Isolation of antibacterial constituent from rhizome of *Drynaria quercifolia* and its sub-acute toxicological studies

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ABSTRACT

Background and the purpose of the study: The rhizomes of *Drynaria quercifolia* have antibacterial properties and are used traditionally for the treatment of cough, tuberculosis and typhoid fever. In the present study an attempt was made to isolate microbiologically active constituents from the rhizome of *D. quercifolia* and to determine their antibacterial and toxicological effects.

Methods: Bioassay-guided investigations was employed for isolation of the active constitute of the rhizome of *Drynaria quercifolia* J. Smith. Disc diffusion technique and serial tube dilution technique were used to determine *in vitro* antibacterial activity and MIC, respectively. Sub-acute toxicities (body weight, hematological, biochemical and histopathological) were studied in albino mice upon 14 days treatment.

Result and major conclusion: Bioassay-guided investigations led to isolation of 3,4-dihydroxybenzoic acid whose *in vitro* antibacterial activity, minimum inhibitory concentration (MIC) and sub-acute toxicities were studied. The 3,4-dihydroxybenzoic acid showed significant antibacterial activity against four Gram-positive and six Gram-negative bacteria. The MIC values of 3,4-dihydroxybenzoic acid against these bacteria ranged from 8 to 64 µg/mL. In sub-acute toxicities studies 3,4-dihydroxybenzoic acid showed no significant effect in comparison to that of control group. In addition, acetyl lupeol was isolated from rhizome of this plant whose *in vitro* antibacterial activity was insignificant. Isolation of 3,4-dihydroxybenzoic acid and acetyl lupeol are the first report from this plant.

Keywords: *Drynaria quercifolia*, 3,4-dihydroxybenzoic acid, Bacteria, Mice.

INTRODUCTION

The frequency of life threatening infections caused by pathogenic microorganism is increased worldwide and is becoming an important cause of morbidity and mortality in immunocompromised patients in the developing countries (1, 2). Although huge numbers of antibacterial agents have been discovered, the pathogenic microorganisms are developing resistance against these agents day by day (3, 4). In the third world countries like Bangladesh, irrational use of antimicrobial agents is a major cause of such resistance (4). In recent years, attempts have been made to investigate the indigenous drugs against infectious diseases (5). Research in the field of indigenous plant is a significant aspect to develop a safe antimicrobial principle through isolation, identification and biological studies (5).

Drynaria quercifolia J. Smith (syn. *Polypodium quercifolium*, Fam. Polypodiaceae), locally known as Gurar, is a parasitic fern (6, 7) that is widely distributed in Bangladesh, India and Thailand (7, 8). The rhizomes of the plant have antibacterial properties and are used traditionally for treatment of cough, tuberculosis and typhoid fever (6-7,9). ASEAN Centre for Biodiversity mentioned in their Checklist of Medicinal Plant in Southeast Asia that rhizome decoction or drink of *D. quercifolia* rhizome uses as antipyretic preparation (8). Isolation of Friedelin, epifriedelinol, *β*-amyrin, *β*-sitosterol, *β*-sitosterol 3-*β*-D-glucopyranoside and naringin from this plant has been reported (9). Although the plant is widely used for remission of several ailments related to microorganism, no significant microbiologically active constituent has been yet isolated. Therefore, in the present study an attempt was made to isolate microbiologically active constituents from the rhizome of *D. quercifolia* and to determine their antibacterial and toxicological effects.

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MATERIALS AND METHODS

Plant materials

The rhizome of the plant was collected from various part of Lakshmipur district of Bangladesh and identified by Professor A.T.M. Naderuzzaman, Department of Botany, University of Rajshahi, Bangladesh and a voucher specimen (No. 1939) was deposited. The rhizome was cut, air-dried and powdered.

Isolation of compounds

The powder materials (600 g) were extracted with ethanol (3 L) in a Soxhlet apparatus (Quickfit, Staffordshire, England) at 65 $^{\circ}$C for 72 h (10). The extract was filtered through filter paper. The filtrate was concentrated under reduced pressure at 50^{0} C in a rotary vacuum evaporator to afford a blackish green mass (25.4 g). This green mass was further extracted with petroleum ether (3 x 50 mL), chloroform (3 x 50 mL) and ethyl acetate (3 x 50 mL) and dried under reduce pressure to afford petroleum ether (7.5 g), chloroform (7.8 g) and ethyl acetate (5.5 g) fractions, respectively (11, 12).

The ethyl acetate soluble fraction showed significant antibacterial activity and was subjected to column chromatography using chloroform and methanol of increasing polarity. Column chromatography yielded 32 fractions. The fractions eluting with 10-25% methanol in chloroform showed significant antibacterial activity and were subjected to preparative TLC (mobile phase 15% methanol in chloroform) to give compound **1** (89 mg). In solubility test compound **1** was sparingly soluble in water and freely soluble in ethyl acetate, methanol and acetone.

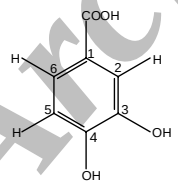

Figure 1. Structure of compound **1** (3, 4-dihydroxy benzoic acid)

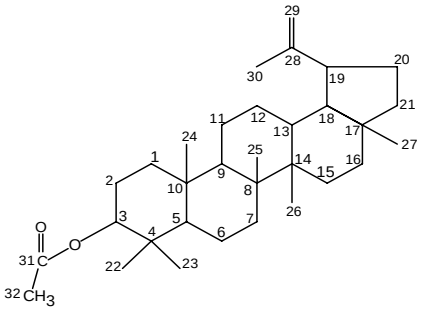

Figure 2. Structure of compound **2** (acetyl lupeol)

The chloroform soluble fraction showed moderate antibacterial activity and was subjected to column chromatography using n-hexane, chloroform and methanol of increasing polarity. Column chromatography yielded 41 fractions. The fractions eluting with 5-10% chloroform in n-hexane showed antibacterial activity and were subjected to preparative TLC (mobile phase 40% chloroform in n-hexane) to give compound **2** (25 mg) whose antibacterial effect was insignificant. The TLC analysis also indicated that compound **1** is also present in these fractions (5-10% chloroform in n-hexane) but in minor quantity. The antibacterial activity of the petroleum ether fraction was insignificant.

Antibacterial screening

In vitro antibacterial screening was carried out against four Gram-positive bacteria (*Bacillus subtilis*, *Bacillus megaterium*, *Staphylococcus aureus*, *Streptococcus β-haemolyticus*) and six Gram-negative bacteria (*Escheichia coli*, *Shigella dysenteriae*, *Shigella sonnei*, *Shigella flexneri*, *Pseudomonus aeruginosa*, *Salmonella typhi*). These organisms were available in the Microbiology Research Laboratory of Pharmacy Department, Rajshahi University, Bangladesh. The pure cultures of these bacteria were collected from the Microbiological Laboratory of the Institute of Nutrition and Food Science (INFS) and Department of Microbiology, University of Dhaka, Bangladesh.

Disc diffusion method, a qualitative to semi quantitative test, was used for antibacterial screening (13, 14). Briefly, 20 mL quantities of nutrient agar were plated in petridish with 0.1 mL of a 10^{-2} dilution of each bacterial culture (18 h old). Filter paper discs (6 mm in diameter) impregnated with respective samples (compound **1** and **2**, of the chloroform and ethyl acetate fraction) were placed on the test organism seeded plates (Table 1). Methanol was used to dissolve the sample and was completely evaporated before application on the test organism seeded plates. Blank disc impregnated with solvent methanol followed by drying off was used as negative control. The activity was determined after 18 h of incubation at 37 °C. The diameters of zone of inhibition produced by samples were then compared with the standard antibiotic kanamycin 30 µg/disc. Each sample was used in triplicate for determination of antibacterial activity.

Minimum inhibitory concentration (MIC) determination

Serial tube dilution technique was used to determine MIC of compounds against tested bacteria (3, 4, 15, 16, 25). Compound **1** (1.024

mg) was dissolved in 2 mL of distilled water (few drops Tween 80 was added to facilitate dissolution) to obtain stock solution having concentration 512 µg/mL. Serial dilution technique was used to obtain 256 up to 2 µg/mL dilutions. After preparation of suspensions of test organisms (10^7 organism per mL), 1 drop of suspension (0.02 mL) was added to each broth dilution. After 18 h incubation at 37^0 C, the tubes were then examined for the growth. The MIC of compound **1** was taken as the lowest concentration that showed no growth. Growth was observed in those tubes where the concentration of the compound **1** was below the inhibitory level and the broth medium turbid (cloudy). Distilled water with few drops of tween 80 was used as negative control and kanamycin was used as positive control.

Experimental animals for sub-acute toxicity studies

The experiment was carried out on albino mice (Swiss strain). They were 2-3 months old of both sexes weighing between 20 -27 g (average weight was 22.13 g). They were collected from the International Center for Diarrhoeal Diseases and Research, Bangladesh (ICDDR,B). The animals were housed in iron cages (17) under temperature and light controlled condition (18). They were fed a balanced diet (19) and tap water. The animals were maintained under this condition for 15 days before experiment in order to adjust with food and environment. The mice were divided into two groups, experimental and control groups. Number of mice in each group was four (17, 20, 21, 31).

Experimental procedure for sub-acute toxicity studies

The animals were supplied with measured amount of fresh food and sufficient water daily, throughout the study. Each mouse of experimental group was administered 0.2 mL of sample solution (containing 300 µg of compound **1**) daily, for 14 consecutive days and each mouse of control group was administered 0.2 mL of solvent (it was used to dissolve compound **1**) daily, for 14 consecutive days (21). Intraperitoneal route was used for administration of compound **1** and solvent.

The behavior of the animals, throughout the study was observed daily. The body weight of each mouse was measured before administration of the compound and after completion of the treatment. For hematological study (total and differential blood cell count, percent hemoglobin determination), blood was drawn from the tail vein of both groups before drug administration, at 7th day and after completion of treatment. For biochemical studies, blood was collected at 15th day after completion of treatment from the jugular veins of each mouse. Biochemical parameters (serum glutamate oxaloacete transaminase, serum glutamate pyruvate transaminase, serum alkaline phosphatase, urea, uric acid and creatinine) were determined using standard procedures (21) and reagents supplied by Boehringer Mannheim GmbH Diagnostica. After finishing blood collection for hematological and biochemical studies the mice were sacrificed for histopathological studies. Histopathological studies of liver, kidney, heart, lung and spleen were performed using a haematoxylin and eosin stain and D.P.X. mounting fluid (21). The samples were observed under microscope at the Department of Pathology, Rajshahi Medical College Hospital, Bangladesh.

RESULTS

Structures of isolated compounds

The compound **1** obtained as brownish color needle like crystal with melting point 199-200 °C. In solubility test compound **1** was sparingly soluble in water and freely soluble in ethyl acetate, methanol and acetone. The liquid chromatography/electrospray-mass spectroscopy (LC/ES-MS) in the positive ion mode of compound **1** showed molecular $[M+H]^+$ peak at *m/z* 154.8 corresponding to a molecular formula of $C_7H_6O_4$. The IR spectrum exhibited bands at 1240, 1375, 1739, 2877, 2908 and 2985 cm^{-1}. The ^{1}H-NMR, ^{13}C-NMR, HSQC and HMBC spectral data of compound **1** was in good agreement with spectral data of 3,4-dihydroxybenzoic acid published in literature (22). The compound **2** was obtained as needle like crystal with melting point $218\text{-}220^0$ C. The liquid chromatography/ electrospray-mass spectroscopy (LC/ES-MS) in the positive ion mode of **2** showed molecular $[M+H]^+$ peak at *m/z* 469.5 corresponding to a molecular formula of $C_{32}H_{52}O_2$. The IR spectrum exhibited bands at 1240, 1650, 1700, 1735, and 3065 cm^{-1}. The NMR spectral data of compound **2** was in good agreement with spectral data of acetyl lupeol reported in literature (23). These finding indicated the isolated compound **1** and **2** are 3,4-dihydroxybenzoic acid and acetyl lupeol, respectively. Isolation of both of these compounds from *Drynaria quercifolia* are reported for the first time

Antibacterial screening

The compound **1** has shown significant antibacterial activity against tested Gram-positive and Gram-negative bacteria compared with standard kanamycin (Table 1). The extract and different fractions containing compound **1** also exhibited significant antibacterial activity. The antibacterial activity of acetyl lupeol against these bacteria was insignificant (Table 1).

Table 1. *In vitro* antibacterial activities of chloroform and ethyl acetate fractions, kanamycin as standard and compound **1** and **2**.. Data are presented as Mean ± SEM

Test organism	Diameter of zone of inhibition (mm)					
	Chloroform fraction 150 µg/disc	Ethyl acetate fraction 150 µg/disc	Compound **1** 30 µg/disc	Compound **1** 80 µg/disc	Compound **2** 80 µg/disc	Kanamycin 30 µg/disc
Gram-positive						
Bacillus subtilis	11 ± 2.5	20 ± 1.7	12 ± 0.8	27 ± 2.4	6 ± 0.5	30 ± 2.3
Bacillus megaterium	9 ± 1.2	21 ± 2.1	10 ± 1.2	22 ± 1.1	8 ± 1.1	24 ± 1.3
Staphylococcus aureus	14 ± 0.7	24.5 ± 1.7	13 ± 0.9	26 ± 1.4	0	31 ± 1.5
Streptococcus-β -haemolyticus	15 ± 1.2	24 ± 1.5	8 ± 0.6	23 ± 1.9	7 ± 0.4	25 ± 0.9
Gram-negative						
Escheichia coli	12 ± 2.5	19 ± 1.3	14 ± 1.5	22 ± 1.3	7 ± 0.5	24 ± 2.1
Shigella dysenteriae	10 ± 0.6	21 ± 1.6	13 ± 1.2	19 ± 1.5	10 ± 0.8	30 ± 1.4
Shigella sonnei	11 ± 1.2	12 ± 0.8	14 ± 1.1	25 ± 1.8	8 ± 1.0	32 ± 1.6
Shigella flexneri	9 ± 0.7	17 ± 1.4	11 ± 1.2	21 ± 0.8	11 ± 0.9	28 ± 1.1
Pseudomonus aeruginosa	13 ± 1.0	22 ± 1.1	13 ± 1.6	25 ± 2.2	0	31 ± 1.6
Salmonella typhi	8 ± 1.2	11 ± 0.7	14 ± 1.2	25 ± 1.4	0	28 ± 1.4

The control disc used for solvent showed no zone of inhibition, so their data was omitted from the above data. Data are represented in the form of mean of three tests ± SEM of the standard group.

Table 2. Minimum inhibitory concentration of the compound **1** and kanamycin as standard.

Bacteria	MIC (µg/mL)	
	Compound **1**	kanamycin
Bacillus subtilis	8	2
Bacillus megaterium	16	4
Staphylococcus aureus	32	8
Streptococcus β-haemolyticus	32	8
Escheichia coli	16	8
Shigella dysenteriae	32	2
Shigella sonnei	16	4
Shigella flexneri	32	16
Pseudomonus aeruginosa	64	16
Salmonella typhi	16	4

The control used for solvent showed no MIC values, so their data was omitted from the above data.

Table 3. Effect of compound **1** on body weight of mice after intraperitoneal administration.

Group of mice	Body weight (g) before treatment (n = 4, $M_1 \pm SD_1$)	Body weight (g) after treatment (n = 4, $M_2 \pm SD_2$)	% change	Calculated 't' value	Remark
Control	23.48 ± 1.41	23.9 ± 1.42	+1.79	0.42	NS
Experimental	23.55 ± 1.5	23.93 ± 1.5	+1.6	0.36	NS

M_1 and M_2 = mean weight of mice; SD_1 and SD_2 = standard deviations; n = number of mice; '+'= increase, '-' = decrease; t = significance of difference between experimental and control group and NS = not significant. Student's t-test was used for comparison between the experimental and control groups. $p<0.05$ was considered to be statistically significant.

Minimum inhibitory concentration (MIC) determination

The MIC values of compound **1** against tested Gram-positive and Gram-negative bacteria ranged from 8-32 and 16-64 µg/mL, respectively (Table 2). These MIC values indicate the potency of compound **1** against Gram-positive bacteria in comparison with Gram-negative bacteria is higher.

Body weight changes (sub-acute toxicities studies)

Average body weights of all mice before and after treatment are presented in Table 3. After 14 days control group gained weight 1.79% and experimental group gained weight 1.6%. The change in body weight for both control and experimental group were insignificant.

Hematological profiles (sub-acute toxicities studies)

The hematological profiles of the experimental and control group mice were determined before at 7th day and after treatment and were compared to check the hematological disorders after intraperitoneal administration of compound **1**. No noticeable changes in the values of RBC, WBC, platelet and differential WBC count and haemoglobin percentage in experimental mice were observed when compared with those of control group mice (Table 4) were observed.

Biochemical parameters of blood (sub-acute toxicities studies)

Biochemical parameters of blood (SGOT, SGPT, alkaline phosphatase, urea, uric acid and creatinine) were determined after treatment by compound **1** and compared with values of control group mice to check any change in these parameters. It was found that most of the parameters were slightly changed with respect to control group mice but remained within the normal range (Table 5). These findings indicate insignificant adverse effect of compound **1** on liver and kidney functions.

Table 4. Hematological profiles of experimental group and control group mice before treatment, at 7th day of treatment and after treatment.

Parameters		Control			Experiment		
		Day 1 M ± SD	Day 7 M ± SD	Day 15 M ± SD	Day 1 M ± SD	Day 7 M ± SD	Day 15 M ± SD
RBC (million/cu. mm)		5.08 ± 0.07	5.13 ± 0.07	5.16 ± 0.09	5.10 ± 0.08	5.17 ± 0.09	5.37 ± 0.12
WBC (th./cu. mm)		5.86 ± 0.32	5.94 ± 0.33	6.04 ± 0.28	6.08 ± 0.21	6.15 ± 0.23	6.19 ± 0.22
Differential WBC count (No/cu. mm)	Neutrophil	3637.7± 198.7	3681.3± 204.9	3746.3± 176.5	3766.5± 132.4	3813 ± 143.8	3836.25 ± 139.3
	Lymphocyte	1760.3 ± 96.1	1781.3 ± 99.1	1812.8 ± 85.3	1822.5± 64.08	1845 ± 69.5	1856.25 ± 67.4
	Monocyte	310.75 ±16.98	314.25 ± 17.5	320.50± 15.1	321.5 ± 11.23	325.5 ± 12.3	327.75 ± 11.84
	Eosinophil	134.5 ± 7.5	136 ± 7.6	139 ± 6.7	139.25 ± 5.07	141 ± 5.2	142.25 ± 5.58
Platelets (No/cu. mm)		310916 ± 228	311935 ± 308	320913 ± 568	324142 ± 208	326578±329	325643 ± 367
Haemoglobin (gm/100 mL)		15.6 ± 0.46	15.8 ± 0.59	15.9 ± 0.63	15.12 ± 0.43	15.16 ± 0.57	15.75 ± 0.44

M = mean value; SD = standard deviation and n = 4. Student's t-test was used for comparison of the experimental and control groups. $p<0.05$ was considered to be statistically significant.

Table 5. Effects of compound **1** on biochemical parameters of blood of mice after i.p. administration at a dose of 300 µg/mouse/day for 14 consecutive days.

Biochemical parameters	Control group (n = 4, $M_1 \pm SD_1$)	Experimental group (n = 4, $M_2 \pm SD_2$)	Percentage of change	Calculated 't' values	Remark
S.G.O.T. (IU/L)	11 ± 1.22	11.5 ± 1.5	+4.5	+0.29	NS
S.G.P.T. (IU/L)	13.75 ± 0.43	15.5 ± 0.87	+12.7	+3.60	NS
Serum alkaline phosphatase (IU/L)	0.48 ± 0.03	0.48 ± 0.03	00	00	NS
Creatinine (mg/dL)	0.54 ± 0.02	0.55 ± 0.03	+1.85	+0.55	NS
Uric acid (mg/dL)	7.12 ± 3.26	7.31 ± 1.78	+2.45	+0.10	NS
Blood urea (mg/dL)	28.5 ± 3.35	30 ± 3.7	+5.2	+0.60	NS

M_1 and M_2 = mean values; SD_1 and SD_2 = standard deviations; n = number of mice; '+' = increase; '-' = decrease; t = significance of difference between experimental and control group and NS = not significant. Student's t-test was used for comparison between the experimental and control groups. $p<0.05$ was considered to be statistically significant.

Histopathological studies (sub-acute toxicities)
After 14 day of drug administration, the animals of both control and experimental groups were sacrificed and their liver, kidney, lung, spleen and heart were isolated and examined under a microscope. No abnormalities were detected in the organs of both control and experimental animals, indicating that compound **1** has no significant adverse effect on cellular structures.

Statistical analysis
Antibacterial activity is presented as mean ± standard error mean (Mean ± SEM) and toxicological data are presented as mean ± standard deviation (Mean ± SD). Student's t-test was used for comparison of the experimental and control groups. $p<0.05$ was considered to be statistically significant.

DISCUSSION

Isolation of 3,4-Dihydroxybenzoic acid from *Drynaria quercifolia* (Syn. *Polypodium quercifolium*, Fam. Polypodiaceae) is reported for the first time. 3, 4-dihydroxybenzoic acid has been previously isolated from *Polypodium leucotomos* (Polypodiaceae) (24), bark of *Cananga odorata* (Annonaceae) (22, 25), stem and fruits of *Vismia parviflora* (Guttiferae) (26) and *Illicium verum* (Illiciaceae) (27). Also isolation of acetyl lupeol is reported from the genus *Drynaria* as well as from the family Polypodiaceae for the first time. Acetyl lupeol has previously been isolated from roots of *Parahancornia amapa* (Apocynaceae) (28), bark of *Ficus macrophylla* (Moraceae) (29), *Amsonia grandiflora* (Apocynaceae) (30) and *Himatanthus articulata* (Apocynaceae) (23). This is the first report for occurrence of the above two compounds concurrently in a plant species.

The traditional uses of the rhizome of *Drynaria quercifolia* for the treatment of cough, tuberculosis and typhoid fever (6, 7) might be related to antibacterial constituent(s) of the plant. The antibacterial property of *Drynaria quercifolia* rhizome has also previously been reported in literature (9). Antibacterial activity of 3, 4-dihydroxybenzoic acid and fractions containing 3,4-dihydroxybenzoic acid indicate that this is a principal compound for which the rhizome of *Drynaria quercifolia* showed antibacterial properties. MIC values of 3, 4-dihydroxybenzoic acid against tested bacteria (8-64 µg/mL) also indicated that this compound has some antibacterial activity. Sub-acute toxicity studies of compound **1** showed no significant effect on body weight, hematological and biochemical parameters of mice. In histopathological study, compound **1** also has shown no significant effect on liver, kidney, heart and lung of mice. These results of sub-acute toxicity studies indicate its safety for clinical trial.

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