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ANTIMICROBIAL, CYTOTOXICITY, FREE RADICAL SCAVENGING ACTIVITIES AND COMPOUNDS ISOLATED FROM THE ROOTS OF *HEMIDESMUS INDICUS* (LINN.) R. BR.

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ABSTRACT: *Hemidesmus indicus* Linn. R. Br. is a twining shrub traditionally used for the treatment of various diseases and ailments in the rural area of Bangladesh. Different extracts (methanol, hexane, ethyl acetate, n-butanol and water) from the roots of this shrub tested for their antimicrobial, cytotoxicity and free radical scavenging activities. All extracts showed moderate free radical scavenging activity and SC_{50} values ranging from 24.98µg/mL to 93.14µg/mL, and sufficient potency for their brine shrimp lethality activity and IC_{50} values from 1.32µg/mL to 8.67µg/mL. The n-hexane extract showed significant antimicrobial activity against all tested organisms, with a zone inhibition ranging from 10 mm to 27 mm at the concentration of 400µg/disc. Bioassay guided fraction of the extracts has led to isolation of two triterpeniods, 3β-O-acetyl β-amyrin, lupeol 3- β -acetate and a phenolic compound, 2-hydroxy-4-methoxy benzaldehyde. The structures of the compounds were elucidated on the basis of spectroscopic methods as well as comparison with available data in the literature.

INTRODUCTION: A large number of medicinal plants are now widely used all over the world including Bangladesh for production of both traditional and modern drugs and development of new drugs. It is estimated that the current annual demand of medicinal plants in Bangladesh for the manufacture of only traditional medicines is more than one thousand metric tons. *Hemidesmus indicus* (Linn.) R.Br. medicinal plant is being traditionally used for the treatment of various diseases and aliments which is belongs to the Periplocaceae family locally known as Anantamul.

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H. indicus is a twining shrubby climber with small opposite lanceolate sessile leaves, small flowers in axillary cluster and tortous stout long roots, grows in the Sal forests of Dhaka and Mymensingh and also in the other places in Bangladesh [1]. It is used economically for the production of low-cost medicines locally.

Root is a valuable alterative, tonic, purgative, demulcent, diaphoretic, and diuretic, antipyretic, aphrodisiac, antidiarrheal and blood purifier [2]. It is employed in nutritional disorders, loss of appetite, gonorrhea, syphilis, leucoderma, itching, chronic rheumatism [3, 4] gravel and other urinary diseases, leucorrhoea, fever, asthma, bronchitis and skin diseases; useful in hemicranias, pain in the joints and piles; juice is given to children in tonsillitis[5]. Some earlier phytochemical studies showed that the roots of *H. indicus* contain hexatriacontane, lupeol, its octacosanoate, α-amyrin, β-amyrin, its acetate[6]

and sitosterol [7]. It also contains coumarino-lignoid-hemidesminine [8], hemidesmin I and hemidesmin II, six pentacyclic triterpenes including two oleanenes, and three ursenes.

It also afforded 3-keto-lup-12-en-21, 28-olide along with lupanone [9], lupeol-3-β-acetate, hexadecanoic acid, 4-hydroxy-3-methoxybenzaldehyde and 3-hydroxy-4-methoxybenzadehyde [10], glycosides-indicine and hemidine and the roots of *H. indicus* are aromatic and possess the crystalline compound 2-hydroxy-4-methoxybenzaldehyde as the major compound (> 90%) in their volatile oils.

Although some information is available on the traditional uses of this species in herbal medicine as well as its chemical composition. To best of our knowledge a few works has done on the roots of *H.indicus* that is locally available in Bangladesh.

This paper deals with the antimicrobial, cytotoxicity and free radical scavenging activities of different extracts of the roots of *H. indicus* and isolation and identification of compounds from this species through bioactivity guided fraction of its extract. This is with the view to validate its usage against microbial infections as well as other diseases.

MATERIALS AND METHODS:

Plant material: The roots of *Hemidesmus indicus* L. R.Br. were collected from Modhupur, Tangail, Bangladesh in December 2012. The plant was identified and confirmed by Prof. Dr. Md. Abul Hassan, Department of Botany, University of Dhaka. A voucher specimen (NO DACB36524) was prepared and deposited at Bangladesh National Herbarium (BNH).

The fresh roots were taken into laboratory and cut into small pieces and were air dried. The roots were ground to powder by a Cyclotec grinding machine. The powders were stored in air tight bottle and these were used throughout the investigation.

General experimental procedure: Melting points were measured on Stuart Scientific SMP3 melting point apparatus and were uncorrected. The IR data for all compounds were obtained from dissolved sample in methanol solvent using a Shimadzu FT-IR 8400S spectrometer. UV spectra and absorbance were performed with a Perkin Elmer Lambda 25 UV-visible spectrophotometer.

^{1}H (400 MHz) and ^{13}C (100.60 MHz) NMR spectra were recorded on a Bruker DPX- 400 (400 MHz) instrument, with chemical shift data reported in ppm relative to the solvent used. General laboratory solvents were distilled from glass before use. Column chromatography (CC) and Vacuum liquid chromatography (VLC) were performed using Merck silica gel (0.063–0.2 mm) and silica ge 60H (15µm), respectively. Silica gel 60 F_{254} coated on aluminum plates for thin layer chromatography (TLC) was supplied by Merck. The reagents used in the present work were of analytical grade (Merck and BDH). Vitamin C was purchased from Sigma Aldrich Chem Co.

Extraction and Isolation: The dried, ground root (600g) was extracted with methanol at room temperature for three days. The extract was filtered and evaporated to dryness using a rotary evaporator under reduced pressure. The methanol extract (40g) was suspended in water and partitioned between water and n-hexane, aqueous part was further portioned between ethyl acetate and water then n-butanol and water. The amount of n-hexane, ethyl acetate, n-butanol and water extracts were found to be 1.5g, 6.8g, 10.54g and 2.8g respectively.

All extracts were tested for their DPPH, antimicrobial inhibition activities and cytotoxicity test **(Table 1-3)**. The n-hexane extract (0.5g) was fractioned by column chromatograph over silica gel (0.063-0.2 mm), eluting with n-hexane-dichloromethane (0-100%) and then with dichloromethane-Methanol (0-15%). A total of 12 fractions (50 ml) were collected.

The fraction eluted with 50% and 60% dichloromethane in n-hexane was combined to give compound **1** (13.1 mg). The ethyl acetate extract (5.0 g) was fractioned by VLC over silica gel 60 H (15µm), eluting with n-hexane-EtOAc (0-100%) and then with EtOAc-MeOH (0-50%). A total of 20 fractions (200 mL) were collected. The fraction eluted with 60-80% ethyl acetate in n-hexane were same and combined (110 mg) that was found to be a mixture of two compounds and chromatograph over silica gel (0.063-0.2 mm) using n-hexane dichloromethane (4:6) followed by methanol to

give seven fractions. The sub-fractions 2 and 5 afforded compound **2** (9.1 mg) and **3** (6.5 mg) respectively.

Free radical-scavenging activity*:* The free radical scavenging activity of the roots of *H. indicus* were assayed spectrophotometrically and these were carried out as previously described method [11]. The ascorbic acid was used as a positive control. Each treatment was replicated thrice.

Antimicrobial Screening: Antimicrobial activities of all extracts (methanol, n-hexane, ethyl acetate and n-butanol) of the roots of *H. indicus* were carried out by the disc diffusion methods [12, 13]. Five bacterial species which include two *Gram*-positive and three *Gram*-negative bacterial strains and two fungus *Aspergillus niger* and *Aspergillus flavus* were taken for the test. The bacteria were *Bacillus cereus (*ATCC-10876*), Staphylococcus aureus (*ATCC-9144*), Escherichia coli (*ATCC-25922*), Pseudomonas aeruginosa (*ATCC-27853*),* and *Salmonella typhi (*ATCC-6539*).* Each organism was maintained on nutrient agar slant. The samples were dissolved separately in chloroform and applied to sterile filter paper disc at a concentration of 400µg/disc. Kanamycin disc (30µg/disc) was used at standard in antibacterial and Ketoconazole disc (30µg/disc) was used at standard in antifungal study.

The sample disc, standard disc and control discs were placed gently on the previously marked zones in the agar plates pre-inoculated with test bacteria. The plates were then kept in the refrigerator at 4^0C for about 24 hours upside down to allow sufficient diffusion of the materials from discs to the surrounding agar medium. The plates were then inverted and kept in an incubator at 37^0C for 24hours.

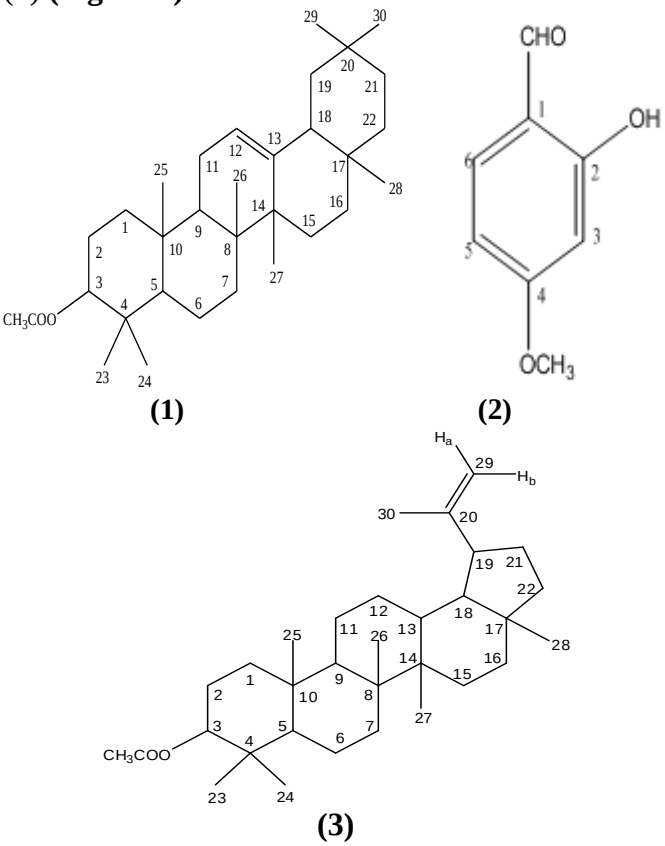
The antimicrobial potency of the test agents was measured by their activity to prevent the growth of the microorganisms surrounding the discs which gives clear zone of inhibition expressed in mm. The experiment was carried out in triplicate and the mean values were taken. The zones of inhibition were calculated as mean ±S.D. (n=3).

Brine Shrimp Lethality Bioassay: Brine Shrimp Lethality Bioassay of all the extracts (methanol, n-butanol, n-hexane and ethyl acetate) were carried out against *Artemia sarina* in a 1-day in vivo assay[14].

For the experiment 4mg of each of the extracts was dissolved in dimethyl sulfoxide (DMSO) in vials to get stock solutions. Solutions of varying concentrations such as 400, 200, 100, 50, 25, 12.50, 6.25, 3.125, 1.563, 0.781µg/ml were obtained by serial dilution technique. The median lethal concentration LC_{50} of the test samples after 24 hours was obtained by a plot of percentage of the shrimps killed against the logarithm of the sample concentration. Here vincristine sulphate was used as a standard.

RESULTS AND DISCUSSION:

The n-hexane and ethyl acetate extracts of the roots of the *H. indicus* were repeatedly fractionated using column chromatography over silica gel and led to the isolation of three known compounds (**1-3**). The known compounds were identified by comparisons of their physical and spectral data with literature values as 3β-O-acetyl β-amyrin[15] (**1**), 2-hydroxy-4-methoxy benzaldehyde[16] (**2**), lupeol 3- β -acetate[17] (**3**) **(Figure 1)**.



(1) **(2)** **(3)**

FIG 1: STRUCTURES OF THE COMPOUNDS ISOLATED FROM THE ROOTS OF *H. INDICUS*

Free radical-scavenging activity*:* The antioxidant acts either by scavenging various types of free radicals derived from oxidative processes by preventing free radical formation through reduction precursors or by chelating metals.

The free radical scavenging activity assay was carried out using the DPPH method. The free radical scavenging activity of the extracts was measured at concentration (3.125- 100µg/mL) and the results were shown in **Table 1**. The free radical scavenging activity was expressed as SC_{50}. The concentration needed to reduce 50% of DPPH. In this study all the extracts were shown to possess significant DPPH radical scavenging activity except n-hexane extract and SC_{50} of ethyl acetate, methanol, n-butanol and water extracts was 24.15, 46.63, 24.98 and 36.06 µg/ml, respectively that was compiled in **Table 1**.

Antimicrobial Screening: The bacterial and fungal strains used for the experiment were collected as pure cultures from the Institute of Nutrition and Food Science (INFS), University of Dhaka. The four extracts from the roots of *H. indicus* exhibited poor to significant antimicrobial activity against the test organisms.

TABLE 1: FREE RADICAL SCAVENGING ACTIVITY OF THE EXTRACTS OF THE ROOTS OF *H. INDICUS*

Test materials	Inhibition (%)				
	100 µg/mL	50 µg/mL	12.5 µg/mL	3.125 µg/mL	SC_{50} (µg/mL)*
Vitamin C	98.23	90.14	85.21	37.67	5.84
Methanol Extract	80.03	53.71	12.38	6.34	46.63
n-Hexane Extract	54.46	21.95	8.07	6.23	93.14
Ethyl acetate Extract	64.83	58.51	17.85	11.26	42.15
n-Butanol Extract	81.25	74.83	37.61	21.55	24.98
Water Extract	64.39	62.91	28.19	5.04	36.06

*SC_{50}: Concentration (µg/mL) at which the inhibition % of DPPH radical scavenging activity is 50%.

The methanol extract was inactive against *Gram*-positive bacteria and showed moderate activity against *Gram*-negative bacteria. It was also inactive against *Aspergillus niger* and *Aspergillus flavus.* The n-hexane extract showed significant antbacterial activity against all the test microorganisms **(Table 2)** specially *Salmonella typhi* (13 mm) and *Pseudomonas aeruginosa* (14 mm) compared to standard antibiotic Kanamycin. It showed promising antifugal activity against *Aspergillus niger* and *Aspergillus flavus*, with a zone inhibition 16 mm and 27 mm respectively at the concentration of 400 µg/disc.

TABLE 2: ANTIMICROBIAL ACTIVITY OF THE EXTRACTS OF THE ROOTS OF *HEMIDESMUS INDICUS*.

Test Organism	**Determination of zone of inhibition (mm)**					
	Methanol 400 µg/disc	**Hexane 400 µg/disc**	**Ethylacetate 400 µg/disc**	**Butanol 400 µg/disc**	**Blank 0 µg/disc**	**Kanamycin 30 µg/disc**
Gram Positive bacteria						
Bacillus cereus	---	11	11	08	---	20
Staphylococcus aureus	---	10	10	08	---	22
Gram Negative bacteria						
Salmonella typhi	08	13	08	---	---	28
Pseudomonas aeruginosa	11	14	---	08	---	18
Escherichia coli	05	11	07	10	---	25
Fungi						**KET 30 µg/disc**
Aspergillus niger	---	16	---	---	---	34
Aspergillusflavus	---	27	--	---	---	44

Note: --- = No Activity
KET= ketoconazole (Standard)

Ethyl acetate and n-Butanol extracts were found to be moderately active against *Gram*-positive bacteria and did not show any activity against both tested fungi. Ethyl acetate extract was poorly active against *Salmonella typhi, Escherichia coli* and inactive against *Pseudomonas aeruginosa.* n-Butanol extract was poorly active against *Gram*-negative bacteria except *Salmonella typhi.*

Brine Shrimp Lethality Bioassay: The degree of the lethality was found to be directly proportional

to the concentration ranging from the lowest concentration to the highest concentration. **Table 3** showed that the brine shrimp lethality testing after 24hours of exposure to the samples and the positive control, vincristine sulphate. The LC_{50} values obtained from the best fit line slope were given in the **Table 3**.

TABLE 3: CYTOTOXIC ACTIVITY OF THE EXTRACTS FROM THE ROOTS OF *H. INDICUS*

Tested materials	LC_{50} (µg/ml)
Methanol extract	2.55
n-hexane extract	1.32
Ethylacetate extract	8.67
n-Butanol extract	6.15
Vincristine sulfate (Positive control)	0.35

In comparison with the positive control, the cytotoxicity exhibited by Ethyl acetate extract shows the highest activity and the n-butanol extract was significant. Comparison with positive control, methanol and n-hexane extracts show moderate cytotoxic activity. Therefore, the positive response obtained in this assay suggests that the roots of the plant may contain antitumor, antibacterial or pesticidal compounds

CONCLUSION: The antimicrobial screening of different extracts of the roots of *H. indicus* was done by disc diffusion method using both *gram*-positive, *gram*-negative bacteria and fungi. From this result it can be reported that n-hexane extract has significant antimicrobial activity.

The free radical scavenging activity of five extracts of the roots of *H. indicus* was studied using 1, 1-diphenyl-2-picrylhydrazyl, which showed good antioxidant potency specially ethyl acetate and n-butanol extracts showed the significant efficacy. From the results of the brine shrimp lethality bioassay it reveals that the crude extracts have moderate to potent cytotoxic activity. n-Hexane and ethyl acetate extracts led to the isolation of three known compounds such as two triterpeniods (**1, 3)** and a phenolic compounds (**2**).

Triterpeniods and phenolic compounds are important plant secondary metabolites with many biological and pharmacological activities. It has been reported that 2-hydroxy-4-methoxybenzaldehyde has significant antimicrobial and antioxidant activities which could potentially be developed as an antimicrobial and antioxidant agent in future [18]. The present study has further supported the traditional uses of this plant as antimicrobial and antioxidant agents.

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