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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 micro-organisms 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*, *S. dysenteriae* and *V. cholera*. In brine shrimp lethality bioassay, the highest lethality was showed by crude methanol extract having LC_{50} 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 area[1]. Leaves of *L. aspera* is traditionally used for the remedy of colds, coughs, chronic skin eruption, painful swelling, wound healing and even used as insecticide[2]. Alcoholic extract (90%) of *L. aspera* showed antiulcer effect which significantly reduced acid secretion[3] and ethanol extract capable of exhibiting antihyperglycemic activity[4]. Phytochemical examination is revealed the presence of terpenoids in whole plant[5]. Nicotine[6], sterols[7] and other alkaloids[8] have been isolated from the aerial part of the plant. Novel phenolic compounds such as [4- (24-hydroxy-1-oxo-5-n-propyltetracosanyl)-phenol][9], aliphatic ketols, (28-hydroxypentatriacontan-7-one and 7-hydroxydotriacontan-3-one)[10] 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 dominant[11] 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 procedure[12] 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 F_1 to F_{13}. Among the fractions, F_6 showed single spot on TLC plate and it was remarked as LA-2. Fraction F_9 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 F_{10} 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 P_1 to P_7 where fraction P_6 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.

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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[13, 14].

III. Results and Discussion

Characterization of compound LA-1

Compound LA-1 was white crystalline solid having R_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. ^{1}H NMR spectrum (400 MHz, $CDCl_3$) 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 ^{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_2-) and methine (>CH-) protons. ^{13}C NMR spectrum (100 MHz, $CDCl_3$) of compound LA-1 showed peaks at δ 37.27, 31.69, 31.69, 42.34, 140.78, 121.72, 31.92, 31.92, 50.16, 36.52, 19, 39.79, 42.33, 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 ^{13}C NMR spectrum (100 MHz in $CDCl_3$) 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 (^{1}H NMR and ^{13}C NMR) data of the compound LA-1 and comparing the reported value[15] of ^{1}H NMR and ^{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_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^{-1}. IR spectrum showed characteristic absorption frequencies at 3392 and 1188 cm^{-1} typical of the O-H and C-O bond vibrations, respectively; the absorption at 889 cm^{-1} was indicative of an unsaturated out of plane C-H vibration; the absorption at 1748 cm^{-1} was indicative of the C=C vibrations. ^{1}H NMR spectrum (400 MHz, $CDCl_3$) 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 ^{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. ^{13}C NMR spectrum (100 MHz, $CDCl_3$) 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 ^{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 ^{1}H NMR and ^{13}C NMR spectral data of LA-2 was in good agreement with the reported data[16] of lupeol and the compound LA-2 was establish as lupeol.

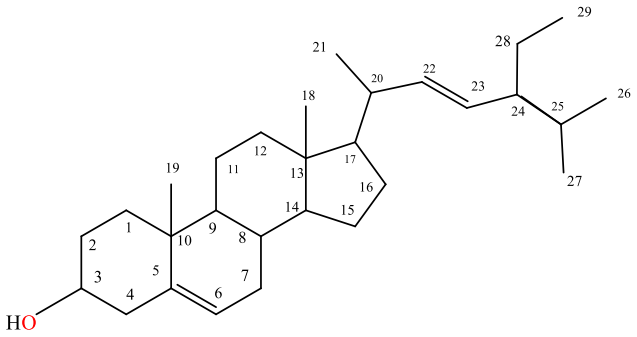

Fig. 1. Structure of LA-1 as stigmasterol

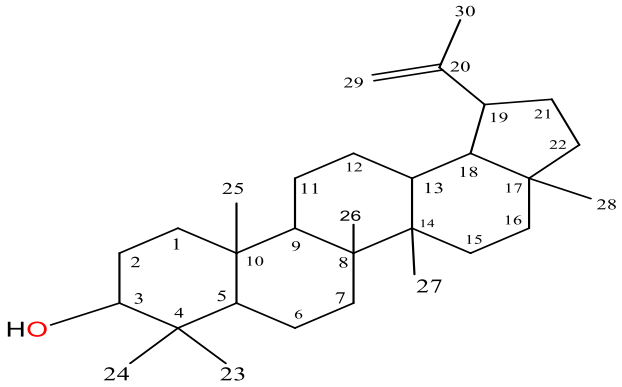

Fig. 2. Structure of LA-2 as lupeol

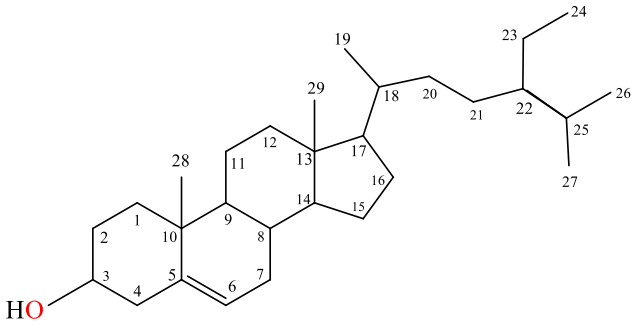

Fig. 3. Structure of LA-3 as β-sitosterol

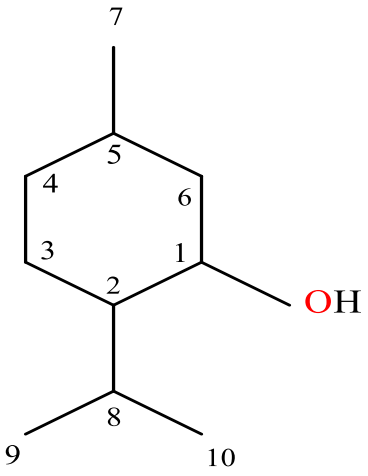

Fig. 4. Structure of LA-4 as menthol

Characterization of compound LA-3

The compound LA-3 (~ 7.0 mg) was white crystalline solid, its melting point was found to be 148-150˚C and having R_f value 0.50 (in 98% DCM:2% methanol). It was soluble in chloroform and dichloromethane. The IR spectrum of the compound LA-3 revealed absorption band at 3450, 2920, 2872, 1650, 1450, 1365 and 1040 cm^{-1}. IR absorption band at 3419 cm^{-1} and at 1027 cm^{-1} were assignable to O-H group and C-O stretching, respectively. The absorption band at 1683 cm^{-1} due to C=C bond stretching. ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound LA-3 showed peaks at δ 5.27, 3.60, 1.12, 0.90, 0.84, 0.71 and 1.07-2.08 ppm. The proton signals at δ 3.60 and 5.27 ppm were indicative of proton connected to the C-O group and olefinic proton, respectively. The signals between δ 1.07-2.08 ppm were characteristic of steroidal nucleus. ^{13}C NMR spectrum (100 MHz, $CDCl_3$) of compound LA-3 showed peaks at δ 38.76, 31.82, 79.05, 41.13, 143, 122.68, 32.68, 32.43, 46.49, 37.08, 24.73, 39.29, 41.68, 55.24, 27.2, 28.11, 47.64, 33.82, 18.33, 33.06, 27.69, 45.9, 25.9, 15.55, 29.7, 23.57, 23.02, 17.05 and 15.33 ppm. The ^{13}C NMR spectrum of compound LA-3 showed the presence of twenty nine (29) carbons among them the signal at δ 79.05 ppm was due to one oxymethine carbon and signals at δ 143.0 and 122.68 ppm were due to two olefinic carbons. From the physical characteristics and spectral analysis (^{1}H NMR and ^{13}C NMR) data of the compound LA-3 and comparing the reported value[17] of ^{1}H NMR and ^{13}C NMR spectral data of β-sitosterol, the structure of the compound LA-3 was established as β-sitosterol.

Characterization of compound LA-4

The compound LA-4 (~ 30 mg) was a waxy, crystalline substance, clear white in color, which was solid at room temperature and melts slightly above having R_f value 0.6 (80% DCM and 20% n-hexane). It was soluble in chloroform and melting point was found to be 36-38˚C. The FT-IR spectrum of the compound LA-4 provided absorption band at 3266, 2959, 2872, 1448, 1383 and 1078 cm^{-1}. ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of 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 –OH 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[18] 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[13]. 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, 9.36, 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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