ABSTRACT

The chemical examination of methanolic extract of the plant, L. citriodora yielded two known compounds; oleanolic acid (1) and saccharose (2). The structures of the compound were established based on physical and chemical data (UV, IR, $^1$H and $^{13}$C NMR and mass) and also co-comparison with an authentic compounds. The crude extract, fractions and isolated compounds were studied for elastase inhibition and dendrite elongation activity. The compound, oleanolic acid showed good elastase inhibition activity.

Key words: Lippia citriodora, chemical constituents, oleanolic acid, elastase inhibition

INTRODUCTION

Before 20$^{th}$ century treatment for infections and diseases were based primarily on folklore medicine. Combination of plant materials with microbial properties were used to treat various infections.$^{[1]}$ The pharmaceutical industry did not exist in early 1900 BC and there were no synthetic drugs or medicines for treating skin disorders, instead people turned to nature and looked for natural remedies. Many cosmetic industries are showing great interest on natural products to treat various skin disorders.$^{[2]}$ The use of plant materials can lead to the identification of new bio-active skin care agents for the benefit of consumers.$^{[3]}$ There are several agents have been identified, tested and have given promising results under laboratory conditions.$^{[4]}$

*Lippia citriodora* Kunth belongs to Verbenacea, widely distributed in tropical, subtropical, central to South America, as well as in Africa. The shrub is commonly grown in Indian gardens, especially on hills. The plant is propagated from seeds and cuttings and thrives well on loamy soil. Generally, the leaves are being used for flavoring beverages, desserts, fruit salads and jellies and also for seasoning food. A decoction made from the leaves and flowers is given as febrifuge, sedative and anti-flatulent.$^{[5]}$ The plant showed antispasmodic, antimicrobial properties and is traditionally used to treat *Candida*.$^{[6]}$ The volatile oil obtained from the leaves collected from different places showed differently.$^{[7]}$ The essential oil obtained from the leaves was analyzed by GC-MS showed mixture of several terpenes and the major compounds are 1,8-cineole (23.66%), α-curcumene (14.83%), geranial (13.74%), limonene (13.4%) and caryophyllene oxide (6.6%).$^{[8]}$ Several class of compounds, flavonoids, terpenoids, phenolics have been reported from this plant. These are flavonoids like., apigenin, chrysoeriol, cirsimaritin, luteolin, salvigenin, artemetin, hesperidin and vitexin, terpenoids like., citral, citronellal, linalool, borneol, cineol, eugenol, nerol, limonene, 3-caryophyllene, myrcenene, pyrollic acid, isovaleric acid.$^{[6]}$ The essential oil obtained from the leaves of lemon verbena (*L. citriodora*) could be used as a potential control agent against stored product insects.$^{[6]}$

Based on the above information and our continuous interest on the isolation of biologically active molecules from Indian medicinal plants for personal care applications.$^{[9-17]}$ we have undertaken the leaves of *L. citriodora* for chemical examination. In this article, we report the isolation and structure elucidation of two known secondary metabolites, 1-2 (Figure 1) and their biological studies. Structures of the compound were established based on...
physical and chemical data and also comparison with an authenticated compound. According to our knowledge, no study has been reported previously relating to the elastase inhibition activity on this plant.

MATERIALS AND METHODS

General
UV: Shimadzu UV spectrophotometer; IR: Prestige 21 FT IR (Shimadzu); NMR: ¹H and ¹³C NMR (Bruker AMX 400); Mass spectrum: Jeol SX 102/DA 600 mass spectrometer. Column chromatography (CC) was carried on a silica gel column (100-200 mesh). Purity of the samples was checked by TLC on pre-coated aluminum sheets and coated with silica gel 60 F254 (20 X 20 cm, 0.2mm thickness, Merck) and compounds were detected under UV light (254 & 366 nm) and spraying with 5% sulphuric acid in methanol followed by heating the plates at 110°C for 5 min. The chemical shift values are reported in ppm (δ) units and the coupling constants (J) are in Hz.

Plant material
The leaves of *Lippia citriodora* (1.5 kg) were collected from Kalvarayan hills, near Salem, Tamil Nadu, India in July 2008 and it was identified by Prof. D. Subramaniam (Retd), Taxonomist, Department of Botany, Annamalai University, Annamalai Nagar, Tamil Nadu India. A voucher specimen of this plant was deposited in Department of Botany, Annamalai University, Annamalai Nagar, Tamil Nadu, India.

Extraction and Isolation procedure
The leaves of *Lippia citriodora* (1.5 kg) were exhaustively extracted with methanol (3.0 L) by using soxhlet apparatus. The solvent was removed by rotary evaporator under reduced pressure at ~40°C to get 76g crude methanolic extract. The crude extract (45g) was suspended in methanol: water (2:8), partitioned with n-hexane, chloroform, ethyl acetate and saturated n-butanol to get corresponding fractions 17.64g, 5.5g, 3.5g and 4.7g respectively. The hexane fraction showed moderate elastase inhibition activity and compound C showed potent activity. Fraction C showed mixture of two amorphous powder, mp.270-272°C. Other fractions did not yield any compound. The n-butanol fraction showed solid nature after keeping at room temperature. The fraction was washed with excess of methanol, the colorless solid was obtained which was identified as saccharose (2, 120mg). Both compounds were submitted for elastase inhibition activity and compound 1 showed good inhibition.

Oleanolic acid (1, Fig.1): Colorless amorphous powder, mp: 270-272°C; UV (MeOH)nm: end absorption; IR (KBr) cm⁻¹: 3382 (br), 1710, 1485, 1114, 1070 and 833cm⁻¹; ¹H NMR (400 MHz, CD2OD): δ 0.74, 0.77, 0.87, 0.89, 0.94, 1.11 (each 3H, s), 2.80 (1H, br d, J=11.93 Hz, 18-H), 3.12 (1H, br s, 3-H), 5.22 (1H, br s, 12-H); ¹³C NMR (100 MHz, CD2OD): 618.9, 19.3, 20.5, 22.0, 26.5, 27.1, 27.2, 29.5, 30.4, 30.5, 31.7, 34.4, 36.4, 36.6, 40.8, 42.5, 43.1, 45.6, 50.2, 59.9, 61.6, 62.7, 63.5, 68.1, 68.4, 69.5, 70.1, 74.8, 75.5, 79.0, 89.8, 103.9, 169.4, 169.6, 169.8, 169.9, 170.0, 170.4, 170.6.

Saccharose (2, Fig. 1): Colorless crystals, mp: 271-272°C; UV (MeOH)nm: end absorption; IR (KBr) cm⁻¹: 3322 (br), 1066 and 835cm⁻¹; ¹H NMR (400 MHz, D2O): δ 5.17 (1H, d, J=3.8 Hz) 3.98 (1H, d, J=8.8 Hz), 3.81 (1H, t, J=8.6Hz), 3.70-3.60 (2H, m), 3.60-3.55 (4H, m), 3.51 (1H, t, J=11.4Hz), 3.43 (2H, s), 3.30 (1H, dd, J=3.8, 13.8 Hz), 3.25 (1H, t, J=18.4 Hz); ¹³C NMR (100 MHz, D2O): δ 59.9, 61.1, 62.2, 69.0, 70.6, 72.2, 72.3, 73.8, 76.2, 81.2, 92.0, 103.5; EIMS m/z (rel. int.): 456.

Saccharose octaacetate (2a): 20 mg of compound 1 was acetylated with pyridine and acetic anhydride at room temp for 24hrs. After usual work up, obtained saccharose octaacetate as colorless solid, yield is 24 mg. ¹H NMR (400 MHz, CDCl₃): δ 5.68 (1H, s) 5.44 (2H, s), 5.37 (1H, br t), 5.07 (1H, t, J=9.4 Hz), 4.87 (1H, d, J=9.9Hz), 4.40-4.25 (4H, m), 4.25 – 4.10 (4H, m), 2.17 (3H, s), 2.11 (12, s), 2.10 (3H, s), 2.04 (3H, s), 2.02 (3H, s); ¹³C NMR (100 MHz, CDCl₃): δ 61.6, 62.7, 63.5, 68.1, 68.4, 69.5, 70.1, 74.8, 75.5, 79.0, 89.8, 103.9, 169.4, 169.6, 169.8, 169.9, 170.0, 170.0, 170.4, 170.6.

G Venkateswara Rao et al

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RESULTS AND DISCUSSION

The compound 1 was isolated as colorless amorphous powder from chloroform and methanol. It was readily recognized as triterpene derivatives based on its physical and spectral data. Its molecular formula was fixed as $C_{30}H_{40}O_3$ based on its mass spectrum. The IR spectrum showed the presence of hydroxyl and carboxylic acid groups. The $^1H$ NMR spectrum clearly showed the presence of seven tertiary methyls at $\delta$ 0.74, 0.77, 0.87, 0.89, 0.89, 0.94 and 1.11 (each as singlet) as oleanane skeleton. Further, the spectrum showed the presence of a broad doublet at $\delta$ 2.80 and a broad singlet of olefinic proton at $\delta$ 5.22 which were assigned to H-18 and H-12 suggesting the presence of an olean-12-ene basic skeleton. Additionally, the spectrum also showed one $\alpha$-methine proton which connected to hydroxyl group at $\delta$ 3.12 (1H, br s). Its $^{13}$C NMR spectrum showed a total of 30 carbons signals, of which one carboxylic carbon at $\delta$ 184.6, two olefinic carbons at $\delta$ 126.2, 147.7, one oxygenated carbon at $\delta$ 82.4 and remaining are saturated carbons. Based on the above spectral data, a literature search revealed that the physical and spectral data of the compound 1 agreed perfectly with oleanolic acid reported from several plants.$^{[18,20]}$

Elastase inhibition: The elastase inhibition activity of crude extract, different fractions and compounds along with ursolic acid were studied on cell free system. The assay method is most reliable method and reported in the literature.$^{[21]}$ Fresh solution of 300 µl (0.6 mg) of succinyl-L-alanyl-L-alanyl-L-alanyl-p-nitroanilide (enzyme substrate), 1200 µl of buffer and varying amounts of the elastase inhibitor under testing are incubated at 37°C for 20 minutes. The hydrolysis is measured by the spectrophotometric measurement of the release of p-nitroaniline at a wavelength of 410 nm. The crude methanolic extract, fractions and isolated compounds were tested and results were mentioned in the table 1.

Table 1: Comparative data of biological activity

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Extract/ Fraction / Compound</th>
<th>Elastase Inhibition (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control, Urosolic acid</td>
<td>IC$_{50}$=13.1</td>
</tr>
<tr>
<td>2</td>
<td>Methanolic extract</td>
<td>45.0% at 40</td>
</tr>
<tr>
<td>3</td>
<td>Hexane fraction</td>
<td>68.0% at 40</td>
</tr>
<tr>
<td>4</td>
<td>Oleanolic acid, 1</td>
<td>IC$_{50}$=15.5</td>
</tr>
</tbody>
</table>

CONCLUSION

Both compounds are reported first time from this plant. Even though several compounds have been reported from this plant, the triterpenoid, oleanolic acid is the first report from this plant The biological study, elastase inhibition is also first time for this plant.

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