ISSN 0975-6299 Vol 2/Issue 1/ Jan-Mar 2011

International Journal of Pharma and Bio Sciences

RESEARCH ARTICLE

PHARMACOGNOSY

PHYTOCHEMICAL SCREENING AND ANTIOXIDANT ACTIVITY STUDIES OF *CERBERA ODOLLAM* GAERTN.

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ABSTRACT

A total of three compounds i.e. one 14β (H) steroid and two benzoic acid derivatives have been isolated from the carbon tetrachloride soluble fraction of a methanol extract of the stem bark of *Cerbera odollam* Gaertn (Family-apocynaceae). The structure of 14β(H) steroid was established to be triticusterol (**1**) on the basis of spectroscopic data. This is the second example of a naturally occurring compound with a 14β(H)-steroid skeleton. The benzoic acid derivatives were tentatively characterized as 2,6-dihydroxy-4-methoxy benzoic acid (**2**) and 2-hydroxy-4-methoxy-6-methyl benzoic acid (**3**). The *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble fractions of methanolic extract of the bark showed moderate to potent antioxidant activity, of which the chloroform soluble fraction demonstrated the strongest antioxidant activity with IC50 value 21.0 µg/ml.

KEY WORDS

Antioxidant activity, Apocynaceae, *Cerbera odollam* Gaertn, Triticusterol, 2,6-dihydroxy-4-methoxy benzoic acid, 2-hydroxy-4-methoxy-6-methyl benzoic acid.

INTRODUCTION

Cerbera odollam Gaertn. is a mangrove plant belonging to the apocynaceae family and distributed widely in the coastal areas of South East Asia and the Indian Ocean [1]. The seeds are excessively toxic, containing cerberin as the main active cardenolide. The odollam tree is responsible for about 50% of the plant poisoning cases and 10% of the total poisoning cases in Kerala, India. It is used both for suicide and homicide [2]. Seeds exhibit antiproliferative as well as antiestrogenic activities [3] in mammals. The leaf extract of this plant has toxic effects on central nervous system of mice [4]. Previous phytochemical investigations on the different parts of the plant have revealed the presence of cardenolides [5-7], lignans [8], iridoid monoterpenes [9] and cardenolide glycoside [1]. We herein report second time isolation of triticusterol **(1)** from natural source and first time isolation of 2,6-dihydroxy-4-methoxy benzoic acid (**2**), 2-hydroxy-4-methoxy-6-methyl benzoic acid (**3**) from the carbon tetrachloride soluble fraction as well as antioxidant activities of the extractives of *C. odollam.*

MATERIALS AND METHODS

General:

The ESIMS spectra were recorded using Applied Biosystems API 2000 (Ionization Mode: ESI). The ^{1}H NMR spectra were recorded using a Bruker AMX-400 (400 MHz) instrument and the spectra were referenced to the residual nondeuterated solvent signal in NMR laboratory of BCSIR, Dhaka. PTLC was carried out using Merck Si gel 60 F_{254} on glass plates (20 cm X 20 cm) at a thickness of 0.5 mm. TLC was conducted on normal-phase Merck Si gel 60 F_{254} as glass plates. Spots on TLC and PTLC plates were visualized by spraying with vanilllin sulfuric acid followed by heating for 5 minutes at 110 ºC.

Plant Material and Authentication:

Barks of *C. odollam* were collected from Sundarban, Khulna in August 2008. The plant was authenticated by Bangladesh National Herbarium and a voucher specimen (DACB-31302) for this collection has been deposited in Bangladesh National Herbarium, Mirpur, Dhaka.

Extraction and Isolation:

The air-dried and powdered material (533 g) was soaked in 1.5 liter of methanol in a large conical flask for 7 days with occasional shaking and stirring. The whole mixture was then filtered off through a cotton plug followed Whatman filter paper no.1 and the filtrate thus obtained was concentrated at 40 °C with a rotary evaporator. A portion (5.0 g) of the concentrated methanol extract was fractionated by the modified Kupchan partitioning protocol [10] which afforded *n*-hexane (850.0 mg), carbon tetrachloride (1.0 g), chloroform (750 mg) and aqueous (1.5 g) soluble materials.

An aliquot of the *n*-hexane soluble partitionate (300 mg) was fractionated by Sephadex (LH-20) column chromatography (CC) using pet-ether and chloroform mixture in order of increasing polarities. A total of 10 fractions were collected, each 20 ml. Preparative TLC of column fractions eluted with 20% petroleum ether in chloroform, over silica gel using toluene : EtOAc (80:20) afforded compound **1** (4.0 mg). Again, Preparative TLC of column fractions eluted with 12% petroleum ether in chloroform, over silica gel using toluene : EtOAc (85:15)

afforded compound **2** (3.5 mg) and **3** (3.5 mg).

Triticusterol (1): (4.0 mg, 0.08% yield); white crystal, ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.88 (3H, s, H-18), 0.92 (3H, d, *J* = 6.4 Hz, H-21), 0.96 (3H, s, H-19), 0.99 (3H, d, *J* = 6.8 Hz, H-28), 1.01 (3H, d, *J* = 6.8 Hz, H-26), 1.02 (3H, d, *J* = 6.8 Hz, H-27), 3.09 (1H, m, H_α-3), 4.66, 4.72 (2H, s, H-24^1); Electrospray MS: *m/z* [M + $CH_3CN]^+$ 453.7, $C_{29}H_{48}O$ + CH_3CN

2,6-Dihydroxy-4-methoxy benzoic acid (2): (3.5 mg, 0.07 % yield); white crystal; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.85 (3H, s, 4-OMe), 6.16 (1H, s, 6-OH), 6.30 (1H, d, *J* = 2.4 Hz, H-5), 6.45 (1H, d, *J* = 2.4 Hz, H-3), 11.11 (1H, s, 2-OH).

2-Hydroxy-4-methoxy-6-methyl benzoic acid (3): (3.5 mg, 0.07 % yield); white crystal; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.48 (3H, s, 6-Me), 3.91 (3H, s, 4-OMe), 6.22 (1H, br. s, H-3), 6.26 (1H, br. s, H-5), 11.68 (1H, s, 2-OH).

Antioxidant Activity:

The antioxidant activity (free radical scavenging activity) of the extracts on the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was determined by the method of Brand-Williams [11]. In the experiment, 2.0 mg of each of the extract was dissolved in methanol. Solution of varying concentrations such as 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.50 µg/ml, 31.25 µg/ml, 15.62 µg/ml, 7.8125 µg/ml, 3.91 µg/ml, 1.95 µg/ml and 0.98 µg/ml were obtained by serial dilution technique. The methanol solution of the extract (2.0 ml) of each concentration was mixed with 3 ml of a DPPH-methanol solution (20 µg/ml) and was allowed to stand for 20 minutes for reaction to occur. Then the absorbance was determined at 517 nm and from these values the corresponding percentage of inhibitions were calculated by using the following equation:

$$\% \text{ inhibition} = [1 - (ABS_{sample} / ABS_{control})] \times 100$$

Then % inhibitions were plotted against respective concentrations used and from the graph IC_{50} was calculated by using *tert*-butyl-1-hydroxytoluene (BHT), a potential antioxidant, was used as positive control.

Statistical Analysis:

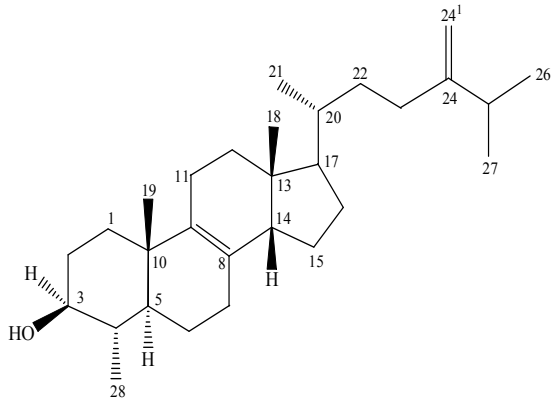
For each of the extracts, three samples were prepared for each of the bioassays. The IC_{50} values were calculated as mean ± SD (n=3) for the antioxidant activity.

RESULTS AND DISCUSSION

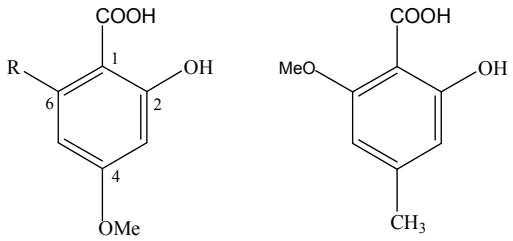
Repeated chromatographic separation and purification of the carbon tetrachloride soluble partitionate of a methanol extract of *C. odollam* provided three compounds (Fig. 1), the structures of which were determined by careful interpretation of NMR and mass spectral data.

Compound **1** was isolated as white crystal from the carbon tetrachloride soluble fraction of *C. odollam.* The Electrospray mass spectrum gave a pseudomolecular ion [M + $CH_3CN]^+$ peak m/z 453.7 corresponding to the formula $C_{29}H_{48}O$. The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound **1** showed two signals at δ = 0.88 and 0.96 ppm for angular methyl groups at C-13 and C-10, respectively. It also revealed four doublets centered at δ 0.92 (3H, d, *J* = 6.4 Hz), 0.99 (3H, d, *J* = 6.8 Hz), 1.01 (3H, d, *J* = 6.8 Hz) and 1.02 (3H, d, *J* = 6.8 Hz) which could be assigned to the methyl substituents at C-20, C-4 and C-25, respectively. The spectrum further revealed a one proton multiplet at δ 3.09, indicative of H_α-3 of the steroid nucleus. Two downfield singlets were appeared at δ = 4.66 and 4.71 ppm demonstrating the presence of two exomethylene protons, H_2-24^1. All the above NMR values and mass spectrum are close in agreement with those reported for triticusterol (**1**) [12]. In case of 14β (H) steroids the angular methyl at C-13 appears within 0.82-0.86 ppm, whereas in14α (H) steroids this methyl appear within 0.61-0.69 ppm [12].

Triticusterol is the first example of naturally occurring steroid with a 14β (H) configuration. Although, this compound was previously isolated from the germ oil of wheat (*Triticum aestivum* L; Graminaea) [12], this is the second report of its isolation from natural source.



(**1**)



2: R = - OH;
3: R = -CH_3

4

Figure 1: ***Structure of compounds isolated from the stem bark of C. odollam***

The ^{1}H NMR spectrum of compound **2** displayed two doublets centered at δ 6.45 (1H, *J* = 2.4 Hz) and 6.30 (1H, *J* = 2.4 Hz). The splitting pattern and coupling constant of these signals revealed these to be *meta* oriented indicating that it is tetrasubstituted benzoic acid. It showed a three proton singlet at δ 3.85 for methoxy group and chelated hydrogenated proton at δ 11.11. A broad singlet of one proton intensity at δ 6.16 suggested the presence of a hydroxyl proton. The comparison of NMR values indicates that the compound **2** is a structural analogue of 2-hydroxy-4-methyl-6-methoxy benzoic acid (**4**) [13]. In compound **4,** the methoxy group is more deshielded (δ = 4.04) while the methyl group present at C-4 appears at δ 2.34. But in compound **2,** the methoxy group appears at δ 3.85 which indicates that this group is shielded and present at the *para* position of benzene ring. On this basis, compound **2** was tentatively characterized as 2,6-dihydroxy-4-methoxy benzoic acid.

The ^{1}H NMR spectrum of compound **3** is very similar to that observed for compound **2** suggesting a close structural similarity. The spectrum displayed a three proton singlet at δ 3.91 for a methoxy group at C-4 position of an aromatic ring and a broad singlet at δ 11.68 for a hydroxyl proton at C-2. A singlet integrating for three protons at δ 2.48 was demonstrative of a methyl group at C-6 of the aromatic nucleus. The ^{1}H NMR spectrum of **3** further showed two broad singlets at δ 6.22 and 6.26 which could be assigned to H-3 and H-5, respectively. On this basis, compound **3** was tentatively characterized as 2-hydroxy-4-methoxy-6-methyl benzoic acid which is a structural isomer of 2-hydroxy-4-methyl-6-methoxy benzoic acid (**4**) [13]. In compound **4,** the three proton singlet appears at δ 4.04 and 2.34 indicating the presence of a methoxy group and a methyl group at C-6 and C-4 of the benzene ring respectively. But here, the methyl group appears at δ 2.48 which is more deshielded due to the anisotropy of carbonyl (CO) moiety of carboxylic acid group. Therefore, the methyl group and methoxy group of **3** are present at C-6 and C-4 of the benzene ring respectively. Due to the paucity of the compound **3**, no further spectral data could be taken as such the structure could not be confirmed.

In antioxidant screening (Table 1), the chloroform soluble fraction showed the highest antioxidant activity with IC_{50} value of 21.0 µg/ml. At the same time, the carbon

tetrachloride soluble fraction of the methanolic extract also exhibited significant antioxidant activity (IC_{50} = 26.0 µg/ml), where the crude methanol extract and aqueous soluble fraction showed mild free radical scavenging activity with the IC_{50} values 46.0 and 62.50 µg/ml, respectively. These results denote the presence of antioxidant principles in the extractives.

Table 1
IC_{50} data of test samples of C. odollam

Samples	IC_{50} (µg/ml)
BHT	14.50 ± 0.32
MEBP	46.0 ± 1.29
HSF	135.0 ± 0.96
CTSF	26.0 ± 1.33
CFSF	21.0 ± 1.25
AQSF	62.50 ± 1.14

The values of IC_{50} are expressed as mean ± SD (n=3); BHT: Butylated Hydroxy Toluene (Std.). MEBP: Crude methanolic extract of the bark of the plant, HSF: n-hexane soluble fraction of the methanolic extract; CTSF: carbon tetrachloride soluble fraction of the methanolic extract; CFSF: chloroform soluble fraction of the methanolic extract; AQSF: aqueous fraction of the methanol extract.

CONCLUSION

We have successfully isolated one 14β (H) steroid and two benzoic acid derivatives from the plant, *Cerbera odollam*. On the basis of these spectral data, the structures of the isolated compounds were characterized as triticusterol **(1),** 2,6-dihydroxy-4-methoxy benzoic acid (**2**) and 2-hydroxy-4-methoxy-6-methyl benzoic acid (**3**).

ACKNOWLEDGEMENTS

The authors wish to thank Professor Hiroaki Ozaki, Department of Chemistry and Chemical Biology, Graduate School of Engineering, Gunma University, Kiryu, Gunma 376-8515, Japan, for providing with ^{1}H NMR and MS measurements

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