Research Article

Marker–based standardization and investigation of nutraceutical potential of Indian propolis

Nanaware Sadhana, Sathiyanarayanan Lohidasan, Kakasaheb Ramoo Mahadik
Department of Pharmaceutical Chemistry, Bharati Vidyapeeth University, Poona College of Pharmacy, Pune 411038, Maharashtra, India

ABSTRACT

OBJECTIVE: Propolis, a resinous material collected by honey bees from various plants, has been explored globally for its medicinal and nutritional properties. However, research over Indian propolis is at infancy. This study was designed to investigate nutraceutical potential of Indian propolis.

METHODS: In the present study, propolis extract was standardized with respect to markers caffeic acid phenethyl ester, caffeic acid, galangin, luteolin, curcumin, apigenin, pinocembrin and quercetin by new high-performance thin-layer chromatographic (HPTLC) methods. The physico-chemical analysis, residues analysis and in vitro antioxidant activity analysis were performed. Nutraceutical value was examined in terms of fats, fibers, minerals, proteins, polysaccharides, total carbohydrates, and energy value.

RESULTS: The developed HPTLC methods were found to be simple, reliable accurate, and the validation parameters were within the limits of the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use guidelines. Macerated ethanolic extract of propolis (MEEP) was found to have polyphenolic content of (20.99 ± 0.24) mg/g and flavonoids content of (8.39 ± 0.04) mg/g. MEEP was found to comprise of (283.33 ± 51.31) g/kg fats, (30.07 ± 7.30) g/kg fibers, (102.56 ± 2.84) g/kg proteins and (389.36 ± 57.50) g/kg carbohydrate with a calorie value of (38 409.33 ± 6 169.80) kJ/kg. It was found that Indian propolis exhibited high nutraceutical value and showed absence of pesticides and heavy metals. The MEEP showed in vitro antioxidant activity with inhibitory concentration of (12.24 ± 4.64) μg/mL.

CONCLUSION: The present work explores Indian propolis as a potential nutritious candidate. The proposed analytical methods can be applied in future screening of the quality of Indian propolis.

Keywords: propolis; high-performance thin-layer chromatography; nutraceutical value; antioxidant activity


1 Introduction

Propolis, a complex resinous material, is a mixture of wax and other substances collected from various plant species by stingless honey bees.1–3 It is used as a sealant for unwanted spaces in the bee hive. Propolis is composed of resin and balsams (50%–60%), pollen (5%–10%), polyphenols, flavonoids, phenolic acids and their esters, terpenoids, steroids and other constituents, including amino acids, minerals and vitamins A and B complex.4,5
Due to its high content of various amino acids, vitamins and minerals, propolis is believed to have nutraceutical value. More than 200 compounds have been reported in propolis, including rutin, galangin (GAL), quercetin (QUR), caffeic acid phenethyl ester (CAPE), caffeic acid (CA), apigenin (API), pinocembrin (PINO), luteolin (LUT) and curcumin (CUR). Many biological activities have been attributed to chemical constituents found in propolis. For example, propolis is extensively studied for potential antitumor effects from the activity attributed to components such as CAPE, artepillin C, p-coumaric acid and ferulic acid. Among the various chemical constituents, CAPE is one of the main biologically active components in propolis. Huang et al. showed CAPE’s ability to suppress the growth of human tumor cells in their in vitro experiments. CAPE is reported to inhibit nuclear factor-κB (NF-κB) activation through multiple immunomodulatory and anti-inflammatory pathways. It has also been used for cytotoxicity on oral submucosal fibroblast, neck metastasis of gingival carcinoma, tongue squamous cell carcinoma cells and treatment in oral cancer. GAL and API were identified as the principal monoamine oxidase (MAO)-inhibitory constituents. Cotherapies of CAPE and LUT, as well as CAPE and QUR, isolated from propolis, have been reported to have antibacterial activity. The highly variable composition of propolis may thus influence its medicinal activity, so there is need for standardization of propolis for clinical use.

A literature survey identified that there are reports for nutraceutical value of bee products from different sources, including Indian mustard bee pollen, Italian pollen, Argentinean propolis and Spanish propolis. However, the study of Indian propolis has just begun and is not yet extensively reported. A handful of studies have reported the chemical composition of Indian propolis, or its medicinal properties, such as anti-atherosclerotic, anti-inflammatory, antioxidant, anticancer, antimicrobial, antiplatelet and pro-oral health activities. Some methods have been reported for the analysis of Indian propolis including high-performance thin-layer chromatography (HPTLC), high-performance liquid chromatography (HPLC), gas chromatography-mass spectroscopy (GC/MS) and gas chromatography-flame ionization detection (GC-FID) methods.

However, no studies have been reported for standardization of Indian propolis in terms of marker compounds such as CA, CAPE, GAL, LUT, CUR, API, PINO and QUR. Furthermore, no attempt has been made to investigate the nutraceutical potential of Indian propolis yet. Hence, the aim of the present work was to optimize a method for extraction, standardization and analysis of physicochemical compounds and their activities, to determine the heavy metal and pesticide content and to investigate the nutraceutical potential of Indian propolis.

2 Materials and methods

2.1 Materials and reagents

Indian propolis, made by bee primarily visiting poplar trees, was collected from a local bee keeper in Bharatpur, Rajasthan, India, in the month of December. It was authenticated from the Central Bee Research and Training Institute, Pune, Maharashtra, India. The raw materials were packed in plastic bags and stored in a domestic freezer at −10 °C.

Analytical standards of LUT, GAL, CUR, API and QUR were procured from Natural Remedies Pvt. Ltd, Bangalore, Karnataka, India. PINO was procured from Mira Biotechnology, China. The pure CAPE and CA were procured from Sigma Aldrich, Bangalore, Karnataka, India. All chemicals and solvents used were of analytical grade (E. Merck, Ltd, Mumbai). Double distilled water was used throughout the study.

2.2 Preparation of extracts

2.2.1 Maceration

An accurately weighed quantity of 50 g of crude propolis was macerated with 150 mL of ethanol and kept for 10–15 d. The maceration was filtered, evaporated and the extract obtained was designated as macerated ethanolic extract of propolis (MEEP).

2.2.2 Microwave-assisted extraction

An accurately weighed quantity of 30 g of crude propolis was extracted in 200 mL of ethanol and heated in a microwave at maximum power consumption (1 200 Watts, voltage 230–240 V, 3 min, 50 Hz). This solution was frozen, filtered and the extract obtained was designated as microwave assisted ethanolic extract of propolis (MWEEP).

2.2.3 Ultrasound extraction

An accurately weighed quantity of 30 g of crude propolis was mixed with 200 mL of ethanol and sonicated for 1 h at room temperature (100% duty cycle and at constant frequency and power). The resulting mixture was filtered, evaporated and the extract obtained was designated as the sonicated ethanolic extract of propolis (SNEEP).

2.2.4 Soxhlet extraction

An accurately weighed quantity of 30 g of crude propolis was extracted using 200 mL of ethanol and a Soxhlet apparatus (Standard Scientific Glass I Industries, Mumbai, consists of borosilicate glass extractor, condenser and round bottom flask). The obtained extract was filtered and evaporated. It was designated as soxhlet ethanolic extract of propolis (SXEEP).
2.3 Instrumentation and chromatographic conditions

An HPTLC system (Camag, Switzerland) equipped with a Linomat V sample applicator fitted with a 100 µL syringe (Hamilton, Switzerland), a TLC Scanner III and running Wincats 4.02 integration software (Camag, Switzerland) was used for the analysis. The analysis was performed on precoated silica gel 60 F254 aluminum-coated TLC plates (20 cm × 10 cm). Two mobile phase systems, comprised of n-hexane:ethyl acetate:glacial acetic acid, 5:3:1 (v/v/v) and toluene: methanol:formic acid, 8:2:0.2 (v/v/v), were used for the simultaneous determination of CAPE, CA, GAL, LUT and CUR and API, PINO and QUR, respectively. The optimized chamber saturation time for the mobile phase was 45 and 20 min respectively, for the two solvent systems, at room temperature of (25 ± 2) °C and a relative humidity of 50% ± 5%. TLC plates were allowed to run up to 8 cm. After development, the plates were dried using a hair dryer and densitometric scanning was performed. The slit dimension was 5 mm × 0.45 mm, and a scanning speed of 20 mm/s was employed. Quantitative evaluation of the plates was performed in the absorption mode at 366 and 276 nm respectively.

2.4 Method validation

Validation parameters such as linearity, limit of detection (LOD), limit of quantification (LOQ), precision, specificity, accuracy and robustness were carried out as per International Conference on Harmonisation guidelines. The serial dilutions of standard solutions were subjected to HPTLC analysis in triplicate. The peak areas were plotted against the corresponding concentrations to obtain calibration curves. In order to ascertain the linearity, residual analysis was performed. The LOD and LOQ were determined from the multipoint calibration curve using standard deviation (SD) and slope of the calibration curve. The intraday and interday precision studies of all markers were carried out by estimating the corresponding responses three times on the same day and on three different days respectively. Measurement of peak area for active compounds was expressed in terms of percent relative standard deviation (% RSD). Specificity was studied by comparing peaks of marker compounds in propolis sample against different samples, including honey and pollen. Further, specificity was ascertained by overlaying their UV absorption spectra with those of the standards. The HPTLC method recovery study was carried out by spiking a known amount of standard drug corresponding to 90%, 100% and 110% (w/w) to sample (standard addition method). Accuracy was expressed as percent recovery. Robustness was evaluated by introducing small changes in volume of the mobile phase, proportion of one solvent in mobile phase, presaturation time and time from chromatography to scanning.

2.5 Selection of extraction method

Selection of extraction method was done by optimizing various methods on the basis of percent yield, amount of analyte in extracts, total polyphenolic content and total flavonoid content. The selected extract was used for further analysis.

2.5.1 Percent yield

The percent yield of dry extract was determined by calculating amount of extract yielded per unit of crude propolis. It was expressed in percent w/w.

2.5.2 Determination of analytes in extracts

The amount of each marker (CAPE, CA, GAL, LUT, CUR, API, PINO and QUR) was quantified for each extract using the proposed methods of HPTLC.

2.5.3 Total polyphenolic content

Total polyphenol content (TPC) of each extract was quantified with the Folin-Ciocalteu colorimetric method and it was expressed in terms of mg gallic acid equivalent (GAE)/g of extract.

2.5.4 Total flavonoid content

Total flavonoid content (TFC) of each extract was determined with the aluminum chloride colorimetric method and it was expressed in terms of mg quercetin equivalent (QE)/g of extract.

2.6 Physicochemical analysis

2.6.1 Moisture content

The moisture content of each extract was determined with a halogen moisture analyzer. Readings were taken in triplicate.

2.6.2 Total ash

Ash content of dried extract was determined by drying the sample at (550 ± 20) °C in a muffle furnace until constant weight was obtained. The ash content was calculated.

2.7 Residue analysis

2.7.1 Pesticide content

Pesticide content was determined using 410 Proster Binary LC with a 500 mass spectrometry ion trap analyzer photodiode array (MS IT PDA) detector, Varian Inc. with atmospheric pressure chemical ionization (APC) or electrospray ionization (ESI). To 2 g of sample, 8 mL of water and acetonitrile (10 mL in 1% acetic acid) were added. Next, 6 g of anhydrous magnesium sulfate and 1.5 g anhydrous sodium sulfate were added. After heating at 150 °C for 5 min, the mixture was kept in a desiccator until cool. This was then vortexed for 3 min and centrifuged at 4 000 r/min (radius of centrifuge: 7.2 cm) for 5 min. A 5 mL sample of the supernatant was transferred to a 15 mL polypropylene centrifuge tube containing 25 mg of primary secondary amine, shaken for 30 s and centrifuged for 5 min at 10 000 r/min. A 2 mL volume from this supernatant was mixed with 200 µL of 10% diethylene glycol solution.
and evaporated to dryness under nitrogen at 35 °C. This solution was reconstituted with 1 mL of methanol and 1 mL of 0.1% acetic acid, filtered through 0.2 µm membrane filter and injected (5–20 µL) into the LC-MS/MS.

2.7.2 Heavy metal content
Heavy metals analysis included arsenic (As), copper (Cu) and lead (Pb), following the Perkin Elmer Corporation (1982) modified method.[45,46]

2.8 Nutritional analysis
2.8.1 Total fat content
Fat or lipid content was determined by extracting a propolis sample with petroleum ether in a Soxhlet extractor at about 100 °C/12 h.[47] The extraction flask was subjected to a heating and cooling cycle to evaporate the solvent completely and the mass of the extract was determined; the difference in weight of the flask before and after extraction gave the fat content of the sample.

2.8.2 Fiber content
Total fiber content was measured following previously reported methods.[48] For fiber determination, a fat-free sample was used. The sample was boiled with sulfuric acid, followed by hot water. The resulting residue was boiled with sodium hydroxide, and washed with hot water. The resulting residue was dried in an oven at 100 °C for 2 h and cooled. The weight of the residue was recorded (W2).[47]

2.8.3 Mineral content
A sample of 2 g was kept in muffle furnace at 45–55 °C for 4–16 h to remove organic matter from the sample through thermal decomposition. Ash residue was dissolved in concentrated 10% nitric acid. The resulting extract was then used to determine mineral content with the ICP/MS instrument.[50]

2.8.4 Total protein content
Calculation of total protein content was carried out using the Lowry method. A standard calibration curve of bovine serum albumin (BSA) was prepared and used to calculate the protein content of samples in units of mg BSA/g.[49]

2.8.5 Total polysaccharide content
Polysaccharide content of propolis extract was determined using the phenol-sulfuric acid method.[50] This method employed a standard curve plotted using D-glucose in a concentration range of 60–90 µg/mL. It was expressed in terms of g equivalents of glucose/kg (g EG/kg).

2.8.6 Total carbohydrates
Total carbohydrate content of the propolis extract was calculated using the following formula[51] based on calculating nutrient values from other components:

\[ \text{Carbohydrates (g)} = 100 - (\text{protein (g)} + \text{fat (g)} + \text{moisture (g)} + \text{ash (g)}) \]

2.8.7 Energy value
The energy value of the propolis extract was calculated based on Atwater numbers using the following formula:[52]

\[ \text{Energy (kJ)} = 1/0.239 \times [4 \times (\text{protein (g)} + \text{carbohydrate (g)}) + 9 \times \text{fat (g)}] \]

2.9 In vitro antioxidant activity of MEEP
The antioxidant activity of propolis extract was assayed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity.[53] Extract solutions were prepared in different concentrations (10–60 µg/mL). The 0.1 mmol/L solution of DPPH was prepared in methanol. One mL of DPPH solution was mixed to 3 mL of different concentrations of extract. The mixture was shaken vigorously and kept for 30 min at room temperature. The absorbance was measured using UV-VIS spectrophotometer at 517 nm. DPPH-scavenging effect was calculated using the equation: Scavenging effect (%) = ((A_{DPPH} - A_s)/A_{DPPH}) × 100, where A_s is the absorbance of the solution when the sample extract has been added at a particular level and A_{DPPH} is the absorbance of the DPPH solution.

2.10 Statistical analysis
All samples were analyzed in triplicate unless otherwise stated and the results were expressed as mean ± standard deviation. The statistical analysis was carried out using the Prism 5.0 version of GraphPad software.

3 Results
3.1 Chromatographic condition and optimization
Several modifications in the mobile phase composition were conducted in order to study the possibilities of improving the performance of the chromatographic system. These modifications included: the type and ratio of the polar and nonpolar solvents, peak shape modifier, scanning at different wavelengths and the effect of saturation time.

Different polarity solvents were used for all tested analytes in different proportions, such as toluene, ethanol, methanol, ethyl acetate, n-hexane, n-butanol. As simultaneous determination of all markers in a single method could not be achieved, two different methods were tried. When the proportion of n-hexane was increased in the first method, bands of marker compound moved toward lower RF value with lower resolution and improper peak shape. Addition of glacial acetic acid improved peak shape. When the proportion of ethyl acetate was increased, marker bands moved toward the solvent front. Finally, n-hexane:ethyl acetate:glacial acetic acid (5:3:1, v/v/v) with a saturation time of 45 min and scanning wavelength of 366 nm was determined to be optimal for simultaneous determination of CAPE, CA, GAL, LUT and CUR. In the second method, an increase...
in the toluene proportion resulted in QUR to be close to the application site and other analytes eluted at lower R_f values. When the methanol proportion was increased, PINO moved to solvent front. Formic acid was added to the mobile phase as a peak shape modifier. Finally, the toluene:methanol:formic acid ratio was optimized at 8:2:0.2 (v/v/v), with a saturation time of 20 min and scanning wavelength of 276 nm for simultaneous determination of API, PINO and QUR.

3.2 Method validation

Linearity was determined using six concentration levels with calibration curves plotted over a wide concentration range. The results are shown in Tables 1 and 2. The interday and intraday precisions of both methods were calculated for all marker compounds using three concentrations in the triplicate. The % RSD values were found to be less than 2% and have been shown in Tables 1 and 2.

No interference of other constituents of propolis with the R_f values of standard compounds (CAPE (0.70), CA (0.44), GAL (0.77), LUT (0.32) and CUR (0.60) in first method and QUR (0.26), API (0.33) and PINO (0.49) in second method (Figure 1 and Figure 2) was observed. For specificity testing, the samples were compared with samples of honey and pollens; it was found that there was no interference. Furthermore, the specificity was confirmed by overlaying the UV absorption spectra of compounds with those of the standards. The results showed that the method was specific and effective enough to separate the markers from the four different extracts.

LOD and LOQ (Tables 1 and 2) indicated that the proposed method exhibited a good sensitivity for the quantification of selected marker compounds. The recovery for the selected markers is shown in Table 3. Moreover, no significant effect had been observed on peak areas of markers (% RSD < 2), when slight changes were made in the volume of the mobile phase, proportion of one solvent in the mobile phase, presaturation time and time from chromatography to scanning (Tables 4 and 5).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Method I of validation parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter</td>
<td>CAPE</td>
</tr>
<tr>
<td>Absorption maximum (nm)</td>
<td>330</td>
</tr>
<tr>
<td>Linearity range (ng/band)</td>
<td>200–450</td>
</tr>
<tr>
<td>Correlation coefficient (r^2)</td>
<td>0.997</td>
</tr>
<tr>
<td>Regression equation</td>
<td>y = 5.325x + 5.495</td>
</tr>
<tr>
<td>Limit of detection (ng/band)</td>
<td>16.74</td>
</tr>
<tr>
<td>Limit of quantitation (ng/band)</td>
<td>50.73</td>
</tr>
<tr>
<td>Intraday (% RSD)</td>
<td>≤ 2</td>
</tr>
<tr>
<td>Interday (% RSD)</td>
<td>≤ 2</td>
</tr>
<tr>
<td>Accuracy</td>
<td>99.8–101.5</td>
</tr>
</tbody>
</table>

CAPE: caffeic acid phenethyl ester; CA: caffeic acid; GAL: galangin; LUT: luteolin; CUR: curcumin; % RSD: percent of relative standard deviation.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Method II of validation parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter</td>
<td>API</td>
</tr>
<tr>
<td>Absorption maximum (nm)</td>
<td>268</td>
</tr>
<tr>
<td>Linearity range (ng/band)</td>
<td>20–120</td>
</tr>
<tr>
<td>Correlation coefficient (r^2)</td>
<td>0.998</td>
</tr>
<tr>
<td>Regression equation</td>
<td>y = 26.89x + 451.6</td>
</tr>
<tr>
<td>Limit of detection (ng/band)</td>
<td>5.22</td>
</tr>
<tr>
<td>Limit of quantitation (ng/band)</td>
<td>15.84</td>
</tr>
<tr>
<td>Intraday (% RSD)</td>
<td>≤ 2</td>
</tr>
<tr>
<td>Interday (% RSD)</td>
<td>≤ 2</td>
</tr>
<tr>
<td>Accuracy</td>
<td>97.9–102.6</td>
</tr>
</tbody>
</table>

API: apigenin; PINO: pinocembrin; QUR: quercetin; % RSD: percent of relative standard deviation.
Figure 1  Densitogram of markers of standards and samples with their $R_f$ values
Densitogram of markers caffeic acid phenethyl ester (CAPE, 0.70), caffeic acid (CA, 0.44), galangin (GAL, 0.77), luteolin (LUT, 0.32) and curcumin (CUR, 0.60) as standards (A) and in macerated ethanolic extract of propolis (B), microwave assisted ethanolic extract of propolis (C), sonicated ethanolic extract of propolis (D) and soxhlet ethanolic extract of propolis (E).

Figure 2  Densitogram of markers standard and sample and their $R_f$ values
Densitogram of markers quercetin (QUR, 0.26), apigenin (API, 0.33) and pinocembrin (PINO, 0.49) as standards (A) and in macerated ethanolic extract of propolis (B), microwave assisted ethanolic extract of propolis (C), sonicated ethanolic extract of propolis (D) and soxhlet ethanolic extract of propolis (E).
3.3 Selection of extraction method

Factors considered for the optimization of the extraction methods were percent yield, quantity of markers, TPC and TFC. The percent yield of dried extract was found to be higher in MEEP than in other extracts (Figure 3). The quantity of each marker (CAPE, CA, GAL, LUT, CUR, API, PINO and QUR) was estimated in the four different extracts by the proposed methods (Table 6). The percentage of each marker was found to be higher in the maceration extract, except galangin, which was found to be higher in MWEEP compared to other extracts.

The results of the TPC and TFC analyses for all extracts are shown in Table 7. The TPC was found to be higher in MEEP and TFC was found to be higher in MWEEP. The total polyphenolic content was expressed in mg equivalent of gallic acid/g while total flavonoid content was expressed in mg equivalent of quercetin/g. TPC was estimated with the linear regression equation \( y = 0.158x - 0.006 \), which was obtained from the standard calibration curve of gallic acid. TFC was estimated with the linear regression equation \( y = 0.050x - 0.082 \), which was obtained from the standard calibration curve of quercetin.

3.4 Physicochemical analysis

The moisture content of the MEEP sample was found to be \((52.60 \pm 4.61)\) g/kg, using the moisture analyzer. Ash content of MEEP was found to be \((55.05 \pm 2.81)\) g/kg.

### Table 3 Results of recovery study

<table>
<thead>
<tr>
<th>Marker compound</th>
<th>Amount in sample (ng/spot)</th>
<th>Total amount after addition of standard (%) (ng/spot)</th>
<th>Amount found after addition of standard (%) (ng/spot)</th>
<th>Recovery after addition of standard (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAPE</td>
<td>600 540 600 660</td>
<td>548.31 598.84 660.40</td>
<td>101.5 ± 10.78 99.80 ± 0.98 100.0 ± 2.11</td>
<td></td>
</tr>
<tr>
<td>CA</td>
<td>400 360 400 440</td>
<td>362.55 370.90 429.76</td>
<td>100.7 ± 6.13 92.72 ± 1.57 97.67 ± 1.96</td>
<td></td>
</tr>
<tr>
<td>GAL</td>
<td>600 540 600 660</td>
<td>534.40 596.23 661.93</td>
<td>98.96 ± 3.14 99.37 ± 2.80 100.1 ± 2.08</td>
<td></td>
</tr>
<tr>
<td>LUT</td>
<td>600 540 600 660</td>
<td>534.40 596.23 661.93</td>
<td>98.96 ± 3.14 99.37 ± 2.80 100.1 ± 2.08</td>
<td></td>
</tr>
<tr>
<td>CUR</td>
<td>600 540 600 660</td>
<td>534.40 596.23 661.93</td>
<td>98.96 ± 3.14 99.37 ± 2.80 100.1 ± 2.08</td>
<td></td>
</tr>
<tr>
<td>API</td>
<td>600 540 600 660</td>
<td>510.06 596.30 677.42</td>
<td>97.87 ± 3.10 99.38 ± 1.21 102.6 ± 4.75</td>
<td></td>
</tr>
<tr>
<td>PINO</td>
<td>200 180 200 220</td>
<td>179.90 200.40 222.38</td>
<td>99.94 ± 1.90 100.20 ± 2.34 101.1 ± 2.00</td>
<td></td>
</tr>
<tr>
<td>QUR</td>
<td>700 630 700 770</td>
<td>611.94 672.53 754.13</td>
<td>97.13 ± 1.90 96.07 ± 1.58 97.93 ± 2.64</td>
<td></td>
</tr>
</tbody>
</table>

Recovery values are represented with mean ± standard deviation (n = 3). CAPE: caffeic acid phenethyl ester; CA: caffeic acid; GAL: galangin; LUT: luteolin; CUR: curcumin; API: apigenin; PINO: pinocembrin; QUR: quercetin; % w/w: percent weight by weight.

### Table 4 Method I of robustness testing

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SD of area (n = 3)</th>
<th>% RSD for area (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CAPE</td>
<td>CA</td>
</tr>
<tr>
<td>Volume of mobile phase (± 1 mL)</td>
<td>18.60</td>
<td>65.35</td>
</tr>
<tr>
<td>Proportion of n-hexane in mobile phase (± 0.1 mL)</td>
<td>19.51</td>
<td>53.85</td>
</tr>
<tr>
<td>Time from spotting to chromatography</td>
<td>18.43</td>
<td>35.54</td>
</tr>
<tr>
<td>Time from chromatography to scanning</td>
<td>17.75</td>
<td>16.03</td>
</tr>
</tbody>
</table>

Values are represented with standard deviation (SD) and % relative standard deviation (% RSD) in three independent experiments. CAPE: caffeic acid phenethyl ester; CA: caffeic acid; GAL: galangin; LUT: luteolin; CUR: curcumin.

### Table 5 Method II of robustness testing

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SD of area (n = 3)</th>
<th>% RSD for area (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>API</td>
<td>PINO</td>
</tr>
<tr>
<td>Volume of mobile phase (± 1 mL)</td>
<td>42.84</td>
<td>29.77</td>
</tr>
<tr>
<td>Proportion of toluene in mobile phase (± 0.1 mL)</td>
<td>28.69</td>
<td>32.34</td>
</tr>
<tr>
<td>Time from spotting to chromatography</td>
<td>33.52</td>
<td>55.51</td>
</tr>
<tr>
<td>Time from chromatography to scanning</td>
<td>42.94</td>
<td>23.28</td>
</tr>
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</table>

Values are represented with standard deviation (SD) and % relative standard deviation (% RSD) in three independent experiments. API: apigenin; PINO: pinocembrin; QUR: quercetin.
3.5 Residues analysis

The identification of residues was done by comparison of retention time (Rt) and absorbance spectrum of standards and samples. By measuring the peak area of the selected analytes, with respect to the internal standard control, quantification of residues was conducted. This ratio was plotted onto the linear calibration curves for each standard solution. Pesticide content and heavy metals were not detected in MEEP. However, copper was found in concentrations of 0.28 mg/L parts per million (ppm). About 113 pesticides were tested including phorate, ediphenphos, dimethoate, tricyclazole, phosphamidon, malaoxon, atrazine, malathion, triazophos, bifenazate, mandipropamid, azadiractin, spiридiclofen and butachlor. All pesticides were found to be absent from the sample.

3.6 Nutritional analysis

The fat content of MEEP was (283.33 ± 51.31) g/kg, while the total fiber content was (30.07 ± 7.30) g/kg.

The mineral analysis of propolis revealed that the sample was rich in calcium (19.2 ppm), zinc (4.72 ppm), iron (1.3 ppm), manganese (0.09 ppm), boron (0.19 ppm), rubidium (0.08 ppm), strontium (0.01 ppm), molybdenum (0.02 ppm), barium (2.84 ppm), aluminium (3.2 ppm) and lithium (0.03 ppm).

Total protein content was estimated using the linear regression equation \[ y = 0.005x + 0.024, \] which was obtained from the calibration curve of standard BSA. The protein content was found to be (102.57 ± 2.84) g/kg.

Total polysaccharide was estimated to be (6.79 ± 0.19) g EG/kg from the linear regression equation \[ y = 0.005x - 0.267, \] which was obtained from the standard calibration curve of glucose. MEEP was higher in carbohydrates with (493.6 ± 54.69) g/kg. It also had a high energy value (40 406.14 ± 4 801.12 kJ/kg).

![Figure 3](image-url)

**Figure 3** Comparison of percent yield of four different extracts
MEEP: macerated ethanolic extract of propolis; MWEEP: microwave assisted ethanolic extract of propolis; SNEEP: sonicated ethanolic extract of propolis; SXEEP: soxhlet ethanolic extract of propolis.

### Table 6 Content of markers found in different extracts

<table>
<thead>
<tr>
<th>Marker</th>
<th>Content in extracts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MEEP</td>
</tr>
<tr>
<td>CAPE</td>
<td>1.47 ± 0.11</td>
</tr>
<tr>
<td>CA</td>
<td>0.99 ± 0.18</td>
</tr>
<tr>
<td>GAL</td>
<td>9.30 ± 0.08</td>
</tr>
<tr>
<td>LUT</td>
<td>1.26 ± 0.06</td>
</tr>
<tr>
<td>CUR</td>
<td>1.31 ± 0.03</td>
</tr>
<tr>
<td>API</td>
<td>2.62 ± 0.12</td>
</tr>
<tr>
<td>PINO</td>
<td>8.07 ± 0.34</td>
</tr>
<tr>
<td>QUR</td>
<td>1.05 ± 0.22</td>
</tr>
</tbody>
</table>

Values are represented with mean ± standard deviation (n = 3). CAPE: caffeic acid phenethyl ester; CA: caffeic acid; GAL: galangin; LUT: luteolin; CUR: curcumin; API: apigenin; PINO: pinocembrin; QUR: quercetin; MEEP: macerated ethanolic extract of propolis; SNEEP: sonicated ethanolic extract of propolis; SXEEP: soxhlet ethanolic extract of propolis.

### Table 7 Summary of TPC and TFC in four different extracts

<table>
<thead>
<tr>
<th>Extract</th>
<th>TPC (mg GAE/g)</th>
<th>TFC (mg QE/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEEP</td>
<td>20.99 ± 0.24</td>
<td>8.39 ± 0.04</td>
</tr>
<tr>
<td>MWEEP</td>
<td>8.40 ± 0.39</td>
<td>13.66 ± 0.82</td>
</tr>
<tr>
<td>SNEEP</td>
<td>8.76 ± 0.37</td>
<td>14.07 ± 0.09</td>
</tr>
<tr>
<td>SXEEP</td>
<td>5.15 ± 0.39</td>
<td>13.26 ± 0.16</td>
</tr>
</tbody>
</table>

Values are shown as mean ± standard deviation (n = 3). TPC: total polyphenolic content; TFC: total flavonoid content; MEEP: macerated ethanolic extract of propolis; MWEEP: microwave assisted ethanolic extract of propolis; SNEEP: sonicated ethanolic extract of propolis; SXEEP: soxhlet ethanolic extract of propolis.
3.7 DPPH antioxidant activity

The MEEP showed a scavenging effect between 38.0% and 95.63% (Figure 4). The IC50 values for the MEEP extract and standard ascorbic acid solution were 12.23 and 17.17 μg/mL respectively. In the present study, MEEP exhibited in vitro antioxidant activity at each of the different concentrations.

![Figure 4](image)

**Figure 4**: 2,2-Diphenyl-1-picrylhydrazyl-scavenging effect of macerated ethanolic extract of propolis (MEEP) and standard ascorbic acid

4 Discussion

Over the past decades, interest on nutraceuticals and natural products has been constantly growing. Nutraceuticals have become targets of high-commercial and research interests on account of their nutritional and therapeutic benefits. A number of antioxidant formulations from different sources, especially from natural products, have emerged on the market. One such natural product, Indian propolis, is known for its wide range of medicinal properties, including antioxidant activity. In the present study we have reported the nutraceutical potential of Indian propolis for the first time, along with its physiochemical properties and some aspects of standardization.

Marker-based standardization is the recent trend in standardization of natural products. As natural products contain many chemical constituents, there is always uncertainty about their safety and efficacy. Thus, well-defined chemical or biologically active markers can be selected and standardization with respect to those markers can be achieved using sophisticated analytical techniques. Standardized extracts help quantify the percentage of selected marker compounds, which are responsible for its biological activity. Thus, considering their biological activities, CAPE, CA, GAL, LUT, CUR, API, PINO and QUR were selected as markers for Indian propolis. Several TLC or HPTLC methods have been reported for the estimation of the marker compounds CAPE, CA, GAL, LUT, CUR, API, PINO and QUR either individually or in combinations. However, there has not yet been a single HPTLC report for the simultaneous separation of more than two markers in Indian propolis. Therefore, in this study, two methods for simultaneous estimation of selected markers have been developed. Among the different solvent systems investigated, mobile phases consisting of n-hexane:ethyl acetate:glacial acetic acid, 5:3:1 (v/v/v) and toluene:methanol:formic acid, 8:2:0.2 (v/v/v) were found to be suitable for the simultaneous determination of CAPE, CA, GAL, LUT and CUR and API, PINO and QUR, respectively. The validation results showed that the proposed method can be used for routine determination of all tested compounds in Indian propolis extract.

Over the past few years, investigations for phenolics in medicinal herbs have gained importance due to their high antioxidant properties. Variations in the TPC and TFC of propolis have been reported to depend upon the geographical source, bee species and type of plants accessible to the bees. The Indian propolis sample examined possessed considerable TPC and TFC, as compared with Korean and Brazilian propolis samples. These findings indicate that propolis with high polyphenol and flavonoid contents should be preferred for commercial propolis products because of the biological significance of the polyphenols and flavonoids.

Keeping this in mind, in the present study, extraction optimization was done using four different extraction methods to maximize the yield of biologically active components from Indian propolis. Overall, MEEP was found to have high percent yield, with higher content of markers and higher TPC. But TFC was found to be higher in SXEEP extract. Considering the TPC, percent yield, higher marker content and easier method of preparation, the MEEP was selected for further analysis.

Heavy metal analysis showed arsenic and lead levels below the detection limit. Copper was found in a low concentration, but it was within normal range, so it could not be considered as contaminant. Absence of pesticides and heavy metals in the MEEP sample suggested that MEEP can be considered to be safe for internal use.

Danert et al. reported that Argentinean propolis contained macro-elements (glucose, fructose, sucrose and proteins), microelements (sodium, potassium, calcium, phosphorous and magnesium) and trace elements (iron), whereas Spanish propolis has been reported to contain minerals such as aluminum, calcium, iron, potassium, magnesium and phosphorus. In the present study in Indian propolis mineral content of sodium, magnesium, phosphorous, sulfur, potassium, selenium and cobalt was found to be within acceptable limits. Nutraceutical parameters, such as fat and fiber content, were also found to be in standard range. Further, it was found that samples of MEEP were rich in protein. MEEP was high in carbohydrates and had high energy value.
DPPH radical scavenging test is the most used method for determining antioxidant activity. It has a high reproducibility and low cost, and it can be made quickly. Thus, the antioxidant activity of MEEP was studied using the DPPH radical scavenging test. Banskota et al.[65] and Moreno et al.[66] reported that water and alcoholic propolis extracts have DPPH free radical scavenging activity, and the activity was attributed to the flavonoid content of propolis.[67] Our results showed significant DPPH radical scavenging activity by MEEP, which can be attributed to the considerable content of phenolics and flavonoids.

In conclusion, the present work explores Indian propolis as a potential nutraceutical agent. Our sample was comprised of 10.33% (w/w) of protein, 49.36% (w/w) of total carbohydrates, 28.33% (w/w) of fats and 3% (w/w) of fiber. The propolis can also be considered as a rich source of high calories. This study showed the absence of heavy metals and pesticides in MEEP, thus it can be considered as safe for use. The study also demonstrated high polyphenol and flavonoid content, suggesting its potential for use in treating oxidative stress. Further, the proposed analytical methods are simple, precise and accurate and specific for quantification and identification of caffeic acid phenethyl ester, caffeic acid, luteolin, galangin, curcumin, apigenin, pinocembrin and quercetin in MEEP, which can be applied for regular quality control analysis of Indian propolis.

5 Acknowledgements

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6 Competing interest

None declared.

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