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Isolation of Stigmasterol and β-Sitosterol from Methanolic Extract of Root Bark of *Calotropis gigantea* (Linn)

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Abstract: Aim of this study is to identify and characterize the bioactive principles from the root bark of *Calotropis gigantea*. It has wide folk medicinal use. For isolation of the compounds, the dried root bark's powder of *Calotropis gigantea* were subjected to hot extraction and then the crude methanol (MeOH) extract was fractionated with petroleum ether, chloroform and ethyl acetate. Two compounds were isolated and purified from petroleum ether fraction of crude methanol extract and the structures were determined as stigmasterol and β-sitosterol by analysis of physical, chemical and spectral characteristics (1D NMR and mass spectrometry).

Key words: Stigmasterol, β-sitosterol, *Calotropis gigantea*

INTRODUCTION

Research studies leading to extraction, isolation and biological study of plant constituents have now formed the major field of study. The plant *Calotropis gigantea*, locally known as Boro Akanda belongs to the Asclepiadaceae family and it grows in tropical region and most abundant in Bangladesh, India, Burma, Pakistan and in the sub Himalayan tract (Kiritikar and Basu, 1994). It is reported that root extract of *Calotropis gigantea* showed very strong cytotoxic effect against KB cell lines *in vitro* (Ekramul Haque, 1994). Cardenolide glycosides, calotropin, frugoside and 4′-O-beta-D-glucopyranosyl frugoside were isolated from the root of *Calotropis gigantea* which are toxic to cell lines of human origin (Kiuchi *et al*., 1998). Some bioactive compounds such as giganticine (a novel insect antifeedant nonprotein amino acid), isorhamnetin-3-O-rutinoside, isorhamnetin-3-O-glucopyranoside, taraxasteryl acetate, calotroposides (A and B) etc. were also isolated and purified from *Calotropis gigantea* (Pari *et al*., 1998; Sen *et al*., 1992; Kitagawa *et al*., 1992). Recently two new cardenolides, 19-Nor and 18, 20-epoxy-cardenolides having inhibitory effect against KB, BC and NCI-H187 cancer cell lines, are isolated from the leaves of *Calotropis gigantea* (Lhinhatrakool and Sutthivaiyakit, 2006). The root extract of *Calotropis gigantea* has potential antipyretic activity against both yeast-induced and TAB (Typhoid) vaccine-induced fever (Chitme *et al*., 2005). Alcoholic root extract of *Calotropis gigantea* also has analgesic, anticonvulsant, anxiolytic and sedative effect (Argal and Pathak, 2006). But these studies are not enough for identifying and characterizing the bioactive compounds in this plant. The purpose of this study is to identify and characterize the bioactive principles from the root bark of *Calotropis gigantea*.

MATERIALS AND METHODS

Preparation of plant material: The roots of *Calotropis gigantea* (Linn) were collected during the month of May-June, 2005 from the relevant area (Meherchandi) of Rajshahi University campus. The plant was taxonomically identified by Professor A.T.M Naderuzzaman, Department of Botany, University of Rajshahi and a voucher specimen (No. 1A. Alam, collection date 15.08.2004) was kept in the Department of Botany, University of Rajshahi. The dried root bark powder (1.5 kg) was subjected to hot extraction with MeOH by Soxhlet extractor and after evaporation of the solvent 140 g crude extract was found. Twenty gram of the crude MeOH extract was fractionated into petroleum ether fraction (15 g), chloroform fraction (2 g), ethyl acetate fraction (1 g) and aqueous fraction (2 g) (Bahl and Bahl, 1992).

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Isolation and purification of compounds: A small portion of Petroleum Ether fraction (PE) of hot and cold extraction was dissolved in petroleum ether and the solution was spotted on TLC plates. Then the TLC plates were run by specific solvent system and were viewed individually under UV light and also with the vanillin-H_2SO_4 reagent (Bobblt, 1963). Through several pilot experiments, it was found that the compounds of petroleum ether fraction were separated by the solvent system of n-Hexane and ethyl acetate in the proportion of 9:1. The petroleum ether fraction, 10 g, was subjected to column chromatography on a silica gel (60-120 mesh) with gradient elution using n-hexane : ethyl acetate and finally with 100% methanol (Srivastave and Srivastave, 1987). Two fractions were found homogeneous on TLC plate by using n-hexane : ethyl acetate (9:1), n-hexane : chloroform (10:1), petroleum ether : ethyl acetate (9:1) and petroleum ether : methanol (7:3) solvent systems. These fractions were crystallized (Bahl and Bahl, 1992) and named as CG-1(Calotropis gigantea-1) and CG-2 (Calotropis gigantea-2), respectively.

Test for alcohol: Four gram of ceric ammonium nitrate was dissolved in 10 mL^{-1} of 2N HNO_3 on mild heating. A few crystals of CG-1 and CG-2 were dissolved in 0.5 mL^{-1} of dioxane. The solution was added to 0.5 mL^{-1} of ceric ammonium nitrate reagent, diluted to 1 mL^{-1} with dioxane and shaken well. Both CG-1 and CG-2 developed yellow to red colour indicating the presence of a alcoholic hydroxyl group.

Test for steroid: Salkowski Reaction: A few crystals of CG-1 and CG-2 were dissolved in chloroform and a few drops of concentrated sulfuric acid was added to the solution. For both CG-1 and CG-2, a reddish colour was seen in the upper chloroform layer.

Liebermann-Burchard reaction: A few crystals of CG-1 and CG-2 were dissolved in chloroform and a few drops of concentrated sulfuric acid was added to it followed by the addition of 2-3 drops of acetic anhydride. Solutions for both CG-1 and CG-2 turned violet, blue and finally green.

Spectroscopic characterization: Different spectroscopic methods were used to elucidate the structure of CG-1 and CG-2. Among the spectroscopic techniques DI (Direct ionization)-mass, ^{1}H-NMR and ^{13}C-NMR were carried out. Mass spectrum was recorded at high resolution on a mass spectrometer (Shimadzu) at Kawakami Laboratory of Japan Advanced Institute of Science and Technology (JAIST), Japan and the data are given in m/z values. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Varian-750 MHZ NMR spectrometer (Shimadzu) at Japan Advanced Institute of Science and Technology (JAIST). The ^{1}H-NMR spectra were recorded at 4.5 MHZ and the ^{13}C NMR spectra recorded at 31.4 MHZ using $CDCl_3$ as solvent with Tetramethylsilane (TMS) as an internal standard.

RESULTS AND DISCUSSION

The melting point of CG-1 and CG-2 were 176 and 133°C, respectively and both of them showed positive results in alcohol test and in all the tests of steroid. DI-mass spectrum of CG-1 and CG-2 showed a parent molecular ion $[M+H]^+$ peak at m/z 412 and 414, respectively which corresponds to the molecular formula $C_{29}H_{48}O$ and $C_{29}H_{50}O$. The ^{1}H-NMR and ^{13}C-NMR data of CG-1 and CG-2 are summarized in Table 1.

From the positive tests for steroids and alcohols given by the CG-1 and CG-2, they were assumed to be a sterol. The melting point of CG-1 (176°C) and CG-2 (133°C) were in a good agreement with the melting point given for stigmasterol and β-sitosterol in the literature

Table 1: ^{1}H-NMR and ^{13}C-NMR data of CG-1 and CG-2

	CG-1		CG-2	
Position	δ_H	δ_C	δ_H	δ_C
1		32.9		32.4
2		34.5		36.1
3	3.25 (tdd J = 4.5 MHZ)	79.0	3.25 (tdd, J = 4.5 MHZ)	71.8
4		42.0		42.3
5		154.6		140.7
6	5.14 (1H, m)	124.4	5.31 (1H, m)	121.7
7		31.2		31.6
8		28.7		28.2
9		42.0		42.3
10		39.6		39.7
11		19.4		20.9
12		31.9		31.8
13		40.8		40.4
14		47.7		45.8
15		21.4		21.2
16		21.3		21.0
17		48.2		50.1
18	1.07 (3H, s)	18.3	1.16 (3H, s)	20.5
19	1.26 (3H, s)	18.2	1.25 (3H, s)	20.2
20		33.4		29.6
21	0.91 (3H, s)	17.4	0.91 (3H, s)	18.7
22	4.62 (1H, m)	107.1		33.7
23	4.61 (1H, m)	139.5		29.1
24		47.7		42.3
25		30.6		30.5
26	1.01 (3H, s)	20.2	1.01 (3H, s)	19.8
27	1.00 (3H, s)	20.2	0.98 (3H, s)	19.3
28		25.4		24.2
29	0.97 (3H, s)	12.2	0.96 (3H, s)	11.8

δ_H = chemical shift values in ^{1}H-NMR spectrum; δ_c = chemical shift values in ^{13}C-NMR spectrum

(Holland *et al.*, 1 976). In ^{1}H-NMR spectrum of CG-1, H-3 proton appeared as a triplet of a double doublet (tdd) at δ 3.25 (J = 4.5 and 1.1 MHZ) and H-6 olefinic proton showed a multiplet at δ 5.14. Two olefenic protons appeared downfield at δ 4.14 (m) and δ 4.61 (m) which were identical with the chemical shift of H-22 and H-23, respectively of stigmasterol (Li *et al.*, 2006). Six methyl protons also appeared at δ 1.07, δ 1.26, δ 0.91, δ 1.01, δ 1.00 and δ 0.97.

Similarly from ^{1}H-NMR data of CG-2 it was seen that H-3 proton appeared at δ 3.29 as a triplet of a double doublet with a J value of 4.5 and 1.1 MHZ and H-6 olefinic proton showed a multiplet at δ 5.31. Moreover, Six methyl protons appeared at δ 1.16, δ 1.25, δ 0.91, δ 1.01, δ 0.91 and δ 1.26 (3H each, s, CH_3). These assignments are in good agreement for the structure of β-sitosterol. ^{13}C-NMR data of CG-1 and CG-2 were also quite similar with the data in the literature of stigmasterol and β-sitosterol, respectively (Conolly and Hill, 1994).

CONCLUSION

From these physical, chemical and spectral evidences CG-1 and CG-2 were confirmed as stigmasterol (Fig. 1) and β-sitosterol (Fig. 2).

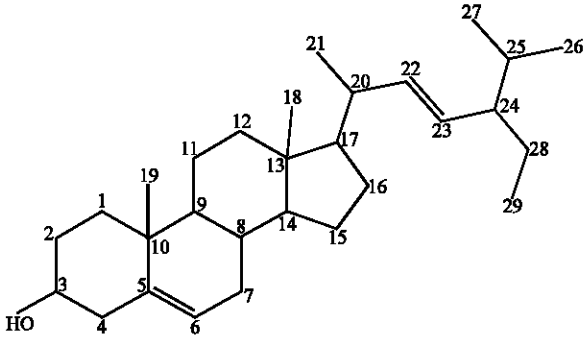

Fig. 1: The chemical structure of CG-1 (Stigmasterol)

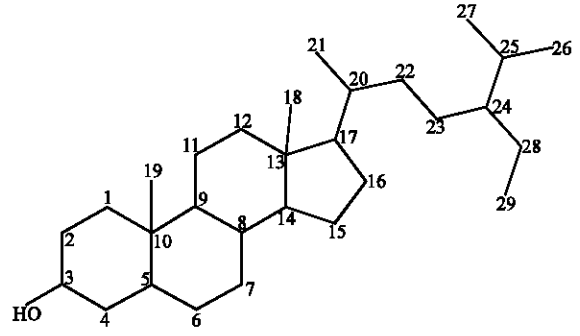

Fig. 2: The chemical structure of CG-2 (β-sitosterol)

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