Novel Phytochemical Constituents Identified from the Seeds of *Mesua ferrea* L: Chemical Characterization and Antimicrobial Activity

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Received: December 12, 2017; Accepted: December 27, 2017; Published: December 29, 2017

Abstract

Novel Phytochemical constituents were isolated from the unsaponifiable matter extracted from seeds of *Mesua ferrea* with the help of n- hexane solvent. The three known compounds, two new aromatic esters and two new oleanane- type pentacyclic triterpenoidal saponins, were identified by spectral (IR,¹H NMR,¹³C NMR spectra, mass spectrum, elemental analysis) and chemical analysis. The identified aromatic esters namely, 5- formyl-2- (propan-2-yl) phenyl acetate, 2,4-diformyl-6-(propan-2-yl) phenyl acetate, and two new oleanane- type pentacyclic triterpenoidal saponins identified were 2α,3β-28 olenolic acid and 2α,3β-28 ursanolic acid (FIG. 1). Antimicrobial activities were assessed for all these isolated compounds. Antimicrobial activities were assessed for the isolated compounds, and variable activities were observed. Among these some of the compounds showed significant zone of inhibition against *B. subtilis* and *P. vulgaris*, while one of these compounds exhibit remarked antifungal activity against *A. niger* and *A. flavus*. To the best of our knowledge, for the first time these novel compounds and being reported along with their antimicrobial properties.

Keywords: *Mesua ferrea* L; Seeds; Calophyllaceae; Novel compounds; Antimicrobial activity.

Introduction

Mesua ferrea L. commonly known as Nagkesar belongs to family Calophyllaceae [1]. The plant is used medicinally in various ailments. The decoction of seeds of M. ferrea is given for the treatment of gastritis, bronchitis and to cure snake bite [2]. Leaves of M. ferrea are antidote for scorpion sting. The different extracts of the plant have shown antiulcer, anti venom, anti protozoal, anti-cancer, anti-oxidant activities [3]. The present study reports the isolation and structural elucidation of four new compounds (FIG. 1) isolated from the seeds of Mesua ferrea.


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Experimental

General procedures
Melting points (mp) are uncorrected. $^1$H NMR was recorded on 300 MHz Varian XL spectrometer, $^{13}$C NMR spectra were recorded on the Varian XL 75 MHz spectrometer, IR spectra were recorded in KBr disk on Perkin Elmer-377 spectrometer, EIMS on Jeol-JMS D 300 mass spectrometer. All chemical shifts (δ) are given in ppm and Me4Si was used as internal the standard. Chemicals used were of the analytical-reagent grade and column chromatography was carried out on alumina grade III and TLC on silica gel G (CDH/Glaxo laboratories). Spots were visualized by exposure to iodine vapor or by spraying with H2SO4-vanillin solution followed by heating at 105°C for 5 min.

1

2

3

4

5
Plant material
The seeds (10 kg) of M. ferrea were collected from the market of Ujjain city and were identified by the authorities of the Institute of Environment Management and Plant Science, Vikram University, Ujjain. A voucher specimen was deposited in the herbarium of the School of Studies in Botany, Vikram University, Ujjain, India.

Extraction and isolation
The seeds (10 kg) were shade dried, cleaned, coarsely powdered and extracted with hexane in soxhlet-extractor for 72 h. The extract was concentrated by rotary evaporator to afford oil (265 mL). The oil was saponified by the alcoholic potash method [4]. Usual work up yielded (32 gm) of an unsaponifiable matter which was separated by repeated column chromatography on alumina grade III. The column was eluted by gradient elution in increasing order of polarity like hexane, benzene, EtOAc and methanol. The fractions were collected in bulk and monitored by TLC. The residue (6.8 g) of hexane fraction was rechromatographed on alumina on the basis of increasing order of polarity of eluents. A well-stirred suspension of alumina III (100-150 g in hexane 60-80) was poured into the column (150 cm long and 50 mm in diameter). When the absorbent was well settled, the excess of hexane was allowed to pass through the column. With silica gel in hexane, the mass was made into slurry and digested in a well stirred column. The column was successively eluted with the hexane, benzene, EtOAc and methanol and their mixtures of increasing polarity. Fractions (a, b and c) (hexane:benzene, 3:1 v/v, and 1:1 v/v, benzene:ether 9:1 v/v) were purified and identified as lawsaritol(1), stigmasterol (2) and β-sitosterol (3). Presence of these sterols were analyzed by IR, $^1$H NMR, $^{13}$C NMR and mass spectrometry and compared with the literature data (Patra et al., 2010, Jain et al., 2009). Fraction (d) afforded a mixture of esters 4 and 5 and other impurities. Fraction (d) was further rechromatographed by eluent (hexane:benzene (3:1 and 9:1 v/v) to give compound (4) and (5) in pure form respectively, fraction (e) yielded compound (6).

Compound 1
Lawsaritol (1). Elution of column with hexane yielded colorless crystals, recrystallize from methanol, 240 mg; m.p 107°C; IR (KBr) vmax: 3429 cm$^{-1}$; EIMS m/z (% intensity) 414 M$^+$ (C$_{29}$H$_{38}$O), 393, 359, 342, 316, 288, 215, 174,146.
**Compound 2**
B-sitosterol (2). Elution of column with hexane yielded colorless crystals, recrystallize from methanol, 80 mg; m.p 136°C; IR (KBr) vmax: 3429 cm⁻¹; EIMS m/z (% intensity) 414 M⁺ (C₂₉H₅₀O), 363, 301, 205, 174, 149.

**Compound 3**
Stigmasterol (3). Elution of column with hexane yielded colorless crystals, recrystallize from methanol, 95 mg; m.p 164°C; IR (KBr) vmax: 3430 cm⁻¹; EIMS m/z (% intensity) 412 M⁺ (C₂₉H₄₈O), 393, 272, 244, 215, 174, 146.

**Compound 4**
5-formyl-2-(propan-2-yl) phenyl acetate (4) ESI-MS m/z (% intensity) 206 (M⁺), 198, 179, 109, C₁₂H₁₄O₃ (45 mg, methanol), m.p 85°C; Elemental analysis (Calculated for C₁₂H₁₄O₃, C= 69.88 %, H= 6.84 %, O= 23.27 %), Observed values (C= 69.60 %, H= 6.94 %, O=23.11 %). Isolated from benzene:methanol (1:2 v/v) fraction using chloroform:methanol:acetic acid (6:4:0.5, v/v) as solvent system. IR Spectrum (KBr) υmax: 2967, 1603, 1429, 1216, 1044, 925, 761, 670 cm⁻¹, ¹³C NMR spectrum (75 Hz, CDCl₃, ppm) 133.5, 131.6, 127.1, 141.7, 192.8, 160.0, 22.1, 12.1 ppm.

**Compound 5**
2, 4-diformyl-6-(propan-2-yl) phenyl acetate (5) ESI-MS m/z (% intensity) 234 (M⁺), 275, 207, 179, 127, C₁₃H₁₄O₄ (70 mg methanol), m.p 95 °C, Elemental analysis (Calculated for C₁₃H₁₄O₄, C= 66.66 %, H= 6.02 %, O= 27.32 %) Observed values; (C= 67.49 %, H= 6.00 %, O=26.12 %). Isolated from (hexane: benzene 1:1 v/v) fraction. On TLC examination it showed a single spot using (hexane: benzene: acetic acid 9:1:0.5, v/v). IR Spectrum (ʎmax, KBr, cm⁻¹): 3022, 1638, 1679, 1436, 1296, 1216, 759, 669, 595, 553 cm⁻¹, ¹³C NMR spectrum (75 Hz, CDCl₃, ppm):126.4, 136.3, 136.0, 117.9, 144.1, 190.24, 195.81, 165, 22.1, 12.1 ppm.

**Compound 6**
2α, 3β-28 olenolic acid and 2α, 3β-28 ursanolic acid (6) ESI-MS 472 (M⁺), 467, 450, 436, 421, 408, 391, 300, 285, 255, 248, 235, 219, 203, 187, 173, 159, 133, 199, 105, 95, C₃₀H₄₈O₄ (45 mg methanol), m.p 80°C, Elemental analysis: (Calculated for C₃₀H₄₈O₄, C=76.23 %, H=10.24 %, O=13.54 %) Observed values; (C=77.11 %, H=10.58 %, O=13.27 %). Isolated from (benzene: ether 3:1 v/v) fraction, TLC chloroform: methanol: acetic acid (5:4:0.5 v/v), IR Spectrum (ʎmax, KBr, cm⁻¹): 3429, 2967, 1384, 1602 cm⁻¹; ¹H NMR spectrum (300 MHz, Pyr, TMS, δ): δ 5.26 (t, -C=CH, J=3.5 Hz),δ 4.24 (d, J=10.3 Hz), δ 2.63 (dd, 1H, J=15.6, 5.2 Hz) for 6(a), δ 2.20 (d, J=11.3 Hz, 1H, C-18) for 6(b).

2.10. Screening of antimicrobial activity

The agar well diffusion method was used for the screening of antibacterial and antifungal activities using the filter paper disc method [5]. For antibacterial activity, the standards are in the form of sterile Hi-Disk cartridges, each disc containing 10 mg of the respective drug. For antifungal activity, Amphotericin B was used as standard. The results are shown in TABLE 1.
### TABLE 1. Antimicrobial assay of compounds (COMP, 1-6)

<table>
<thead>
<tr>
<th>Microbial Strains</th>
<th>Inhibition Zone (mm)</th>
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<tbody>
<tr>
<td></td>
<td>Mesua ferrea L. (seed)</td>
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<tr>
<td></td>
<td>COMP. 1</td>
</tr>
<tr>
<td>G +ve &amp; G –ve Bacteria</td>
<td></td>
</tr>
<tr>
<td>Bacillus Subtilis</td>
<td>21</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>20</td>
</tr>
<tr>
<td>Citobacter freundii</td>
<td>_</td>
</tr>
<tr>
<td>Escherchia Coli</td>
<td>_</td>
</tr>
<tr>
<td>Klebsiella Pneumoniae</td>
<td>_</td>
</tr>
<tr>
<td>Salmonella typhimurium</td>
<td>_</td>
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<tr>
<td>Proteus vulgaris</td>
<td>22</td>
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<tr>
<td>Pseudomonas aeruginosa</td>
<td>_</td>
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<tr>
<td><strong>Fungi</strong></td>
<td></td>
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<td>Candida albicans</td>
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<tr>
<td>Candida tropicalis</td>
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<tr>
<td>Aspergillus candidus</td>
<td>_</td>
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<tr>
<td>Aspergillus flavus</td>
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<td>Aspergillus niger</td>
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</table>

**Result and Discussion**

The novel natural compounds were identified by their IR, $^1$H NMR, $^{13}$C NMR and Mass spectrometry analysis including a comparison with the literature data. The known natural compounds lawsaritol, stigmasterol and β-sitosterol were identified as part of the lipid structures by IR, $^1$H NMR, $^{13}$C NMR spectra and mass spectral analysis and comparison with $^1$H NMR and $^{13}$C NMR data as given in the literature. The mass spectrum and elemental analysis of 5-formyl-2-(propan-2-yl) phenyl acetate indicated the molecular ion peak at m/z 206 suggesting its molecular formula C$_{12}$H$_{14}$O$_3$. IR spectrum showed absorption bands at 761, 862, 1429, 1603 and 121 cm$^{-1}$ which indicated the aromatic nature of compound [4]. $^1$H NMR spectrum showed the signal at δ 10.10 due to the presence of –CHO group in the molecule. The multiplets at δ 7.69 and δ 7.26 showed the presence of benzene ring in the molecule. The singlet at δ 2.59 was due to acetyl group. Doublet at δ 1.26 was assigned to –CH group and a multiplet at δ 3.2 showed the presence of isopropyl group in the molecule [6,7]. The $^{13}$CNMR peaks at 133.5, 131.6, 127.1, 141.7 ppm were due to benzene nucleus in the molecule. The peak observed at 192.8 ppm was due to –CHO group in the molecule. The carbonyl group of acetyl group was resonated at 160.0 ppm and the peaks observed at 22.1 and12.1 ppm were due to methine and methyl carbons of isopropyl group are present in the molecule [6,7].

The ESI-MS showed the molecular ion peak at m/z 206[M$^+$] suggesting its molecular formula as C$_{12}$H$_{14}$O$_3$. The other fragments were obtained at m/z 198, 179,109 were also consistent with the proposed structure [8,9]. Thus on the basis of above spectral evidences, the compound was identified and characterized as 5-formyl-2-(propan-2-yl) phenyl acetate and being reported first time by us.
The elemental and mass spectral analysis of 2,4-diformyl-6-(propan-2-yl) phenyl acetate (5) gave the molecular formula as C_{13}H_{14}O_4. IR spectrum showed the absorption band at 1638 cm\(^{-1}\) due to the presence of carbonyl group of aldehyde in the molecule. Absorption band at 1679 cm\(^{-1}\) indicates –O-CO-CH3 functionality in the molecule. \(^1\)H NMR spectrum showed two aromatic protons were resonated at \(\delta\) 7.26-7.93 as a sharp singlet, which indicates that other protons were derivatized naturally. The peak appeared at \(\delta\) 10.27 as sharp singlet indicates the presence of aldehyde group, in benzene nucleus. The peak appeared at \(\delta\) 10.48 which is deshielded, indicates that two aldehyde groups are present nearby acetoxy group (James and Das, 1976; Silverstein and Webster, 2003). The acetyl (-CO-CH3) methyl group was resonated at \(\delta\) 2.93 as a sharp singlet. It is more deshielded than ketone, which means it is attached as –O-CO-CH3. Two methyl groups were resonated at \(\delta\) 1.24 as a doublet and methane proton was at \(\delta\) 3.34 as a multiplet, which indicates that isopropyl group is present in benzene nucleus. The \(^{13}\)C NMR spectrum showed peaks at 126.4, 136.3, 136.0, 117.9, 144.1ppm, were due to benzene nucleus in the molecule. The peaks observed at 190.24 and 195.81 were due to aldehydic group in the molecule, one is deshielded (195.81) due to the presence of –O-CO-CH3 group present at ortho- position to it. The carbonyl group of acetyl was resonated at 165ppm and the peaks observed at 22.1 and 12.1 ppm suggested that isopropyl group is present in the molecule [8,9]. ESI-MS Spectrum showed the molecular ion peak at 234 [M\(^+\)] which suggested its molecular formula as C_{13}H_{14}O_4. Other abundant fragments were obtained at m/z 275,207,179 and 127 were also consistent with the proposed structure. Thus on the basis of above spectral data compound (5) has been characterized as 2, 4-diformyl-6-(propan-2-yl) phenyl acetate. It is a novel compound and reported first time by us.

The elemental and mass spectral analysis of 2α,3β-28 olenic acid and 2α,3β-28 ursanic acid (6) gave the molecular formula as C_{30}H_{48}O_4. IR Spectrum (\(\lambda_{max}\), KBr, cm\(^{-1}\)) showed band at 3429 cm\(^{-1}\) for the presence of OH group. Bands at 2967 and 1384 cm\(^{-1}\) were due to –CH stretching and bending vibrations. Weak band at 1602 cm\(^{-1}\) showed the presence of unsaturation in the molecule. \(^1\)H NMR spectrum of 6(a) showed the presence of doublet of doublets centered at \(\delta\) 2.63 for one proton at C18. The two sharp singlets at \(\delta\) 1.96 and 62.03 were attributed to two hydroxyl groups present at C-3 and C-2 positions respectively and a ddd centered at \(\delta\) 5.23 was due to hydroxyl bearing proton at C-2 while that of C-3 gave a doublet at \(\delta\) 4.24. Peak at \(\delta\) 5.26 was observed for the olefinic proton resonating for the double bond at C-12:C13 position in 6 (a). Another characteristic of oleananes i.e the appearance of seven methyl signals as sharp singlets at \(\delta\) 0.94, 0.86, 1.21, 0.75, 1.06, 0.85, 0.92 for C-23, C-24, C-25, C-26, C-27, C-29, C-30 respectively, was observed. Thus 6 (a) gave clear evidence to be an oleanane type triterpene with two hydroxyl groups [10].

\(^1\)H NMR spectrum of 6 (b) was characterized to be of ursane type triterpene on the basis of appearance of doublet at \(\delta\) 2.20 for the proton at the C-18 as concluded from the literature [11]. The methyl signals appeared as singlets for the positions at C-23, C-24, C-26, C-27 whereas doublets appeared for the C-29 and C-30 position, typical characteristic pattern of ursane type triterpene at \(\delta\) 0.96, 0.86, 1.21, 0.75, 1.06 and 0.85 (d), 0.94 (d) respectively (Pandey et al., 2013). Hydroxyl bearing protons at C-2 appeared at \(\delta\) 5.23 while that of C-3 gave a doublet at \(\delta\) 4.24. Peaks centered at \(\delta\) 5.26 were observed for the olefinic proton resonating for the double bond at C-12: C-13 positions in 6 (b).

\(^{13}\)C NMR spectrum supported the presence of the two isomers in the (6). The hydroxyl group at C-2 was concluded to be an α and that at C-3 was β as evident from the values observed at 69.4 and 80.3 ppm. The presence of carboxylic acid functionality at C-28 position was confirmed by the signal at \(\delta\)183.2 ppm in the molecule. In \(^{13}\)C NMR spectrum of pentacyclic triterpenes of α- amyrin type (2α, 3β-28 ursanic acid) and β- amyrin type (2α, 3β-28 olenolic acid), the major difference between both series were found to be in chemical shifts for the olefinic carbon atoms (C-12 and C-13) and for the carbon atoms belonging to ring E. A comparison in the ppm values between olefinic carbon signals (C-12 and C-13) for both
series indicated a higher value for the 19β (equatorial) methyl group which is in close proximity to the double bond in the urs-12 ene series, thus there steric effects influence the chemical shifts of these carbons based on this fact the double bond at C-12:C-13 positions in case of 6 (a) was concluded from the peak at 145.4 and 123.4 ppm, whereas for 6 (b) it was observed at 138.5-126.8 ppm [12-14].

ESI-MS Spectrum showed the molecular formula was found to be C30H48O4, M’ 472. The base peak at m/z 248 arising from the Retro Diels Alder fragmentation favored the olenane or ursane type of carbon skeleton of the molecule. The base peak at m/z 248 [203+COOH] and the peak at m/z 203 revealed the presence of carboxylic acid group at C-17 position of the molecule. The other diagnostic peaks were observed at m/z 133, 147, 188, 203 and 391. Thus after the comparison of the data with the literature available, the two compounds in (6) were found to be positional isomers of each other and their structures were established as 2α, 3β-28 ursanolic acid and 2α, 3β-28 olenolic acid [10].

**Screening of antimicrobial activity**

The result has shown that compound 1 and compound 6 showed significant zone of inhibition against *B. subtilis* and *P. vulgaris*. Compound 6 exhibit remarked antifungal activity against *A. niger* and *A. flavus* whereas compound 1 did not exhibit any antifungal activity against any of the tested fungal strains.

**Conclusions**

From the survey of the literature, to the best of our knowledge for the first time, all these compounds are being reported from seeds of *M. ferrea* L. Besides their isolation and chemical characterization, their antimicrobial properties were also accessed for the first time. Further examination of the constituents of this plant is currently in progress.

**Acknowledgements**

Authors are highly thankful to CDRI Lucknow, IIIM Srinagar (J&K) for the use of different techniques of NMR and mass spectra.

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