RHMD)  
Phone: 011-25841143  
E-mail: hbs@niscair.res.in; hbsbhati@yahoo.com  

Dear Ms. Pratibha Nand  

Kindly refer to your letter nil dated 23/09/2011 for identification of one crude drug sample. The sample has been identified as given below.

<table>
<thead>
<tr>
<th>Sl No</th>
<th>Sample Received as</th>
<th>Part</th>
<th>Sample Identified as</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>Camellia sinensis</td>
<td>Leaves</td>
<td><em>Camellia sinensis</em> (L.) Kuntze</td>
<td>O.K.</td>
</tr>
</tbody>
</table>

With regards  

Yours sincerely  

( Dr. H.B. Singh)  

Ms. Pratibha Nand  
E-1024, Saraswati Vihar  
DELHI-110054
APPENDIX B: HPTLC OF MARKER COMPOUNDS
APPENDIX C: LIST OF PUBLICATIONS


Patent Office
INTELLIGENT PROPERTY BOARDING
New No. 82, Lancer 13, Shanti Niketan, Near Shri Hanuman Temple, New Delhi-110 021
Tel No: 011-26580030-31, 26580032, 26580033, 26580034, 26580035, 26580036, 26580037, 26580038, 26580039
E-mail: info@patentindia.gov.in
Website: www.ipindia.gov.in

Application Type: ORDINARY APPLICATION

To
UNIVERSITY SCHOOL OF BIOTECHNOLOGY GURU GOBIND SINGH INDRAPRASTHA UNIVERSITY
SUDHIR KUMAR GAMBIHR
SMD ENTERPRISES 322 R-20 PRATAP COMPLEX MAIN VIKAS MARG SHAKAR
PUR DELHI-110092

References: - CSR NO.: 2207 Dated: 19/03/2012 18:08:53
Received documents purporting to be an application for a patent numbered "S8-ELD2012 " dated
19/03/2012 18:08:53 by "UNIVERSITY SCHOOL OF BIOTECHNOLOGY GURU GOBIND SINGH
INDRAPRASTHA UNIVERSITY" of "SECTOR-15-C DIMAUKA NEW DELHI-110019" relating to "A NOVEL
HERBAL COMPOSITION FOR TREATMENT OF ACNE AND METHOD OF PREPARATION THEREOF"
together with the "Complete Specification" and fee(s) of Rs. 4000 (Four Thousand only)

Note:
1. In case of Patent Applications accompanied by a Provisional Specification, a Complete Specification should be filed within 12 months
   from the date of filing of the Provisional Specification, failing which the application will be deemed to be abandoned under Section 10(2) of the
   Patents Act, 1970.
2. You may withdraw the application at any time before the grant of patent, if you wish so, if in addition to withdrawal, you also wish to
   prevent the publication of application in the Patent Office Journal, the application should be withdrawn within fifteen months from the date of
   priority or date of filing, whichever is later.
3. If not withdrawn, your application will be published in the Patent Office Journal after eighteen months from the date of priority or date of
   filing, whichever is later.
4. If you wish to get your application examined, you should file a request for examination in Form 18 within 48 months from the date of
   priority or date of filing, whichever is earlier, failing which the application will be treated as withdrawn by the applicant under Section 10(2)(b)
   of the Patents Act, 1970.

for Controller of Patents & Designs
<table>
<thead>
<tr>
<th>Date</th>
<th>Patent</th>
<th>User</th>
</tr>
</thead>
<tbody>
<tr>
<td>06 May 2012</td>
<td></td>
<td>userid040082</td>
</tr>
</tbody>
</table>

**ACKNOWLEDGEMENT RECEIPT**

Date/Time : 06/05/2012 23:55:43

Name & Address :
UNIVERSITY SCHOOL OF BIOTECHNOLOGY, GURU GOBIND SINGH INDRAPRASTHA UNIVERSITY,
SUDHIR KUMAR GAMBIHR, OM ENTERPRISES, 202, R-20, PRATAP COMPLEX, MAIN VIKAS MARG, SHAHAR PUR, DELHI-110092

Application Number : 688/DEL/2012

Date of Filing : 09/03/2012 18:08:53

CBR Number : 2207

Title of invention : A NOVEL HERBAL COMPOSITION FOR TREATMENT OF ACNE AND METHOD OF PREPARATION THEREOF

Mode of Payment : Online Banking

Bank : AXIS

Amount in Rs. : 4000

For Controller of Patents & Design
Antimicrobial Investigation of Linum usitatissimum for the Treatment of Acne

Pratibha Naole*, Subhama Drabu* and Rajinder K. Gupta

1Malharjigari Srigujal Institute of Pharmacy, Jamnagar, New Delhi, India
2University School of Biotechnology, Guru Gobind Singh Indraprastha University, 16-C, Dwarka,
New Delhi-12, India

Received: April 23rd, 2011; Accepted: September 30th, 2011

Light petroleum, dichloromethane and methanolic extracts of Linum usitatissimum were investigated using GC/MS. The main components of three sequential extracts were methyl linoleate (31.6-35.9%), methyl linolenate (3.6-8.1%) and linolenic acid (1.4-7.1%). Components possessing antimicrobial activity against acne causing bacteria, namely α-linolenic acid (7.0-7.1%), α-linolenic acid (1.7-3.1%), terpinen-4-ol (1.3-4.6%), eugenol (1.6-7.1%) and 1,8-cineole (1.1-4.1%), were found in varying amounts. Antimicrobial testing indicated that the light petroleum extract was more active against Staphylococcus aureus and Pseudomonas aeruginosa with a MIC value of 2.5 mg/ml and a MBC of 2.5 mg/ml was observed against E. coli.

Keywords: GC-MS, Linum usitatissimum, antimicrobial activity, acne.

Flax, Linum usitatissimum Linn. (Linaceae), a versatile, blue-flowered herb of western Argentina, Russia and the USA [1] is cultivated in various temperate and tropical regions of the world. Egyptians, Hebrews, Greeks and Romans used its seeds as food and its fiber for textiles. Flax is a cool temperate annual herb [2]. Traditionally, it is cultivated for obtaining vegetable oil, which is known as linseed oil or flaxseed oil. This is then used in the manufacture of paints, varnishes and linoleum because of its drying and hardening properties when exposed to air and sunlight. The oil is rich in polyunsaturated fatty acids, particularly α-linolenic acid (α-LA- omega-3 fatty acid) (35-65%), linolenic acid (LA- omega-6 fatty acid) (6-20%), and oleic acid (12-30%) [3]. The two polyunsaturated fatty acids, LA and ALA, are essential for human beings because the body is incapable of synthesizing them and they must be obtained from the fats and oil present in food [4].

ALA accumulates preferentially in the skin and adipose tissues. ALA gets converted into the longer chain fatty acids EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid), whereas LA is converted to another long-chain fatty acid, arachidonic acid (AA). The body further converts AA and EPA into very powerful hormone-like substances called eicosanoids that affect physiological functions such as cell growth and division [5]. Flaxseed has been expected to be effective for a number of medicinal uses, mainly as a laxative, expectorant, and demulcent [6]. Recently it has been reported that flaxseed contains phytoestrogen compounds with weak estrogen or antiestrogenic activity, which have purported health benefits such as mitigation of hormone dependent breast cancer, prostate cancers, osteopenia, cognitive dysfunction, cardiovascular disease [7], immune system dysfunction, inflammation and infertility. Linumus, a major class of phytoestrogens, provide protection against cancer by blocking certain enzymes involved in hormone metabolism and interfering with the growth mechanisms and spread (metastasis) of cancer cells [8].

Flaxseed not only contains lignans but several other bioactive compounds namely phenolic acids, anthocyanin pigments, flavonoids, flavones, and phytic acid, which have been reported for their antimicrobial activity as they neutralize free radicals, which damage the cells [9-11]. Many clinicians believe ALA to be beneficial for overall skin vitality and epidermal integrity [12]. Flaxseed has been reported to possess anti-inflammatory potential [13-15] and the presence of ALA in it decreases eicosanoid synthesis. It is interesting to note that the demand for herbal cosmetics for treatment of acne is increasing due to less observed side effects, and the development of resistance of acne-causing bacteria towards antibiotics.

To the best of our knowledge, although previous reports revealed the composition, antibacterial and anti-inflammatory activity of L. usitatissimum, the literature lacks research concerning the utilization of these extracts...
Table 1: GC/MS profiling of Linnun utilisatissimum extract.

<table>
<thead>
<tr>
<th>Compound ID</th>
<th>RT (min)</th>
<th>% Arom.</th>
<th>LUPE</th>
<th>LUDCM</th>
<th>LUME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Styrene</td>
<td>4.03</td>
<td>0.5</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Benzocyclobutene</td>
<td>4.05</td>
<td>0.5</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>1-Thiophene</td>
<td>4.84</td>
<td>0.7</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>α-Pinene</td>
<td>5.02</td>
<td>1.1</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Decane</td>
<td>6.93</td>
<td>0.8</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Isopropen</td>
<td>7.50</td>
<td>3.1</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>4-Cymene</td>
<td>7.78</td>
<td>7.1</td>
<td>1.6</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Undecane</td>
<td>10.43</td>
<td>1.8</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>α-Terpine</td>
<td>8.96</td>
<td>11.1</td>
<td>1.7</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Terpinen-4-ol</td>
<td>13.30</td>
<td>11.7</td>
<td>1.3</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Dodecane</td>
<td>14.34</td>
<td>1.4</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Aromadendrene</td>
<td>22.43</td>
<td>0.4</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>α-Alloaromadendrene</td>
<td>22.48</td>
<td>0.4</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>β-Cadinene</td>
<td>25.26</td>
<td>0.6</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Hexadecane</td>
<td>27.51</td>
<td>1.3</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Heptadecane</td>
<td>30.44</td>
<td>0.5</td>
<td>8.4</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>1-Nonadecane</td>
<td>33.00</td>
<td>0.4</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>1-Docosene</td>
<td>33.03</td>
<td>0.4</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Octadecane</td>
<td>33.23</td>
<td>0.7</td>
<td>8.4</td>
<td>1.5</td>
<td>--</td>
</tr>
<tr>
<td>Dibromoethyl phthalate</td>
<td>35.09</td>
<td>1.5</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>1-Hipadecane</td>
<td>35.69</td>
<td>0.8</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Methyl palmitate</td>
<td>36.56</td>
<td>1.6</td>
<td>6.7</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>37.54</td>
<td>--</td>
<td>--</td>
<td>2.9</td>
<td>--</td>
</tr>
<tr>
<td>Nonacosane</td>
<td>38.40</td>
<td>0.4</td>
<td>4.3</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Tetratriocane</td>
<td>38.43</td>
<td>0.4</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Methyl linoleate</td>
<td>40.71</td>
<td>3.4</td>
<td>7.2</td>
<td>9.1</td>
<td>--</td>
</tr>
<tr>
<td>Methyl linolenate</td>
<td>40.87</td>
<td>11.9</td>
<td>34.2</td>
<td>33.9</td>
<td>--</td>
</tr>
<tr>
<td>α-Linolenic acid</td>
<td>41.92</td>
<td>7.0</td>
<td>--</td>
<td>7.1</td>
<td>--</td>
</tr>
<tr>
<td>Phytenol</td>
<td>41.16</td>
<td>--</td>
<td>--</td>
<td>1.1</td>
<td>--</td>
</tr>
<tr>
<td>Methyl ricetate</td>
<td>41.46</td>
<td>0.8</td>
<td>3.7</td>
<td>3.1</td>
<td>--</td>
</tr>
<tr>
<td>Methyl noreticolate</td>
<td>41.49</td>
<td>0.8</td>
<td>3.7</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>α-Linolenyl alcohol</td>
<td>41.91</td>
<td>--</td>
<td>--</td>
<td>7.1</td>
<td>--</td>
</tr>
<tr>
<td>Ethyl linoleate</td>
<td>42.30</td>
<td>--</td>
<td>3.7</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Docosane</td>
<td>43.14</td>
<td>--</td>
<td>0.4</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>1-Bromodocosane</td>
<td>43.13</td>
<td>0.4</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Cycloctadecane</td>
<td>44.77</td>
<td>0.8</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>1-Tricosane</td>
<td>45.23</td>
<td>1.1</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Triocane</td>
<td>47.35</td>
<td>--</td>
<td>3.0</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Tetraoctane</td>
<td>47.60</td>
<td>0.6</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Pentacosane</td>
<td>49.55</td>
<td>--</td>
<td>3.0</td>
<td>1.5</td>
<td>--</td>
</tr>
<tr>
<td>Ricosane</td>
<td>51.60</td>
<td>1.9</td>
<td>3.0</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Hemanosane</td>
<td>51.61</td>
<td>1.9</td>
<td>6.2</td>
<td>1.3</td>
<td>--</td>
</tr>
<tr>
<td>Tricosane</td>
<td>51.62</td>
<td>--</td>
<td>1.9</td>
<td>1.3</td>
<td>--</td>
</tr>
<tr>
<td>Hericosane</td>
<td>51.63</td>
<td>--</td>
<td>4.3</td>
<td>1.3</td>
<td>--</td>
</tr>
<tr>
<td>α-Zeocysphrate</td>
<td>52.37</td>
<td>0.8</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Linolenyl aldehyd</td>
<td>54.03</td>
<td>--</td>
<td>--</td>
<td>6.9</td>
<td>--</td>
</tr>
<tr>
<td>Hapicosane</td>
<td>54.04</td>
<td>1.8</td>
<td>6.1</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>54.06</td>
<td>1.0</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Tetratriocane</td>
<td>57.1</td>
<td>3.0</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

LUPE = light petroleum extract; LUDCM = dichloromethane extract; LUME = methanolic extract; RT = retention time of the compounds.

Preliminary phytochemical screening of the light petroleum, dichloromethane and methanolic extracts of Linnun utilisatissimum revealed the presence of terpenes, proteins and fatty acids. The three test extracts obtained were abbreviated as LUPE, LUDCM and LUME, respectively and their composition found by GC/MS analysis is presented in Table 1. Though 50 constituents were identified, the major components present in all the extracts were methyl linolate (11.9% - 33.9%) followed by methyl linolenate (3.4% - 9.1%). The amount of α-linolenic acid ranged from 7.0% to 7.1% in LUPE and LUME, but linoleic acid was not identified in any of the extracts. All the principal components documented for anti-acne activity, namely α-pinene, α-terpinene, terpinen-4-ol [18] and α-linolenic acids [19], were found in LUPE.

The antimicrobial screening was carried out on the three sequential extracts using ciprofloxacin phosphate as a positive control. The highest zone of inhibition was observed in LUPE (14.0 ± 0.08 mm) against P. aeruginosa. LUPE was found to be effective against aerobic and anaerobic test strains with an MIC of 1.25 mg/mL against S. aureus and P. aeruginosa, and 2.5 mg/mL against S. epidermidis. A MBC of 2.5 mg/mL was observed against S. aureus and P. aeruginosa. The MIC of LUDCM against S. aureus and S. epidermidis was 1.25 mg/mL and 2.5 mg/mL, respectively, whereas LUME was found to be active only against S. aureus with a MIC of 2.5 mg/mL.

Clindamycin showed remarkable antimicrobial activity against S. epidermidis and P. aeruginosa with an MIC of 0.08 mg/mL and a MBC of 0.16 mg/mL. Bacterial Susceptibility Index values indicated that S. aureus was susceptible to all three test extracts (100%), followed by S. epidermidis (66.7%), which was not susceptible to LUME and P. aeruginosa (33.3%), which was susceptible only to LUPE.

Experimental

Plant materials: Dried ripe seeds were procured from authorized herbal stores in Delhi, India. Their botanical identities were determined and authenticated at NISCAIR, Pusa Campus, New Delhi. A voucher specimen (NISCAIR/RCM/consul/2008-09/978/09) has been deposited at NISCAIR, New Delhi, India.

Extraction and yield: Flax seeds were pulverized, passed through sieve #10, and then extracted in a Soxhlet apparatus at room temperature. Sequential extraction of 200 g of flax seeds was undertaken with solvents of increasing polarity i.e. light petroleum (PE), dichloromethane (DCM), and methanol (Me). The extracts were evaporated under vacuum using a rotary evaporator and stored at 4°C in airtight containers for further studies. The percentage yield of the extracts is recorded in Table 2.

GC/MS analysis: Three sequential extracts were analyzed for volatile components with an Agilent 6890N gas chromatograph connected to a 5973B mass-selective detector. Chromatographic separation was on a capillary
Antimicrobial activity of *Limon setosissinum*

column of fused silica HP-5 ms (0.25 mm × 30 m × 0.25 µm). One µL of each extract was injected in the split mode (1:50) with an injector temperature of 280°C. The oven temperature was programmed, starting from 70°C (1 min) at 25°C/min to 150°C (10 min), at 3°C/min to 200°C (1 min) and at 8°C/min to a final temperature of 280°C (3 min). Helium was used as carrier gas. The detection was performed in El mode with an ionization energy of 70 eV, source at 230°C and quadrupole at 150°C. Relative percentage amount of the separated compounds were calculated automatically from the peak areas of the total ion chromatogram. The identity of the components was assigned by comparison of their retention time and spectral data with the corresponding data from the NIST 05 library [26].

**Evaluation of antibacterial activity:** The antibacterial activities of LUPE, LUDCM, LUME and standard clindamycin were determined using disc diffusion [21] and microdilution methods [22]. The microorganisms examined included aerobic *Staphylococcus aureus* (MTCC 96), *S. epidermidis* (MTCC 2659) and the anaerobic *Propionibacterium acnes* (MTCC *1951*). Organisms were supplied by the Microbial Type Culture Collection (MTCC) Centre, Institute of Microbial Technology, Chandigarh.

Stock solution of the test extracts having a concentration of 1% w/v was prepared in the solvent used for extraction [21]. One hundred µL of standardized bacterial suspension (5x10⁵ CFU/mL) was inoculated followed by loading of 20 µL of each extract on sterile discs. Overnight incubation was at either 37°C or 30°C depending on the type of aerobic bacteria. Similarly, for *P. acnes* incubation was in anaerobic chamber at 37°C having an atmosphere consisting of 10% CO₂, 10% H₂ and 80% N₂ for 48 h. Standard clindamycin (10 µg/disc) was used as positive control and the solvents as a negative control. Each test was performed in triplicate and the bioactivity was determined by measuring the diameter of inhibition zones in mm (Table 2).

MIC was determined by a microdilution method [22]. The test extracts were dissolved in DMSO (10%) and diluted further with DMSO to give concentrations ranging from 5 - 0.078 mg/mL and these dilutions were then distributed in 96 well plates, along with a growth control. Each test extract and control was analyzed by adding 20 µL of INT reagent solution (0.5 mg/mL) to detect growth. The trays were again incubated at either 37°C or 30°C for 30 min. All the experiments were performed in triplicate and the results were expressed in mg/mL (Table 2).

**Acknowledgments** — We acknowledge the financial support from All India Council for Technical Education 802/3/BOR/RID/RPS-27/2008-09 & UGC-SAP-3-23/2011. We also wish to thank Professor Ranma Chaudhary, HOD, Microbiology, All India Institute of Medical Sciences for providing the antimicrobial facility to carry out antibacterial activity of plant extracts.

**Table 2:** Yield (%) and antimicrobial activity of *Limon setosissinum*.

<table>
<thead>
<tr>
<th>Organism &amp; Extract</th>
<th>LUPE</th>
<th>LUDCM</th>
<th>LUME</th>
<th>Clindamycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yield (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>11.0</td>
<td>12.03</td>
<td>12.9</td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>2.5</td>
<td>5</td>
<td>0.625</td>
<td></td>
</tr>
<tr>
<td><em>Propionibacterium acnes</em></td>
<td>14</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>MIC (mg/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LUPE - light petroleum extract; LUDCM - dichloromethane extract; LUME - methanolic extract; * - zone of inhibition (Mean ± SEM); ** - MIC (mg/mL); *** - MBC (mg/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

References


IN VITRO ANTIBACTERIAL AND ANTIOXIDANT POTENTIAL OF MEDICINAL PLANTS USED IN THE TREATMENT OF ACNE

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ABSTRACT

In the present study, petroleum ether, dichloromethane and methanolic extracts of three medicinal plants namely Camellia sinensis, Glycyrrhiza glabra and Calendula officinalis were analyzed for their antibacterial and antioxidant activity in different test systems. Highest zone of inhibition (7±1) mm was observed with methanolic extract of Camellia sinensis using disc diffusion method. Phytochemical analysis indicated that amongst nine test extracts, methanolic extract of Camellia sinensis had the highest total phenolic content (104.93 ± 1.630 mg GAE/g). Flavanoid (115.50 ± 2.884 mg RGU/g dry extract) and flavonol content (574.446 ± 7.946 mg RGE/g dry extract) of methanolic extract of Glycyrrhiza glabra was also found to be superior among all the extracts. Antioxidant assays revealed highest DPPH scavenging (IC₅₀=44.03 ± 1.704 µg/ml) and metal chelating (EC₅₀=254.64 ± 5.467 µg/ml) effect in methanolic extracts of Camellia sinensis. Similarly, methanolic extract of Glycyrrhiza glabra exhibited highest radical scavenging activity (IC₅₀=21.37 ± 1.422 µg/ml) when reacted with the ABTS +.

Keywords: Acne, Free radicals, Medicinal plants, Total phenolic, Total flavonoid, Total flavonol, DPPH, ABTS, Metal chelating.

INTRODUCTION

Acne vulgaris is the most common skin disorder of pilosebaceous unit, generally characterized by formation of sebaceous comedones, inflammatory lesions and presence of bacteria namely Staphylococcus aureus, Staphylococcus epidermidis and Propionibacterium acnes in the follicular canal. 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. Although oxygen is an important component for human beings, yet it can produce various ROS such as superoxide anion, hydrogen peroxide and hydroxyl radicals etc. Furthermore, ROS play a critical role in irritation and disruption of the integrity of the follicular epithelium and are responsible for the progression of inflammatory acne 5. These toxic ROS can also act as messengers in the induction of several biological responses such as NF-κB, AP-1 and the generation of cytokines. 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 antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT) and glucose-6-phosphate dehydrogenase (G6PDH) existing in the cell, but due to the depletion of immune system and natural antioxidants in different ailments, it becomes necessary to use antioxidants as free radical scavengers for the removal of ROS to reduce cell damage that occurs during acne inflammation 6. Drugs like tetracycline, erythromycin, minocycline and metronidazole are gaining more importance and are preferred over antibiotics 7 because of their antioxidant effect. Moreover, antibiotic resistant acne has now become a critical problem worldwide. Hence, Antioxidants are extensively studied for their capacity to protect organisms and cell from damage that are induced by oxidative stress 1. There are a number of synthetic antioxidants like butylated hydroxy anisole, butylated hydroxy toluene, propyl gallate and gallic acid ester which are available but are suspected to cause negative health effects and are also unstable at elevated temperatures. Hence, the objective of our research work was to investigate antibacterial and antioxidant potential of the following three medicinal plants viz: Camellia sinensis 10, Glycyrrhiza glabra 11, and Calendula officinalis 12.

MATERIALS AND METHODS

Plant material, chemicals and reagents

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, New Delhi, India vide voucher specimen (NISCAIR/RHIM/consult/2007-08/956/120) and (NISCAIR/RHIM/consult/2008-09/978/079). Chlormycetin 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 powdered and passed through sieve #10 and were used for extraction in soxhlet apparatus at room temperature. Sequential extraction of 200 g was carried out with solvents of increasing polarity i.e. petroleum ether (PE), dichloromethane (DCM) and methanol (Me) and the extracts were abbreviated as: Camellia sinensis (CSP, CDD, CSME), Glycyrrhiza glabra (GGE, GDDM, GGDE) and Calendula officinalis (COP, CDDM, COPEM). The extracts were evaporated under vacuum conditions using a rotary evaporator and stored at 4°C in air tight containers for further studies. The percentage yield was recorded. Preliminary phytochemical screening of the test extracts was carried out 11, 12.

Antimicrobial screening of extracts

Microorganisms and media

Aerobic bacteria: Staphylococcus aureus (NTCC: 96), Staphylococcus epidermidis (NTCC: 5639) and anaerobic bacteria: Propionibacterium acnes (NTCC: 1551) 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 reinoculated chlorostaid 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% CO₂, 10% H₂ and 80% N₂ for 48 h.

Disc diffusion method

Antibacterial activity of extracts was tested using agar disc diffusion method 13. 100 µl of fresh culture suspension of test bacteria was evenly spread on nutrient agar and reinforced clostridial agar plates. The concentration of cultures was 5×10⁵ CFU/ml. For screening, 6 mm diameter filter paper discs, 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 39°C for 24 h and 48 h depending upon the type of bacteria under optimum conditions. Clear zones of inhibition were measured in mm and Chloramphenicol (10 µg/disc) was used as positive control.
Phytochemical analysis

Determination of total phenolic content

The total phenolic content of the nine extracts was determined using the Folin-Ciocalteu method with modifications. 100 ml of the diluted extracts containing 500 mg and standard phenolic compound gallic acid (10-50 mg/ml) were mixed separately with 62.5 ml of Folin-Ciocalteu reagent and diluted with 0.287 ml distilled water and 0.375 ml of 20% aqueous NaCO3. The mixtures were then allowed to stand for 2 h and the total phenolic content was determined using spectrophotometer at 765 nm. The concentrations of the total phenolic compounds were calculated using the equation \( y = 0.0276x + 0.0105; r^2 = 0.9964 \) and the total phenolic content was expressed as mg of Gallic acid equivalents (GAE)/g of dried extract.

Determination of total flavonoid content

The total flavonoid content of test extracts was determined using the method of Chang et al. with some modifications. 100 ml of the extracts containing 500 mg was mixed with 300 ml of distilled water and 30 ml of 5% NaNO2. The mixture was kept at room temperature for 5 min followed by addition of 30 ml of 10% AlCl3. 0.2 ml of 1 M NaOH and 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 \( y = 0.00100x - 0.00047; r^2 = 0.9945 \) obtained from the rutin (20-100 mg/ml) calibration curve and the flavonoid content was expressed as mg of rutin equivalents (RE) per g of dried extract.

Determination of total flavonoid content

Total flavonoid content of the test samples was determined using the method of Mikhailova et al. modified method. Calibration curve of the standard was prepared by mixing methanolic rutin solution (20-100 mg/ml) with 1 ml of 2% AlCl3 and 3 ml of 5% sodium acetate. The absorption was read after 2.5 hrs at 470 nm. The same procedure was carried out with 100 ml of plant extract (10 mg/ml) containing 500 mg instead of rutin solution. The flavonoid content was expressed as mg of rutin equivalents (RE) per g of dried extract using the equation \( y = 0.00442x - 0.00445; r^2 = 0.9961 \).

Antioxidant capacity

DPPH assay

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

\[ \text{DPPH scavenging effect} \% = 100 \times (A_0 - A_t)/A_0 \]

where \( A_0 \) is the absorbance of the control reaction and \( A_t \) is the absorbance of reaction mixture containing DPPH and extract at 517 nm. The antioxidant activity of the extract was expressed as IC50 value which is defined as the concentration (µg/ml) of extract that inhibits the formation of DPPH radicals by 50%. This was obtained from linear regression analysis.

ABTS+ radical cation decolorization assay

ABTS+ radical scavenging activity of the test samples was measured using the ABTS method with minor modifications. It measures the reduction of the ABTS radical cation by an antioxidant. ABTS radical cation (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) was produced by reacting 7 mM ABTS solution with 0.45 mM potassium persulfate in 100 mM phosphate buffer solution (pH 7.4) and keeping the mixture in a dark place at room temperature for 12-24 h before use. This solution was further diluted with 100 mM phosphate buffer solution (pH 7.4) to give an absorbance of 0.700 ± 0.02 at 734 nm. For the study of radical scavenging activity, 900 µl of ABTS was added to 100 µl of various concentrations (100-500 µg/ml) of the extracts and acetic acid. The reaction mixture was then incubated for 20 min and the absorbance was measured at 734 nm using methanol as a blank. ABTS+ radical scavenging activity was calculated using the same formula as mentioned in DPPH assay.

Metal chelating effect on ferric ion

The chelation of ferrous ions by test extracts was measured using the method of Dhindsa et al. modified method. To 1 ml of test extracts (100-500 µg/ml) and 50 µl of 2 mM FeCl3, in the reaction mixture, 0.2 ml of 5 mM ferrozine solution was added to initiate the reaction. The reaction mixture was then vigorously shaken and left undisturbed at room temperature for 10 min and then the absorbance of the mixture was measured at 562 nm. The chelating activity of the extracts was evaluated using FeCl3 as standard. The percentage inhibition of ferrozine-Fe3+ complex formation was calculated using the same formula as mentioned in DPPH scavenging activity.

Statistical analysis

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

RESULTS AND DISCUSSION

Antimicrobial screening

In vitro antimicrobial screening using standard discs as a positive control clearly indicated that CSME, GOME and COME showed promising antimicrobial activity against all the three organisms. It was observed that the extracts of C. sinensis and C. glabra showed significant antimicrobial activity against test organisms except E. coli which did not exhibit antimicrobial activity against S. epidermides. ODOD and COME were found to be significantly active against S. epidermis but did not show inhibitory activity against C. sinensis and P. acnes. Highest zone of inhibition, 17.8 ± 0.016 mm, was observed for CSME against S. epidermides (Table 1).

Table 1: Antimicrobial screening of plants against S. aureus (MTCC 96), S. epidermidis (MTCC 2639) and P. acnes (MTCC 1951) using disc diffusion method

<table>
<thead>
<tr>
<th>Zone of inhibition in mm</th>
<th>S. aureus*</th>
<th>S. epidermidis*</th>
<th>P. acnes*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PE</td>
<td>DCM</td>
<td>ME</td>
</tr>
<tr>
<td>Camellia</td>
<td>7.3</td>
<td>9.0</td>
<td>14.4</td>
</tr>
<tr>
<td>Ricinus</td>
<td>±0.28</td>
<td>±0.06</td>
<td>±0.27</td>
</tr>
<tr>
<td>Hydrastis</td>
<td>7.66</td>
<td>9.06</td>
<td>11.5</td>
</tr>
<tr>
<td>Glaucoma (RS)</td>
<td>±0.16</td>
<td>±0.06</td>
<td>±0.28</td>
</tr>
<tr>
<td>Gaulthera</td>
<td>NA</td>
<td>9.06</td>
<td>NA</td>
</tr>
<tr>
<td>Officinalis</td>
<td>±0.12</td>
<td>±0.17</td>
<td>±0.15</td>
</tr>
<tr>
<td>Chlauracyn</td>
<td>14.94</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Phosphate</td>
<td>±0.08</td>
<td>±0.11</td>
<td>±0.05</td>
</tr>
</tbody>
</table>
Le = leaves; R & St = roots and stolons; F3 = Fowers; PE = petroleum ether extract; DCM = dichloromethane extract; ME = methanolic extract; NA = No antibacterial activity. Values are Mean ± SE (n= n) of three measurements; *P< 0.05.

There is lack of reports revealing evidence for the antimicrobial activity of these medicinal plants against acne causing bacteria, still our findings could be based on the antibacterial action of epigallocatechin gallate and epicatechin in C. sinensis (3). Antibacterial and anti-inflammatory effect of G. glabra is attributed to the presence of glycyrrhizin and its hydrolysate glycyrrhetic acid (4). Presence of triterpenoids in C. officinalis is known to provide anti-inflammatory activity. It has also been reported that esters of faradiol-3-myristic acid, faradiol-3-palmitic acid and 4-taracaterol are the three most active compounds to reduce edema and the flavonoid, kaempferol, has antibacterial potential against P. acnes (5).

**Phytochemical analysis**

**Total phenolic, flavonoid and flavanol content**

Phenols are the simplest biactive phytochemicals possessing 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. The results of the present study clearly indicate (Figure 1) that amongst all the test extracts, CSME shows the highest amount of GAE of phenolic compounds (104.59± 1.630 mg/g).

![Fig. 1: Total phenolic content of test extracts](image1)

COPE did not show any phenolic content. Flavonoid and flavanol are polyphenolic compounds which play an important role in stabilizing lipid oxidation and are also associated with antioxidative action (2). Highest and lowest flavonoid content was observed in GGBME (115.503 ± 2.984 mg/g) and COPE (2.488 ± 1.659 mg/g) respectively (Figure 2).

![Fig. 2: Total flavonoid content of test extracts](image2)

Furthermore, flavonoid content of the extracts was between (574.443 ± 7.937 and 15.532 ± 1.229 mg/g). Highest flavonoid content was observed in GGBME (574.443 ± 1.367 mg/g). COPE didn't show any flavonoid content (Figure 3). The total phenolic, flavonoid and flavanol content of extracts was found to be statistically significant (P< 0.05).

Results from this study support the previous findings as *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 and their derivatives, rutin and myricitin, which have been shown to possess high antioxidant and free radical scavenging activity and positive effect on human health (6,7). Even Licorice flavonoid constituents are known to have free radical scavenging effect (8) and antioxidant potential (9). Licorice flavonoid constituents mainly consist of flavones, flavonols, isoflavones, flavanones, dihydroflavones and dihydrochalcones. *Calendula officinalis* also contains various flavonoids which include quercetin, kaempferol, rutin, isorhamnetin, isoquercetin, calendolainiade (10), calendolaviscid and calendoflavohloside thus having a potential role in antioxidant activity.

Highest flavonoid and flavonol content in GSBE and highest total phenolic content in CSME conform phytochemical screening. Basically, flavonoids are a family of polyphenolics which are synthesized by plants and can be categorized into different
subclasses and each subclass comprises of hundreds of different compounds like anthocyanidins, flavonoids, flavones, flavonols, flavanones and isoflavones. The activity of flavonoids is due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls making them responsible for the radical scavenging effect35 and chelating process respectively. It has also been reported44 that phenolic compounds exhibit redox properties by acting as reducing agents, hydrogen donors and singlet oxygen quenchers. Strong relationship35 was observed between 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 can lead to the destruction of pathogens. Furthermore, phenolic compounds directly contribute to the antioxidant action and act as free radical terminators, thereby impairing the inflammatory processes.36

Free radical scavenging assays
In recent years, focus has been on the toxicity related with oxidative stress while treating acne vulgaris because the rate of generation of ROS is more than the rate of its removal. This is due to the continuous production of free radicals even during the normal use of oxygen, as in respiration and other cell mediated immune functions. Free radicals due to environmental pollutants, radiation, chemicals, toxins, deep fried and spicy foods as well as physical stress, cause depletion of immune system antioxidants, change in gene expression and induced abnormal proteins.37 The biological system tries to protect against free radicals with the help of enzymes like SOD and CAT. SOD converts superoxide radicals (O₂⁻) into water and O₂, and similarly if the activity of CAT gets reduced, it leads to the accumulation of superoxide radicals and hydrogen peroxide.38

Effect on DPPH radical
The in vitro antioxidant activity of test extracts was estimated using DPPH assay. All methanolic extracts 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 color 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.

Lower IC₅₀ value indicated a greater antioxidant activity39. Our experimental data indicated that though all the test extracts demonstrated H-donor activity, the highest DPPH radical scavenging activity was observed in CSMB (IC₅₀ = 44.03 ± 1.784 µg/ml) followed by GGME (IC₅₀ = 51.97 ± 3.050 µg/ml) and GOME (IC₅₀ = 111.96 ± 1.129 µg/ml) presented in (Figure 4).

Mann-Whitney U test showed the comparison of ascorbic acid and active methanolic extracts. P value < 0.05 was observed in GOME indicating the data to be significantly different, whereas CSMB and GGME showed P value > 0.05.

![Fig. 3: Total flavonol content of test extracts](image1)

![Fig. 4: DPPH Scavenging effect (%) of methanolic extracts of active medicinal plants and ascorbic acid](image2)
ABTS radical scavenging activity

ABTS assay is an excellent tool to determine the antioxidant activity of hydrogen donating and chain breaking antioxidants. ABTS⁺ is a blue chromophore produced by the reaction between ABTS and potassium persulfate. Addition of the plant extracts to this preformed radical cation reduced it to ABTS in a concentration-dependent manner. Reduction of free radicals by the test extracts using ABTS was measured at 734 nm. Results showed that GGMME, CSME and COME exhibit potent antioxidant activity with IC₅₀ values of 21.37 ± 1.422 µg/ml, 28.99 ± 1.544 µg/ml and 33.03 ± 1.784 µg/ml respectively when the results were compared with standard ascorbic acid with an IC₅₀ value of 1.286 ± 1.066 µg/ml (Figure 5). P value < 0.05 was observed when ascorbic acid and active methanolic extracts were compared.

![ABTS Scavenging effect (%) of methanolic extracts of active medicinal plants and ascorbic acid](image)

**Fig. 5:** ABTS Scavenging effect (%) of methanolic extracts of active medicinal plants and ascorbic acid

Metal chelating activity

Metal chelating capacity of a sample plays an important role as it reduces the concentration of the transition metals which catalyze lipid peroxidation. It has been reported that chelating agents are effective as secondary antioxidants because they decrease the redox potential which ultimately stabilizes the oxidized form of the metal ion. In this assay, Ferrozine forms a violet complex with Fe⁺² but in the presence of a chelating agent, complex formation gets interrupted which results in a decrease in violet color of the complex. The results clearly demonstrated that the formation of the ferrozine-Fe⁺² complex is inhibited in the presence of the test and reference compounds. Amongst the thirteen test extracts used for in vitro antioxidant activity, CSME, GGMME and COME exhibited potent metal chelating activity with IC₅₀ values of 234.64 ± 5.467 µg/ml, 331.16 ± 1.972 µg/ml and 458.53 ± 1.393 µg/ml respectively when the results were compared with standard Na₂EDTA with an IC₅₀ value of 15.01 ± 1.884 µg/ml (Figure 6). Three methanolic extracts and standard Na₂EDTA showed significant difference (P<0.05).

![Metal Chelating effect (%) of methanolic extracts of active medicinal plants and ascorbic acid](image)

**Fig. 6:** Metal Chelating effect (%) of methanolic extracts of active medicinal plants and ascorbic acid

The chelating ability of the test extracts towards ferrous ions was investigated. Our findings revealed that the plant extracts are not as good as standard Na₂EDTA but the decrease in colour formation in the presence of the extract indicated that they possess iron chelating activity. Ferrous ions are the most effective pro-oxidants and are capable of stimulating lipid peroxidation by the Fenton reaction. They also promote peroxidation process by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals which themselves are capable of abstracting hydrogen and thus propagating the chain reaction of lipid peroxidation process.

CONCLUSION

The results clearly indicate that methanolic extracts of *C. sinensis*, *G. glabra* and *C. officinalis* possess broad-spectrum antibacterial activity. Presence of higher concentration of phenolic compounds in these test 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 acne. Therefore, further investigation is needed to explore the parameters essential for formulation so that antibacterial and
antioxidant potential of these medicinal plants can be utilized to provide safe and effective topical herbal formulation for the treatment of acne.

REFERENCES


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 ± 1.639 mg GAE/g) and FRAP value (1040.339±1.948 mg TE/g). Flavonoid content (115.50±3.2.994 mg RutE/g dry extract) of methanolic extract of Glycyrrhiza glabra was found to be superior among all the extracts. Highest DPPH scavenging (IC50=44.03 ±1.594 µg/ml) effect was also observed in methanolic extract of Camellia sinensis. In vitro antimicrobial screening indicated that methanolic extracts showed promising antimicrobial activity against Staphylococcus aureus, Staphylococcus epidermidis and Propionibacterium acnes.

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Key words:
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) [3]. Reactive oxygen species (ROS) namely hydroxyl (OH) and super oxide (O$_2^-$) and reactive nitrogen species (RNS) like nitrous oxide (N$_2$O), 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 [4]. 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, COCDM, COME) and *Linum usitatissimum* (LUPPE, 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 [9]. 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$_2$CO$_3$. 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
Gupta Rajinder K et al: Screening for Antioxidant and Antibacterial potential of common medicinal plants in the treatment of Acne

<table>
<thead>
<tr>
<th>Scientific name</th>
<th>(GAE) Phenolic Content (mg/g)</th>
<th>(RuE) Flavonoid content (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PE</td>
<td>DCM</td>
</tr>
<tr>
<td>Camellia sinensis (le)</td>
<td>14.16 ± 0.91</td>
<td>17.88 ± 1.85</td>
</tr>
<tr>
<td>Glycyrrhiza glabra (root)</td>
<td>60.52 ± 1.49</td>
<td>73.51 ± 1.45</td>
</tr>
<tr>
<td>Calendula officinalis (fl)</td>
<td>ND</td>
<td>52.26 ± 1.28</td>
</tr>
<tr>
<td>Linum usitatissimum (se)</td>
<td>1.62 ± 0.69</td>
<td>2.69 ± 0.25</td>
</tr>
</tbody>
</table>

le (leaves) ; ro (root) ; 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 [3]. 100 μl of the extract containing 500 μg was mixed with 300 μl of distilled water and 30 μl of 5% NaNO₂. The mixture was kept at room temperature for 5 min followed by addition of 30 μl of 10% AlCl₃ 0.2 ml of 1mM NaOH and 1ml distilled water. The absorbance of the reaction mixture was measured at 517 nm with UV spectrophotometer. The concentration of the flavonoid compounds was calculated using the equation (y = 0.0108x - 0.00476; R² = 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-picrylhydrazyl (DPPH) assay using Biot method with some modifications [2]. The reaction mixture contained 100 μl 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 (A₀ - Aᵣ)/A₀

where A₀ is the absorbance of the control reaction and Aᵣ is the absorbance of reaction mixture containing DPPH and extract at 517 nm. The antioxidant activity of the extract was expressed as IC₅₀ value which is defined as the concentration (μg/ml) of extract that inhibits the formation of DPPH radicals by 50%. It was obtained from the 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-tripryrdyl-2-tiazine) solution in 40 mM HCl, 5 mL of 0.05M FeCl₃·6H₂O and 50mL of 300 mM acetate buffer (pH 3.6) and was heated at 37°C. 100 μl of various extracts (100μg/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 [2]. The absorbance of the coloured reaction mixture (ferrous tripyrdyltriazine 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: \( y = 0.0749 + 0.003X, \quad r^2 = 0.9888 \) (Figure 2).

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

![DPPH scavenging effect graph](image)

**Figure 2:** Ferric reducing antioxidant power of test extracts

![Ferric reducing antioxidant power graph](image)

**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), C. 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 \[\cdot\]. 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 ± 1.650 mg/g) and the lowest was observed in LUP (1.520 ± 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 \[\cdot\], 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 \[\cdot\]. 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 \[\cdot\].

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 \textsubscript{50} value indicated a greater antioxidant activity \[\cdot\]. 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 \textsubscript{50} = 44.03 ± 1.784 µg/ml) followed by GGME (IC \textsubscript{50} = 51.07 ± 3.050 µg/ml) and COME (IC \textsubscript{50} = 111.96 ± 1.129 µg/ml) presented in (Fig. 1) whereas LUME (IC \textsubscript{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\textsuperscript{3+} TPTZ) complex and produce a coloured product, ferrous tripyridyltriazine (Fe\textsuperscript{2+} TPTZ) \[\cdot\]. 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 (450.089 ± 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.[24]

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.

Acknowledgments
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References

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21) Oktay M, Cukic I, Kudefioglu OL. Determination of in vitro antioxidant activity of fennel
Phytochemical and antimicrobial screening of medicinal plants for the treatment of acne

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Experiments on Camellia sinensis Linn. leaves, Glycyrrhiza glabra Linn. roots and rhizome and Calendula officinalis Linn. flowers were screened for their in vitro antimicrobial activity using agar disc diffusion method. The antimicrobial activity of petrol ether, dichloromethane and methanolic extracts of different parts of these plants were studied against acne causing bacteria, namely Staphylococcus aureus (MTCC 96), Staphylococcus epidermidis (MTCC 2639) and Propionibacterium acnes (MTCC 1951). Methanolic extract of C. sinensis leaves possessed highest antibacterial activity against S. epidermidis. Lowest minimum inhibitory concentration (0.625 mg/mL) and minimum bactericidal concentration (2.5 mg/mL) against S. epidermidis were also observed for methanolic extract of C. sinensis leaves. Phytochemical screening revealed the presence of alkaloids, flavonoids, glycosides and terpenoids which indicates that these phytoconstituents may be responsible for their anti-acne activity.

Keywords: Antimicrobial activity, Calendula officinalis, Camellia sinensis, Disc diffusion method, Glycyrrhiza glabra, Phytochemical screening.

IPC code; Int. cl. (2011.01)—A61K 36/00, A61P 31/10, A61P 17/10

Introduction

Camellia sinensis Linn. (Family-Theaceae) known as green tea, is the second most common beverage consumed worldwide next to water. It is an evergreen shrub or tree which is mainly cultivated in India and China. Three main varieties of tea have been reported namely green, black, and oolong. Green tea is made from unfermented leaves and contains the highest concentration of powerful antioxidants. Green tea has a number of pharmacological activities such as anticancer, lipid lowering, neurovascular blocking action, immunomodulatory effect, antiviral, antibacterial1, antispasmodic, antioxidant1, anti-inflammatory4, etc. A large number of phytoconstituents like alkaloids (caffeine, theobromine), proteins, enzymes, carbohydrates, lipids, polyphenols, catechins (epicatechin, epicatechin-3-gallate, epigallocatechin and epigallocatechin-3-gallate), carbohydrates, tannins, vitamins and minerals have been reported to be present in this plant5.

Glycyrrhiza glabra Linn. (Family-Fabaceae) known as liquorice, has been used in medicines for more than 4000 years now. It is native to central and south west Asia, Mediterranean basin of Africa, South Europe and India. Various species of liquorice have been reported namely Glycyrrhiza inflata Fisch., Glycyrrhiza uralensis Fisch. and Liquiritia officinalis Moench6. In the traditional system of medicine, liquorice is used as a demulcent, anti-tussive, laxative, sweetener, diuretic, antiarthritic, antibacterial7, anti-inflammatory8, anti-acne8, aphrodisiac, estrogenic, antioxidant, antineoplastic, anticholesterenic and antitussive9. Phytoconstituents isolated from this plant are glycyrrhizin, glycyrrhizinic acid, glabran A and B, glycyrrhetol, glabrolide, isoglaboride, isoflavonoids, coumarins, triterpene sterols liquiritin, liquiritin, flavonoids, chalcones and isofoflavonoids, such as glabridin10, 11.

Calendula officinalis Linn. (Family-Asteraceae) known as Pot marigold is an important medicinal plant for the indigenous people of India, Europe, US and China. Its leaves and flowers have been reported to possess many pharmacological activities which include antioxidant, anti-inflammatory, antibacterial and antiviral activities12-14. Phytoconstituents isolated from this plant are sitosterols, stigmas, lipids, ψ-teraxasterol, lupeol, faradiol-3-O-palmitate,

6Correspondent author: E-mail: rkg67@yahoo.com; Phone: +91 9871263252 (Mob.); Fax: +91 11 2386941/2.
faradiol-3-O-myristate, faradiol-3-O-laurate, quer cetin, iso hamnetin, isoclerentin, calendofloside, a-tocopherol and other sesquiterpenes.

Since the existing literature exhibits only a limited research concerning the utilization of these extracts in the treatment of acne, an attempt was made to evaluate the antimicrobial activity of petroleum ether, dichloromethane and methanolic extracts of leaves, roots, stolons and flowers of these plants, respectively, by agar diffusion and broth microdilution methods.

Materials and Methods

Plant materials

Samples of C. sinensis leaves, G. glabra roots and stolons and C. officinalis flowers were collected from medicinal gardens and authorized herbal stores in Delhi and authenticated by NISCAIR, Pusa Campus, New Delhi with voucher specimen (NISCAIR/RHM/consult/2008-09/778/09) and have been preserved in our department for the future reference.

Extraction and preliminary phytochemical screening

Shade-dried plant leaves, roots, stolons and flowers (200 g) were pulverized separately and subjected to sequential solvent extraction by continuous hot extraction (soxhlet) method. The extraction was done with different solvents in their increasing order of polarity such as petroleum ether (PE), dichloromethane (DCM), and methanol (ME) and the extracts were abbreviated as: Camellia sinensis (CSPE, CSDCM, and CSME), Glycyrrhiza glabra (GGPE, GGDCM, and GGME) and Calendula officinalis (COPE, CODCM, and COME). Every time, the marc was dried in air at room temperature and later used for extraction with other solvents. All the extracts were evaporated using a rotary evaporator and the percentage yield was thus recorded. Dried extracts were stored at 4°C in airtight containers for further studies. Concentrated extracts were subjected to various chemical tests in order to detect the presence of different phytoconstituents.

Microorganisms 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% CO2, 10% H2 and 80% N2 for 48 h.

Antimicrobial screening using disc diffusion method

Antibacterial activity of extracts was tested using agar disc diffusion method. Fresh culture suspension (100 μl) of test bacteria was evenly spread on nutrient agar and reinforced clostridial agar plates. The concentration of cultures was 5 x 10^6 CFU/mL. For screening, 6 mm diam. 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 and 48 h depending upon the type of bacteria under optimum conditions. Clear zones of inhibition were measured in mm, including the diameter of 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.

Minimum inhibitory concentrations using microdilution method

Minimum inhibitory concentration (MIC) of active medicinal extracts (AME) was studied by using broth microdilution method with slight modifications. Extracts were dissolved in (DMSO) dimethyl sulfoxide (10% of total volume) and two fold dilutions were done using pre-sterilized culture broth to give final concentrations ranging from 5-0.078 mg/ml. 100 μl of each dilution was distributed in 96 well plates. Sterility control (sterilized nutrient broth) and growth control (culture broth with DMSO) were also set up. Each test and growth control well was inoculated with 5 μl of a bacterial suspension (5x10^6 CFU/ml). All experiments were performed in triplicate and the microdilution plates were incubated under optimum conditions. Bacterial growth was detected after the addition of 20 μl of 70% alcoholic solution of INT (0.5 mg/ml) into each well followed by incubation for 30 minutes. Colour change from yellow to purple indicated the presence of microbial growth.

Minimum bactericidal concentration determination

The minimum bactericidal concentration (MBC) is defined as the lowest concentration of a compound
that kills the microorganism. The MBCs of the extracts were determined by plating 10 μL of samples from each MIC well without visible growth onto culture media plates. Following the incubation for optimum period, the plates were examined for colony growth and MBCs were recorded.

**Results and Discussion**

In present investigation, percentage yield of nine extracts indicated that GGME showed highest percentage yield (20.11) followed by COME (11.22) and CSME (9.55) (Figure 1). Phytochemical screening showed the presence of the following phytocomponents: C. sinensis (alkaloids, flavonoids, terpenoids and tannins), G. glabra (carbohydrate, glycosides, flavonoids, saponins, terpenes and sterol) and C. officinalis (flavonoids, saponins and terpenoids). *In vitro* antimicrobial screening using clindamycin phosphate as a positive control clearly indicated that CSME, GGME and COME show promising antimicrobial activity against all the three organisms (Table 1). It was observed that all the extracts of C. sinensis and G. glabra showed significant antimicrobial activity against test organisms except GGPE which did not exhibit antimicrobial activity against *S. epidermidis* (Figures 2–4).

![Image](image-url)

**Figure 1**—Percentage yield of plant extracts

**Figure 2**—Antimicrobial activity of plant extracts against *S. aureus*

**Figure 3**—Antimicrobial activity of plant extracts against *S. epidermidis*

<table>
<thead>
<tr>
<th>Plant Extracts</th>
<th>Percentage Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. sinensis</td>
<td>20.11</td>
</tr>
<tr>
<td>G. glabra</td>
<td>11.22</td>
</tr>
<tr>
<td>C. officinalis</td>
<td>9.55</td>
</tr>
</tbody>
</table>

![Table](table-url)

<table>
<thead>
<tr>
<th>Plant Extracts</th>
<th>S. aureus* (MTCC 96)</th>
<th>S. epidermidis* (MTCC 2639)</th>
<th>P. acnes* (MTCC 4905)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PE  DCM  ME  PE  DCM  ME  PE  DCM  ME</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. sinensis (Le)</td>
<td>14.4±0.27 7.4±0.05 10.0±0.06 7.4±0.05 17.8±0.16 7.6±0.18</td>
<td>7.8±0.01 13.8±0.15</td>
<td></td>
</tr>
<tr>
<td>G. glabra (R&amp;S)</td>
<td>9.0±0.06 11.5±0.28 8.0±0.12 11.8±0.15 7.1±0.05 13±0.05</td>
<td>13.9±0.15</td>
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<tr>
<td>C. officinalis (F)</td>
<td>9.0±0.12 11.8±0.15 9.13±0.08 12.4±0.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clindamycin phosphate</td>
<td>18±0.11 18±0.05</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Le = Leaves; R & St = Roots and Stolons; F = Flowers; PE = Petroleum ether extract; DCM = Dichloromethane extract; ME = Methanolic extract; NA = No antibacterial activity. Values are Mean ± SEM (mm) of three measurements; *P< 0.05.
Table 2: Minimum inhibitory and minimum bactericidal concentrations of AMEs and Clindamycin

<table>
<thead>
<tr>
<th>Active methanolic extracts</th>
<th>S. aureus (MTCC 96)</th>
<th>S. epidermidis (MTCC 2639)</th>
<th>P. acnes (MTCC1951)</th>
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<tr>
<td></td>
<td>MIC (mg/ml)</td>
<td>MBC (mg/ml)</td>
<td>MIC (mg/ml)</td>
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<tr>
<td>CSME</td>
<td>1.25</td>
<td>2.5</td>
<td>0.625</td>
</tr>
<tr>
<td>GGME</td>
<td>2.5</td>
<td>&gt;5</td>
<td>2.5</td>
</tr>
<tr>
<td>COME</td>
<td>NA</td>
<td>NA</td>
<td>2.5</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>0.312</td>
<td>0.63</td>
<td>0.078</td>
</tr>
</tbody>
</table>

CSME = Methanolic extract of Camellia sinensis; GGME = Methanolic extract of Glycyrrhiza glabra; COME = Methanolic extract of Calendula officinalis; NA = No antibacterial activity. Results are average of three measurements.

Fig. 4—Antimicrobial activity of plant extracts against P. acnes

CODCM and COME were found to be significantly active against S. epidermidis, however, they did not show inhibitory activity against S. aureus and P. acnes. Highest zone of inhibition (17.8±0.016 mm) was observed for CSME against S. epidermidis. The results, after preliminary antimicrobial screening, revealed that active methanolic extracts have potential in the treatment of acne hence, they were further evaluated for their MICs and MBCs. The lowest MICs against S. epidermidis (0.625 mg/ml), S. aureus (1.25 mg/ml) and P. acnes (1.25 mg/ml) were recorded in CSME (Table 2). Similarly lowest MBCs against S. aureus (2.5 mg/ml), S. epidermidis (2.5 mg/ml) and P. acnes (2.5 mg/ml) were also observed in CSME (Table 2).

Though there is lack of literature to provide evidence for the antimicrobial activity of these medicinal plants against acne causing bacteria, yet our findings could be based on the bactericidal action of epigallocatechin gallate and epicatechin in C. sinensis. Antibacterial and anti-inflammatory effect of G. glabra is attributed due to the presence of glycyrrhizin and its hydrolysis product, glycyrrhetinic acid. Presence of triterpenoids in C. officinalis is known to provide anti-inflammatory activity. It was also reported that esters of faradiol-3-myristic acid, faradiol-3-palmitic acid and 4-tanaxasterol are the three most active compounds to reduce edema and the flavonoid, kaempferol, demonstrated antibacterial activity against P. acnes. Hence, the antimicrobial activity of AMEs showed broad spectrum potential as the active compounds are concentrated more in ME fraction than in PE and DCM fractions which supports phytochemical screening.

Conclusion

The results clearly indicated that scientific studies carried out on these medicinal plants, possessing traditional claims of effectiveness in skin disorders, provided fruitful results. Therefore, methanolic extracts of C. sinensis, G. glabra and C. officinalis, possessing broad-spectrum activity could be utilized in treating acne vulgaris and formulating anti-acne herbal products. The main focus of our work is on the anti-acne potential of herbs which we plan to study further with the ultimate objective of providing scientifically validated herbal remedies against acne. Further in this regard, characterization is required to determine the types of compounds responsible for the antibacterial activity.

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