Isolation of Secondary Metabolites from Leucas aspera and Investigation of Biological Activity

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Abstract

Leucas aspera plant was subjected to isolation of secondary metabolites and screening of their biological activities. Four compounds, stigmasterol, lupeol, β-sitosterol and menthol, were isolated from methanol extract. Sixteen different microorganisms were used for investigating antimicrobial activity of the different extracts of L. aspera where noteworthy zone of inhibition was observed against Gram positive B. subtilis and S. aureus, B. megaterium and Gram negative S. paratyphi, S. typhi, V. mimicus, V. cholera and S. paratyphi. In brine shrimp lethality bioassay, the highest lethality was showed by crude methanol extract having LC50 values of 4.07µg/mL. The total antioxidant capacity of crude methanol fraction was found to be 59.40 mg/g of plant extract which was maximum comparing with other fractions. No significant cytotoxicity was observed on both HeLa and Vero cell at 1mg/mL sample inhibition.

Keywords: Secondary metabolites, Antimicrobial, Lethality bioassay, Antioxidant, Cytotoxicity.

I. Introduction

L. aspera (Family: Lamiaceae) commonly known as 'Dondokalash' is one such medicinal plant which is being used traditionally for antipyretic, analgesic, anti-inflammatory, anti-rheumatic and antibacterial treatment and paste of the plant is subjected to inflamed area1. Leaves of L. aspera is traditionally used for the remedy of colds, coughs, chronic skin eruption, painful swelling, wound healing and even used as insecticide2. Alcoholic extract (90%) of L. aspera showed antifulcer effect which significantly reduced acid secretion3 and ethanol extract capable of exhibiting antihyperglycemic activity4. Phytochemical examination is revealed the presence of terpenoids in whole plant5. Nicotine6, sterols7 and other alkaloids8 have been isolated from the aerial part of the plant. Novel phenolic compounds such as 4-(24-hydroxy-1-oxo-5-n-propyltetracosanyl)-phenol9, aliphatic ketols, (28-hydroxypentatriacontan-7-one and 7-hydroxydotriacontan-3-one10 have been isolated from the roots of L. aspera. The volatiles, u- farnesene, x-thujene and menthol are the major constituents of the leaves and amyl propionate and isoamyl propionate are dominant11 in flower of L. aspera.

Considering the potential bioactivity, the plant materials have been chosen for further studies to find out their unexplored efficacy and isolation of a new compound.

II. Experimental

Collection of sample

The whole plant of L. aspera was collected from Panchagarh, Bangladesh and washed to remove mud and dust particles. Taxonomic identification was confirmed by the renowned plant taxonomist Professor Dr. Mohammad Zashim Uddin, Department of Botany, University of Dhaka. The collected fresh plant crushed into powder and stored in an airtight container.

Phytochemical screening

Phytochemical screening was carried out using standard procedure12 for identifying the chemical constituents. The presence of saponins, tannins, steroids, flavonoids, terpenoids and cardiac glycosides were observed in L. aspera plant.

Extraction

The dried powder (800 g) of L. aspera plant was taken in a clean, round bottomed flask and extracted with n-hexane followed by methanol at room temperature and atmospheric pressure. The extracts were evaporated to dryness at 40°C using a rotary evaporator (Buchi, Switzerland) under reduced pressure. The amount of methanol extract was found to be 4.9 g.

Isolation and characterization of compounds from methanol extract

The methanol extract was subjected to column chromatography over column grade silica gel using hexane as eluting solvent with increasing percentage of dichloromethane, ethyl acetate and methanol, respectively, from where thirty six fractions were collected. Studying on TLC plate similar fractions were combined together and renamed as F1 to F13. Among the fractions, F6 showed single spot on TLC plate and it was remarked as LA-2. Fraction F9 was appeared to contain two spots and fractionated by preparative TLC. The preparative TLC was developed using DCM:EA (9:1) mixed solvent and a pure compound was isolated, which was LA-1. The fraction F10 appeared to contain four spots. This fraction was subjected to sub column for further fractionation. Each of the fractions from sub column was monitored by TLC and similar fractions were combined together and marked as P1 to P7 where fraction P6 was found to be a pure compound. This isolated pure compound was marked as LA-3. The compound LA-4 was isolated by using steam distillation technique.
Extraction of plant for biological activity screening

Freshly prepared whole plant powder was extracted with hexane, chloroform, dichloromethane, ethyl acetate and methanol at room temperature respectively. All the extracts were evaporated to dryness and used for antimicrobial activity screening, brine shrimp lethality bio assay, cytotoxicity assay on cancer cell lines and determination of total antioxidant capacity using their individual standard procedure\textsuperscript{13,14}.

III. Results and Discussion

Characterization of compound LA-1

Compound LA-1 was white crystalline solid having R\textsubscript{f} value 0.78 (in 80% DCM:20% Ethyl acetate) and soluble in chloroform and dichloromethane. The melting point of LA-1 was found to be 157-160°C. \textsuperscript{1}H NMR spectrum (400 MHz, CDCl\textsubscript{3}) of compound LA-1 showed peaks at δ 5.38, 5.13, 5.00, 3.51, 1.01, 0.89, 0.83 and 0.67 ppm. The presence of a multiplet at δ 3.51 ppm in \textsuperscript{1}H NMR indicated the presence of oxymethine proton. The downfield signals at δ 5.0 and 5.13 ppm revealed the presence of olefinic protons. The other signals of the spectrum between δ 1.05-2.30 ppm were due to the presence of different methylene (CH\textsubscript{2}) and methine (CH) protons. \textsuperscript{13}C NMR spectrum (100 MHz, CDCl\textsubscript{3}) of compound LA-1 showed peaks at δ 37.27, 31.69, 31.69, 42.33, 140.78, 121.72, 31.92, 50.16, 36.52, 19, 39.79, 24.33, 56.8, 23.03, 29.18, 56.08, 11.98, 19.04, 39.8, 21.1, 138, 129, 51.24, 31.92, 18.78, 24.31, 11.86 and 21.21 ppm. The \textsuperscript{13}C NMR spectrum (100 MHz in CDCl\textsubscript{3}) of isolated compound LA-1 showed the presence of twenty nine (29) carbon signals. The signals at δ 140.78, 138, 129.0 and 121.72 ppm were observed due to the presence of olefinic carbons. From the physical characteristics and spectral analysis (\textsuperscript{1}H NMR and \textsuperscript{13}C NMR) data of the compound LA-1 and comparing the reported value\textsuperscript{15} of \textsuperscript{1}H NMR and \textsuperscript{13}C NMR spectral data of stigmasterol, the structure of the compound was established as stigmasterol.

Characterization of compound LA-2

Physical appearance of compound LA-2 was white crystalline having R\textsubscript{f} value 0.56 (in 100% DCM) and soluble in chloroform, dichloromethane. The melting point of LA-2 was found to be 120-122°C. FT-IR spectrum of the compound LA-2 showed absorption band at 3056, 2929, 1593, 1450, 1435, 1265 and 898 cm\textsuperscript{-1}. IR spectrum showed characteristic absorption frequencies at 3392 and 1188 cm\textsuperscript{-1} typical of the O-H and C=O bond vibrations, respectively; the absorption at 898 cm\textsuperscript{-1} was indicative of an unsaturated out of plane C-H vibration; the absorption at 1748 cm\textsuperscript{-1} was indicative of the C=C vibrations. \textsuperscript{1}H NMR spectrum (400 MHz, CDCl\textsubscript{3}) of compound LA-2 revealed peaks at δ 4.71, 4.56, 3.2, 2.37, 1.91, 1.67 and 0.69-1.54 ppm. In \textsuperscript{1}H NMR spectrum, a multiplet at δ 3.2 ppm while a pair of broad singlets at δ 4.56 and δ 4.71 ppm (1H, each) was indicative of olefinic protons. The signals between δ 0.69-1.54 ppm were due to several methylene and methane protons and a multiplet signal of one proton at δ 2.37 ascribable to 19β-H is characteristic of Lupeol. \textsuperscript{13}C NMR spectrum (100 MHz, CDCl\textsubscript{3}) of compound LA-2 gave peaks at δ 38.73, 28.0, 79.03, 38.87, 55.32, 18.33, 34.30, 40.85, 50.46, 37.19, 20.95, 25.17, 38.08, 42.85, 27.45, 35.60, 43.01, 48.33, 48.0, 150.98, 29.87, 40.01, 29.7, 15.36, 16.12, 15.99, 14.56, 18.02, 109.32 and 19.31 ppm. The \textsuperscript{13}C NMR spectrum of compound LA-2 showed thirty signals indicating the presence of thirty carbons. The signals at δ 109.32 and 150.98 were characteristic of olefinic carbons. A deshielded signal at δ 79.03 indicated the presence of C-O group. All these \textsuperscript{1}H NMR and \textsuperscript{13}C NMR spectral data of LA-2 was in good agreement with the reported data\textsuperscript{16} of lupeol and the compound LA-2 was establish as lupeol.
compound LA-4 showed peaks at δ 3.38, 2.28, 2.18, 1.96, 1.63, 1.39, 1.41, 0.89, 0.93, 0.80 and 1.16-0.97 ppm. $^{13}$C NMR spectrum (100 MHz, CDCl$_3$) of compound LA-4 showed peaks at δ 71.24, 49.96, 23.02, 34.48, 31.56, 44.95, 22.09, 26.59, 15.93 and 20.91 ppm. IR absorption band at 3266 cm$^{-1}$ assignable to O-H group and bands at 2959 and 2872 cm$^{-1}$ were due to the presence of aliphatic C-H stretching. The absorption band at 1078 cm$^{-1}$ was indicative of C-O stretching. The $^1$H NMR signal at δ 3.38 ppm having coupling constants $J = 4.0$ & 10.4 Hz indicated the presence of oxymethine proton. The broad singlet at δ 2.28 ppm was the indicative of –O–H group. The signals at δ 0.89, 0.93 and 2.18 ppm indicated the presence of isopropyl group. The $^{13}$C NMR spectrum of the isolated compound showed characteristic signals for three methyl carbons at δ 15.93 (C-9), 20.19 (C-10) and 22.09 ppm (C-7), three methylene carbons at δ 23.02 (C-3), 34.48 (C-4) and 44.95 ppm (C-6), three methine carbons at δ 26.59 (C-8), 31.56 (C-5) and 49.96 ppm (C-2). The signal at δ 71.24 ppm (C-1) also indicative of one oxymethine carbon. Comparing the $^1$H NMR and $^{13}$C NMR data of the compound LA-4 with reported value$^{14}$ of $^1$H NMR and $^{13}$C NMR of menthol, the structure of the compound was established as menthol.

Antimicrobial activity screening

Antimicrobial activity of L. aspera was estimated by using disc diffusion method$^{15}$. Crude methanolic extract of L. aspera and its different partitions i.e., n-hexane (HEX), chloroform (CHCl$_3$), dichloromethane (DCM), ethyl acetate (EA) and aqueous (AQ) were subjected to antimicrobial screening. In every case 400 µg sample per disc was applied. Significant zone of inhibition against Gram positive B. subtilis (11 mm) and S. aureus (8 mm), B. megaterium (12 mm) and Gram negative S. paratyphi (8 mm), S. typhi (7 mm), V. mimicus (8 mm), S. dysenteriae (9 mm) and V. cholera (8 mm) was observed.

Brine shrimp lethality bio assay

Brine shrimp lethality bio assay experiment was also carried out by standard procedure$^{14}$. Freshly extracted different fractions of L. aspera were weighed and then a series of solutions of varying concentrations were prepared from the stock solutions by serial dilution method. The LC$_{50}$ values of MeOH, HEX, CHCl$_3$, DCM, EA and AQ fractions were found to be 4.07, 9.56, 8.82, 14.35, 2.40 and 11.39 µg/mL, respectively. Ethyl acetate (EA) and methanol (ME) fraction showed significant lethality whereas HEX and CHCl$_3$ revealed moderate activity. Dichloromethane (DCM) and aqueous (AQ) showed very low activity.

Cytotoxicity assay on cancer and non-cancer cell line

Cytotoxicity test for the different extracts of L. aspera were tested against HeLa cell line (a human cervical carcinoma cell) and Vero cell line (a kidney epithelial cells extracted from an African green monkey) in Center for Advanced Research (CARS), University of Dhaka. 1 mg/mL sample...
of different extracts were applied on both HeLa and Vero cell but no significant cytotoxicity was observed on both HeLa and Vero cell at 1 mg/mL of sample inhibition.

**Total antioxidant capacity: Phosphomolybdenum method**

The total antioxidant capacity was estimated using phosphomolybdenum method and total antioxidant capacity of crude methanol fraction was found to be 59.40 mg/g of plant extract (expressed as ascorbic acid equivalents) which is the highest antioxidant capacity comparing with other fractions. On the other hand, hexane fraction was found to show 19.52 mg/g of plant extract (as ascorbic acid equivalents) which is the lowest antioxidant capacity comparing with other extracts.

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