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Isolation and Structural Characterization of a Steroidal Antimicrobial Agent from *Clerodendrum baronianum*

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Abstract: The preliminary phytochemical screening of the root of *Clerodendrum baronianum* and antimicrobial activities of the crude extract in various solvent systems were determined. A pure colorless needle shape crystal compound (CB) was separated by thin layer and column chromatographic methods. The yield is found to be (10.3 mg %) based upon the ethyl acetate crude extract. The melting point of this compound is 251-253°C. The crude extract and pure compound (CB) were screened for the antimicrobial activity employing several microorganisms. This compound (CB) showed the highest antibacterial activity against *B. subtilis, E. coli, P. aeruginosa* and *St. aureus*. The phytochemical analysis showed that the active agent is a steroid compound. Furthermore, the molecular formula of this pure compound might be assigned as $C_{28}H_{46}O_2N$ based upon the results of several advanced spectroscopic analysis such as IR, ^{1}HNMR, ^{13}CNMR and EI-MS.

Key words: *Clerodendrum baronianum* · IR · NMR · EI-MS · Antibacterial Activity

INTRODUCTION

Despite the availability of different approaches for the discovery of therapeutically, natural products still remain as one of the best reservoir of new structural types. They are used directly as therapeutic agents, as well as starting material for the synthesis of drugs or as models or pharmacologically active compounds [1]. Hence, if history is an indicator, when drugs derived from plants are individual developed, it must be established that the end product will most probably have to be produced commercially by extraction and not by synthesis. If this is true, an abundant and easily accessible source of starting material must be available so that it can be collected and shipped without difficulty or expense [2]. As chemical technology improved, the active constituents were isolated from plants, were structurally characterized and in due course, many were synthesized in the laboratory. Now and then, more active, better tolerated drugs were produced by chemical modifications, or by total synthesis of analogues of the active principles. Research on medicinal plants has continued to be an area of major importance for the last several decades. Industrial chemists began a program in 1995 to look for new drugs in plant [3]. Anti-infective compounds from natural resources are of great interest as the existing drugs are receiving less effective due to improved tolerance of microorganisms. There is a need to look for substances from other sources with proven antimicrobial activity. Therefore this has led to search for new antimicrobial agents among plant origin that can serve as source for the synthesis of new antimicrobial drugs [4-6]. There has been a lot of interest in the investigation of natural materials as sources of new antibacterial agents [7]. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs [8]. In last decades numbers of novel antibiotics have produced, but clinical efficacy of these existing antibiotics is being threatened by the emergence of multi drug-resistant pathogens [9]. The genus *Clerodendrum*. Is very widely distributed in tropical and subtropical regions of the world and is comprised of small trees, shrubs and herbs. The first description of the genus was given in 1753, with identification of *Clerodendrum infortunatum*. *Clerodendrum serratum* is a genus of flowering plants in the Verbenaceae family. Estimates of number of species in *Clerodendrum* vary widely [10]. The genus is native to tropical and warm regions of the world, with most of the species occurring in tropical Africa and southern Asia, but some in the tropical Americas and northern Australia

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and a few extending north into the temperate zone in eastern Asia [11]. The genus is reported to have activities against a wide spectrum of disorders which includes many life-threatening diseases like HIV. Still there are many species of the genus having a potential towards many disorders in their unexplored fold [12]. Also in Myanmar the study of traditional indigenous medicinal plants and their usage in therapy play a very important role. These plants may have biologically active principles. The plant kingdom constituents are invaluable source of new chemical products which may be important due to their bioactive properties and their potential uses in medicines. One of Myanmar indigenous medicinal plants, *Clerodendrum serratum* was used as astringent [13]. The pose of this study is to chemical characterize the main component and to evaluate the antibacterial activities of root of *Clerodendrum baronianum.*

MATERIALS AND METHODS

Sample Preparation: *Clerodendrum baronianum* roots were cut into small pieces and then allowed to dry in air. Those pieces were stored in bottle and used during the conduct experiment.

Extraction, Fractionation and Purification of the Active Compound: The extraction of the natural compounds from plants is achieved according to the typical partitioning scheme using immiscible solvent described below by Shaker *et al.* [14]. The air dried powder rood (300 g) was percolated with (1000 mL) ethanol for about 30 days. The percolated solution was filtered and concentrated under vacuum at 20°C to near dryness. The residue was extracted partitioned first with ethyl acetate (3 × 200 mL). Ethyl acetate extract was filtered and evaporated the crude extract (5.12 g) was fractionated by silica gel flash column chromatography (70 × 3 cm) using in sequence, Chloroform ($CHCl_3$) and $CHCl_3$-Methanol mixtures of increasing polarity (100:0 → 80:20, v/v). Three fractions were obtained based on TLC using successive solvents ($CHCl_3$: MeOH: H_2O, 80:18:2, v/v); (EtOAc: MeOH: H_2O, 80:13:7, v/v); ($CHCl_3$: MeOH, 80:20, v/v). The major fraction (18 mg) was purified by repeated column chromatography over Sephadex LH-20 (2.5 × 50 cm) eluted with MeOH. The active, antibacterial, fraction was further purified by re-crystallization using n-hexane: ethyl acetate (1:1, v/v) to provide pure compound (7.3 mg) as colorless crystal (CB). Pure crystals were powdered and subjected to melting point determination by using electric melting point apparatus (FSA Laboratory Supplies, Leics, UK) according to Thompson and Wolfrom [15].

Fig. 1: *Clerodendrum baronianum*

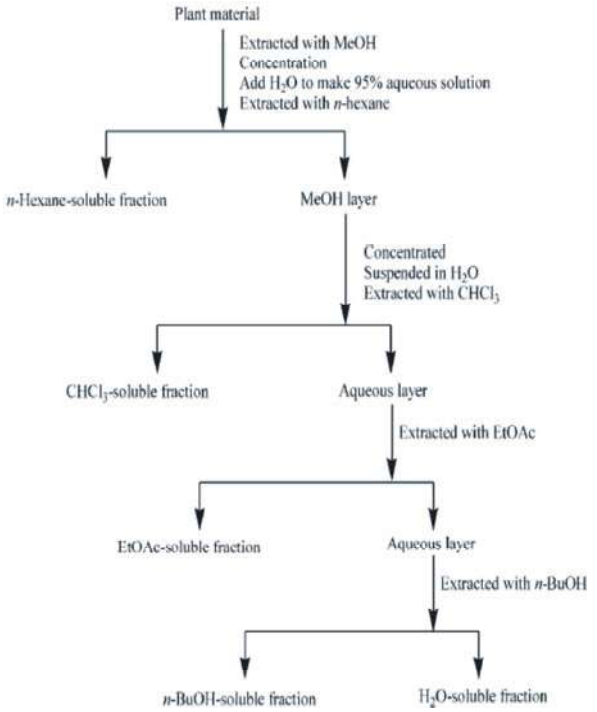

Fig. 2: A typical partitioning scheme using immiscible solvent [15]

Preliminary Phytochemical Analysis: To classify the type of the isolated active compound, qualitative phytochemical tests for the alkaloids, flavonoids, steroids and terpenoids were carried out for the sample by the method described by Harborne [16].

Spectroscopic Studies: Electron impact mass Spectroscopy (EI-MS) was recorded on a Finnegan MAT 95 spectrometer (70 *eV*). The infrared spectra (cm^{-1}) were obtained using a Necolet 205 FT-IR spectrometer connected to a Hewlett-Packard Color Pro plotter. Nuclear Magnetic Resonance ^{1}HNMR and ^{13}CNMR spectra were recorded on a Bruker NM 400 spectrometer operating at 400 MHz (^{1}H) and 100 MHz (^{13}C). All NMR spectra were obtained in DMSO-d_6 using TMS as internal standard, with using chemical shifts expressed in (δ).

Antimicrobial Activities: The antibacterial activities of crud extract and purified compound (CB) were performed by agar-disk diffusion method using cultures of *Bacillus subtilis, Escherichia coli, Psedumonas aeruginosa, Staphylococcus aureus, Aspergillus niger* and *Candida albicans* [17]. The microbial growth inhibition zone was measured after incubation at 30°C, the appearance of the clear microbial free inhibition zones beginning within 24 - 48 hrs for bacteria and yeast and after 48-72 hrs for fungus. The antimicrobial activity was evaluated by measuring the diameter of the inhibition zones against the test organisms. All the experiments were carried out in triplicates.

RESULTS AND DISCUSSION

Isolation and Structure Elucidation of the Active Compound: A number of species from *Clerodendrum* genus were reported to be used as folk medicine by various tribes in Asian and African continents. Many species of the genus have also been documented in traditional systems of medicine practiced in countries i.e. India, China, Korea, Thailand and Japan. The root extract of *Clerodendrum baronianum*, showed a variety of constituents for example flavonoid, terpenoid, alkaloid, glycoside, phenolic compound and steroid in phytochemical analysis. In addition, the ethyl acetate extract of the *Clerodendrum baronianum* showed high activities against four selected microorganisms such as *B. subtilis, E. coli, P. aeruginosa* and *St. aureus*. This compound is obtained through several stages of solvent partitioning, fractionation and various chromatographic technique. The active compound, which symbolized as (CB), was isolated as colorless needle shape crystal could spot on a silica gel TLC when sprayed with anisaldehyde reagent (R_f of 0.8; with solvent system (MeOH:ethyl acetate, 2:8, v/v); melting point was found to be 251-253°C.

The Antimicrobial Activity of (CB): A number of species from the genus *Clerodendrum* were documented in ancient texts for their antimicrobial action. To validate these claims, this research work was carried out using various Gram positive and Gram negative bacterial strains as well as fungi. Agar-disk diffusion method was performed to assess the antimicrobial effect of the crud extract and purified compound (CB) on the test bacterial organisms; *St. aureus, B. subtilis, P. aeruginosa, E. coli andfungal organisms A. niger and C. albicans.* The purified compound (CB) showed the highest level of activity with inhibition zone ranging from 6.4±0.47 mm to 19.6±1.65 mm (Table 1). Also it showed broad-spectrum antibacterial activity against Gram positive and Gram negative bacteria. The results were compared with the standard drug streptomycin. The zone of inhibition was found to be increased with the increase in concentration of the extract and thus exhibiting concentration dependent antibacterial activity [18]. At the same time it did not show any inhibitory effect against fungi. By comparing these results with that published for different species of the genus *Clerodendrum*, dried, aerial parts of *Clerodendrum inerme* showed potent antiviral activity against HBV [19]. Alcoholic extracts of leaves and flowers of *Clerodendrum inerme* also exhibited antibacterial activity against *E. coli* and *St. aureus* [20]. Pectolinarigenin and chalcone glucoside isolated from leaf of *Clerodendrum phlomidis* showed antifungal activity [21]. Two phenyl propanoid glycosides isolated from *Clerodendrum trichotomum* showed potent inhibition of HIV-1 [22]. A new hydroquinone diterpenoid was isolated from *Clerodendrum uncinatum* and was strongly fungi toxic to the spores of *Clerodendrum cucumerinum* Hexane extracts of *C. colebrookianum* showed strong antibacterial activities against *St. aureus*, *St. haemolyticus*, *E. coli*, *P. aeruginosa* [23]. Two flavonoids from roots of *Clerodendrum infortunatum* showed strong antifungal activity [24]. Triterpenoid saponin isolated from the roots of *Clerodendrum wildii*, showed potent antifungal activity [25]. These constituents of family Verbenaceae might have interacted with microorganism and inhibited their growth [26, 27].

Molecular Formula Determination of (CB): The molecular formula of compound (CB) could be determined by using some modern spectroscopic methods such as IR, ^{1}HNMR, ^{13}CNMR and EI mass spectral data, respectively. Hence, IR spectrum showed absorption bands (ν max) are typical from hydroxyl group (OH) 3464 cm^{-1}, amine group (-NH) 3255.7 cm^{-1}, hydrocarbon 3070.3 cm^{-1}, carbonyl group (C=O) 1689 cm^{-1}, cis alkenic and trans alkenic functional groups 887.5 cm^{-1} and the dimethy group that appeared at 1373.4 cm^{-1} and 941 cm^{-1} (Figure 3). The ^{1}HNMR spectrum of (CB) indicates the total number of protons 45 in this compound. ^{13}CNMR spectrum informs the total number of carbons 28 in this compound. ^{1}HNMR and ^{13}CNMR spectral data reveal the partial molecular formula to be $C_{28}H_{45}$ with molecular mass, about 381 (Figures 4 & 5). In the IR spectrum, this unknown compound (CB) should consist of absorption bands for hydroxyl group (-OH), carbonyl group (C=O), isopropyl

Table 1: Antimicrobial activity of crud extract and (CB) of *Clerodendrum baronianum*

		Diameter of inhibition zone (mm)*					
		Bacteria				Fungi	
Sample (ppm)		*E. Coli*	*P. aeruginosa*	*St. aureus*	*B. subtilis*	*A. niger*	*C. albicans*
Curd extract	40	7.4±0.46	6.7±0.38	8.8±0.32	8.5±0.37	00.0±00	00.0±00
	50	10.7±0.33	9.8±0.63	11.3±0.81	10.8±0.91	00.0±00	00.0±00
CB	20	8.1±0.66	6.4±0.47	8.5±0.86	9.8±0.81	00.0±00	00.0±00
	30	12.5±1.33	11.6±1.21	12.8±0.98	13.3±0.97	00.0±00	00.0±00
	40	14.1±1.67	13.5±1.28	14.9±1.42	15.8±1.42	00.0±00	00.0±00
	50	15.4±1.82	14.8±0.98	18.5±1.61	19.6±1.65	00.0±00	00.0±00

*All the values are mean ± S.E. of mean of three determinations.

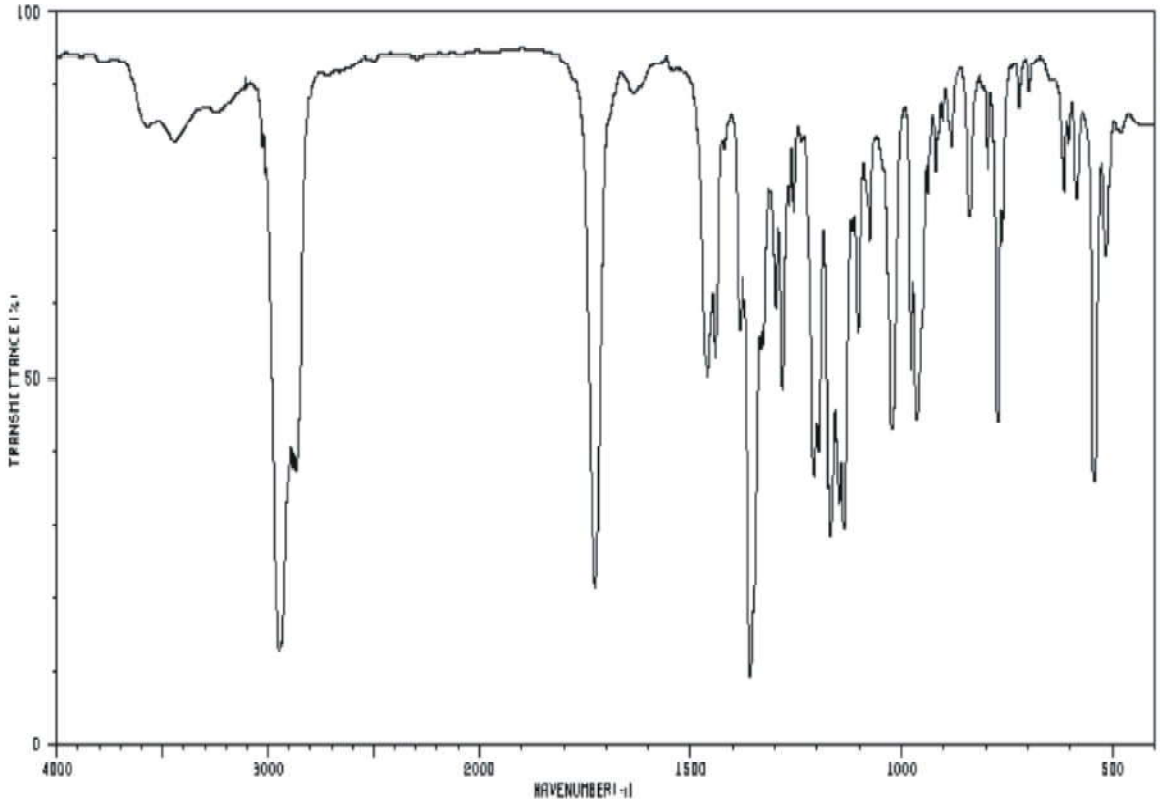

Fig. 3: IR Spectrum of CB

group and exomethylene. As a result, the partial molecular formula could be extended as $C_{28}H_{45}O_2$ with molecular mass, 413.6. In the EI-MS, the molecular ion peak *m/z*, 430 [M^+] represents its molecular mass. Hence, the remaining molecular mass is ~15 (Figure 6). For this reason, the remaining group should be secondary amine (›NH) or (-CH_3). So, the real molecular formula of this unknown compound (CB) can be represented as $C_{28}H_{46}O_2N$. Research reports on the genus denote that the major class of chemical constituents present in the *Clerodendrum* genus are steroids such as β-sitosterol, γ-sitosterol octacosanol, clerosterol, bungein A, acteoside, betulinic acid, clerosterol 3-O-β-Dglucopyranoside, colebrin A-E, campesterol, α-methylsterol, cholesta-5-22-25-trien-3-β-ol, 24-β-cholesta-5-22-25-trine, cholestanol, 24-methyl-22-dihydrocholestanol have been isolated from various *Clerodendron* species such as *Clerodendrum inerme, Clerodendrum phlomidis, Clerodendrum infortunatum, Clerodendrum paniculatum, Clerodendrum cyrtophyllum, Clerodendrum fragrans* [1, 28-32].

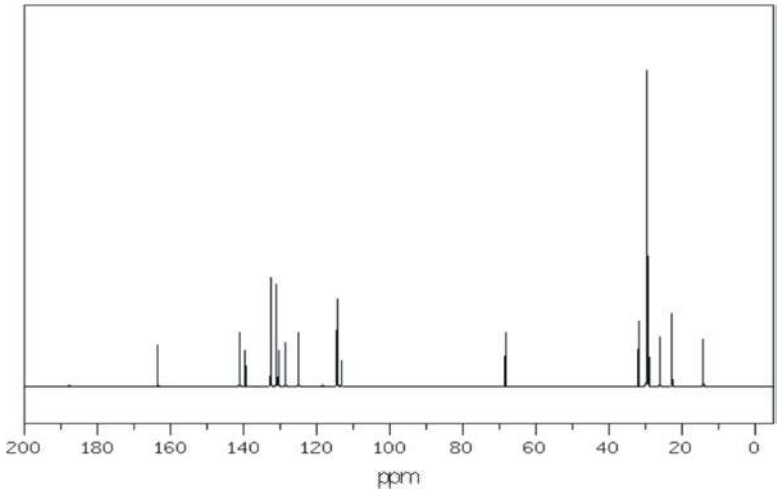

Fig. 4: ^{1}H NMR Spectrum of CB

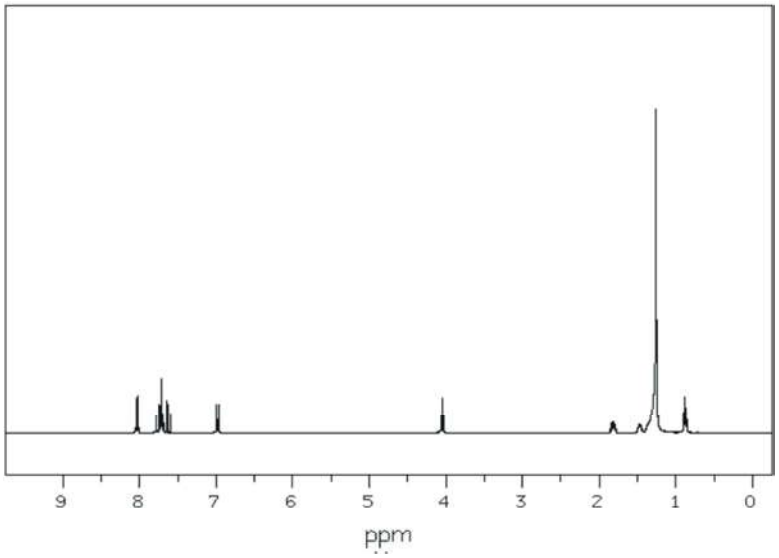

Fig. 5:^{13}C NMR Spectrum of CB

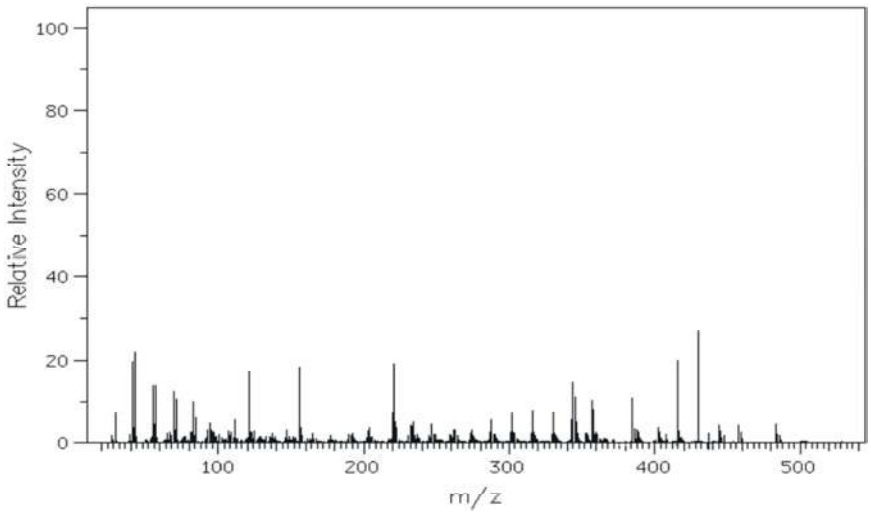

Fig. 6: Mass Spectrum of CB

CONCLUSION

Infectious diseases are caused by pathogenic microorganisms. Many plants are found to contain compound which are used as natural medicines to treat common bacterial infections. Medicinal plants are used in various system of medicine because of minimal side effect and cost effectiveness. Herbal medicines which formed the basis of health care throughout the world since the earliest days of mankind are still widely used and have considerable importance in international trade. In this work, the agar-disk diffusion method was used to evaluate the antimicrobial potential of the crud extract and purified compound (CB) isolated from *Clerodendrum baronianum*. The results obtained indicated that the crud and purified extracts possess antibacterial activity. The purified steroidal compound (CB) had the greatest activity than crud extract. Furthermore, the molecular formula of compound could be determined as $C_{28}H_{46}O_2N$ by some advanced spectroscopic methods such as IR, ^{1}HNMR, ^{13}CNMR and EI-MS, spectral data. The present research work suggests that the *Clerodendrum baronianum* may be considered as a potential source of broad spectrum antibacterial activity.

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