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Antimicrobial and Brine Shrimp Lethality of Fruit Extracts of *Terminalia chebula*. Isolation of a New Compound

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Abstract

The *n*-hexane (HX), carbon tetrachloride (CT), chloroform (CF) and aqueous (AQ) soluble fractions of a crude methanol extract of fruits of *Terminalia chebula* were subjected to antibacterial and antifungal activities and cytotoxicity against brine shrimp nauplii. The *n*-hexane soluble partitionate of the methanolic extract exhibited significant antimicrobial activity and aqueous soluble partitionate showed only mild sensitivity, whereas other partitionates did not show any activity. The brine shrimp lethality with LC_{50} values were 1.254, 0.826, 3.866 and 5.366 µg/mL for HX, CT, CF and AQ soluble partitionates respectively. A pure compound was isolated from the chloroform soluble partitionate and the structure of the compound was elucidated as 2-hydroxy-3-acetoxy-12-oleanen-28-oic acid **(2).** Compound **2** was isolated from *Terminalia chebula* for the first time.

Key words: *Terminalia chebula*, Antimicrobial activity, Cytotoxicity, 2-Hydroxy-3-acetoxy-12-oleanen-28-oic acid.

I. Introduction

Terminalia chebula is a plant species belonging to the genous *Terminalia*, family *Combretaceae*. It is a flowering evergreen tree called in English the black myrobalan and in Bengali Haritaki. The fruit of the tree has been used as traditional medicine for household remedy against various human ailments, since antiquity. *Terminalia chebula* has been extensively used in Ayurveda, Unani and Homoeopathic medicine and has become a cynosure of modern medicine[1].

Terminalia chebula is a medium to large deciduous tree attaining a height of up to 30m, with widely spreading branches and a broad roundish crown. The leaves are elliptic oblong, with an acute tip, cordate at the base, margins entire, glarous above with a yellowish pubescence below. The flowers are monoecious, dull white to yellow, with a strong unpleasant odour, borne in terminal spikes or short panicles. The fruits are glabrous, ellipsoid to ovoid drupes, yellow to orange brown in colour, containg a single angle stone.[2] *Terminalia chebula* is found throughout deciduous forests of the Indian subcontinent and the adjacent areas such as Bangladesh, Pakistan, Nepal, Sri Lanka, Myanmar and the South-West of China[2]. The fruit of *Terminalia chebula* has been reported for various ethno medical uses such as antiviral[3], antifungal[4], antioxidant[5], anticarcinogenic[6], antidiabetic[7] and antibacterial activities[8]. Previous phytochemical studies with *Terminalia chebula* revealed that it is rich in tannin[9], which is composed of chebulic acid, chebulagic acid, corilagin and gallic acid[10].

The current study explores further the antibacterial and antifungal activities of fruits of *Terminalia chebula*, and has included test for cytotoxicity, using brine shrimps. Furthermore, an attempt has been taken to isolate the components from different extracts and we also report here the isolation of a pure compound from the chloroform soluble fraction of methanol extract.

II. Materials and Methods

Plant materials: Fruits of *Terminalia chebula* were purchased from the local market of Comilla in November 2006 and the seeds from individual fruits were removed gently. The fruits were sun-dried for several days and then oven-dried for 24 hrs at 40 °C for better grinding. The dried fruits were then ground into coarse powder. A voucher specimen has been deposited in the Department of Botany, University of Dhaka.

Extraction: About 500 gm of the coarse fruit powder was extracted with methanol for two weeks at room temperature with occasional shaking and stirring. The extract was then filtered off through a cotton plug and followed by Whatman no. 1 filter paper. The volume of the filtrate was reduced using rotary evaporator at low temperature and pressure. A portion (5 gm) of the concentrated methanol extract was fractionated by the modified Kupchan partitioning protocol[11], which afforded of *n*-hexane (0.62 gm), carbon tetrachloride (0.044 gm), chloroform (1.02 gm) and aqueous soluble (2.25 gm) materials.

Chemical investigation of chloroform soluble fraction

The chloroform soluble fraction (1.02 gm) was considered first to investigate chemically as it was the main organic fraction compared to others. TLC screening of the chloroform soluble fraction revealed the presence of a considerable number of spots on TLC plate and thus, 300mg of the chloroform soluble fraction was subjected to column chromatography using Sephadex LH-20 for further fractionation. Then the column fractions were analyzed by TLC and those with satisfactory resolution of components were subjected to PTLC to obtain pure compounds.

Microorganisms: Thirteen bacteria (5 Gram positive and 8 Gram negative) and three fungi, collected from the stock cultures of the Institute of Nutrition and Food Science, University of Dhaka, were used for the antimicrobial assays.

Antimicrobial tests: Antibacterial and antifungal activities were tested by the disc-diffusion method.[12] The extractives were dissolved separately in chloroform and methanol as required and applied to sterile filter paper discs at 400μg/disc and carefully dried to evaporate the residual solvent. Discs containing the test materials were then placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard disc of kanamycin (30μg/disc) and blank discs (impregnated with solvents followed by evaporation) were used as positive and negative controls, respectively. These plates were then kept at low temperature (4 °C) for 24 hours to allow maximum diffusion of test samples. The plates were then incubated at 37 °C for 24 hours to allow maximum growth of the organisms. The test materials having antimicrobial activity inhibited the growth of the microorganisms and a clear, distinct zone of inhibition was visualized surrounding the disc. The antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition in millimeter. The experiment was carried out in triplicate and the average zone of inhibition was calculated.

Table. 1. Antimicrobial activity of the fruits of *Terminalia chebula* extractives

Test Microorganisms	Diameter of zone of inhibition (mm)				
	HX	CT	CF	AQ	KAN
Gram positive bacteria					
Bacillus cereus	11	-	-	9	26
Bacillus megaterium	13	-	-	9	24
Bacillus subtilis	13	-	-	-	25
Staphylococcus aureus	13	-	-	9	23
Sarcina lutea	13	-	-	9	22
Gram negative bacteria					
Escherichia coli	13	-	-	8	25
Pseudomonas aeruginosa	13	-	-	9	23
Salmonella paratyphi	12	-	-	9	25
Salmonella typhi	14	-	-	9	25
Shigella boydii	15	-	-	10	23
Shigella dysenteriae	13	-	-	9	25
Vibrio mimicus	12	-	-	8	24
Vibrio parahemolyticus	13	-	-	8	25
Fungi					
Candida albicans	16	-	-	8	25
Aspergillus Niger	16	-	-	10	25
Sacharomyces cerevacae	15	-	-	8	23

The diameter of zone of inhibition are expressed as mean ± SD (n=3); a diameter less than 8 mm was considered as inactive; HX: *n*-hexane soluble partitionate; CT: carbon tetrachloride soluble partitionate; CF: chloroform soluble partitionate; AQ: aqueous soluble partitionate; KAN: Kanamycin; "-" indicates no activity.

Brine shrimp lethality test: Brine shrimp lethality bioassay technique of Meyer[13] was applied for the determination of cytotoxic property of he fruit extracts of *Terminalia chebula*. The *n*-hexane (HX), carbon tetrachloride (CT), chloroform (CF) and aqueous (AQ) soluble partitionates of the methanolic extract were separately dissolved in DMSO. Four mg of each of the extractives (HX, CT, CF and AQ) was dissolved in DMSO and solutions of varying concentrations such as 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.78125 μg/mL were obtained by serial dilution technique. Vincristine sulphate and DMSO were used as the positive and negative control, respectively. Then the solutions were added to the premarked vials containing ten live brine shrimp nauplii in 5 mL simulated sea water. After 24 hours, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial was

counted. From the data, the percent (%) of lethality of the brine shrimp was calculated for each concentration. The median lethal concentration (LC_{50}) of the test samples was obtained by a plot of percentage of the shrimps killed against the logarithm of the sample concentration.

III. Results and Discussion

Antimicrobial activity : The result of the antimicrobial activities of the *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble partitionates of the methanolic extract of *Terminalia chebula* has been summarized in Table 1. Present investigation showed that the hexane soluble partitionate has the potent to moderate sensitivity and aqueous soluble partitionate has only the mild sensitivity against all the bacteria and fungi, whereas carbon tetrachloride and chloroform soluble partitionates did not show any antimicrobial activity.

The *n*-hexane partitionate of the methanolic extract showed the strongest activity against *Candida albicans* & *Aspergillus niger* having the zone of inhibition of 16 mm for each. The growth of *Saccharomyces cerevacae* (15 mm), *Shigella boydii* (15 mm) and *Salmonella typhi* (14 mm) was also strongly inhibited by the *n*-hexane soluble partitionate while it showed the moderate inhibition activity (11-13 mm) against rest of the microorganisms. On the other hand, aqueous soluble fractionate revealed only mild inhibitory activity against microbial growth with average zone of inhibition 8-10 mm.

Brine shrimp lethality : It was found from the result of the brine shrimp lethality test (Table 2) that the crude MeOH extract exhibited toxicity towards brine shrimp. Test samples showed different mortality rate at different concentrations. The mortality rate of brine shrimp was found to be increased with the increase of the concentration for each sample. The percent mortality of the brine shrimp nauplii was calculated for every concentration for each sample. A plot of log concentration of the sample versus percent of mortality showed an approximate linear correlation between them. The positive control groups showed non linear mortality rates at lower concentrations and linear rates at higher concentrations. There was no mortality in the negative control groups indicating the test as a valid one and the results obtained are only due to the activity of the test samples. LC_{50} obtained from the best-fit line slope were 0.3229, 1.254, 0.826, 3.866 and 5.366 µg/mL for vincristine sulphate (Std.), HX, CT, CF and AQ soluble partitionates respectively. In comparison to positive control (vincristine sulphate), the cytotoxicity exhibited by carbon tetrachloride (CT) soluble partitionate of methanolic extract was promising. On the other hand, *n*-hexane & chloroform soluble partitionates demonstrated moderate activity and aqueous soluble partitionate of methanolic extract showed poor activity.

Table. 2. Brine shrimp lethality of the fruits of *Terminalia chebula* extractives

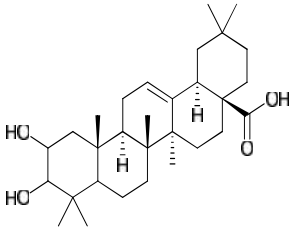
Sample	LC_{50} (µg/ml)	Regression equation	R^2
VS	0.323	y = 29.797x + 64.628	0.927
HX	1.254	y = 24.763x + 47.564	0.978
CT	0.826	y = 22.348x +51.850	0.981
CF	3.866	y = 33.421x + 30.371	0.953
AQ	5.366	y = 27.381x + 30.087	0.844

The values of LC_{50} are expressed as mean ± SD (n=3). VS: vincristine sulphate (Std.); HX: *n*-hexane soluble partitionate; CT: carbon tetrachloride soluble partitionate; CF: chloroform soluble partitionate; AQ: aqueous soluble partitionate.

Isolation of a pure compound from chloroform soluble fraction : We were successful to isolate a pure compound from the chloroform soluble fraction of methanolic extract of *Terminalia chebula* as white powder, which appeared as a proximate spot on TLC plate under UV light at 254 nm. After developing the PTLC plate with Toluene : Ethyl acetate (3:1) as eluent, spraying with vanillin-sulfuric acid followed by heating at 110 °C for several minutes a blue colored spot was observed, which was scrapped out and further purified by column chromatography using chloroform : ethyl acetate (1:1) as eluent. The separated compound was found to soluble in chloroform, acetone, methanol, DMSO and ethyl acetate.

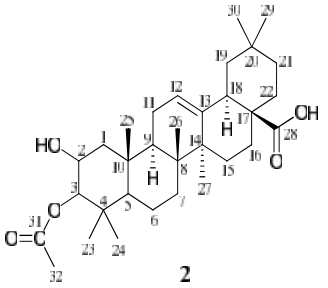
The ^{1}H NMR spectrum (400MHz, $CDCl_3$) displayed the signal of seven tertiary methyl groups at δ 0.73, 0.78, 0.85, 0.87, 0.96, 1.03 and 1.08. It also displayed a triplet at δ 5.23 which indicated the presence of an olefinic proton. A broad singlet at 2.38 indicated the presence of an acetyl group. Except for an acetyl group instead of –OH group, all these spectral features were found to be compatible with the structure of 2,3-dihydroxy-12-oleanen-28-oic acid (**1**)[14]. On the basis of the structure of compound **1**, we have identified the isolated compound as 2-hydroxy-3-acetoxy-12-oleanen-28-oic acid (**2**)[15]. The structures of compounds **1** and **2** are represented in Chart-1.

Chart-1. Chemical structures of compound **1** and compound **2**



1

2,3-Dihydroxy-12-oleanen-28 oic acid



2

2-Hydroxy-3-acetoxy-12-oleanen-28 oic acid

IV. Conclusion

It has been found from the above discussion that the crude methanolic extract along with *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble fractions of the fruits of *Terminalia chebula* have significant antimicrobial and cytotoxic activities, which supports the traditional uses of this fruit for the treatment of bacterial and fungal infections. The highly lethal to brine shrimp nauplii indicates that this fruit contains potential bioactive compounds. In the present study, we have isolated 2-hydroxy-3-acetoxy-12-oleanen-28-oic acid, which will be applied for antitumor, antiproliferative and antidiabatic studies after large scale isolation. A thorough chemical study is also required to isolate the molecules that are responsible for the antimicrobial activities.

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1. (a) Kirtikar, K.R. and B. D. Basu, 1935. *Terminalia chebula*. In: *Indian Medicinal Plants*, **2 (*2nd* Edn.)**, Lalit Mohan Basu Publication, Allahabad (India).

   (b) Sharma, P. V., 1995. Dravya Guna Vigyana. Vol. 2, Chaukhamba Bharati Academy, Varanasi (India).

   (c) Das, B., 1991. Materia Medica of Ayurveda. B. Jain Publishers, New Delhi.

   (d) Chopra, R. N., S. L. Nayar and I. C. Chopra, 1956. Glossary of Indian Medicinal Plants. CSIR, New Delhi.

2. Chattopadhyay, R. R. and S. K. Bhattacharyya, 2007. Terminalia chebula: An update. *Phcog Rev.* (Pharmacognosy Reviews). ***1***, *151-156*.

3. (a) Badmaev, V. and M. Nowakowski, 2000. Protection of epithelial cells against influenza A virus by plant derived biological response modified Ledretan-96. *Phytother. Res*., ***44***, *245-249*.

   (b) Yukawa, T. A., M. Kurokawa, S. Sato, Y. Yoshida, S. Kageyamer, T. Hasegawa, T. Nambo, M. Imakila, T. Hozumi and K. Shiraki. 1996. Prophylactic treatment of cytomegalovirus infection with traditional herbs. *Antiviral Res*. ***32***, *63-70*.

4. Dutta, B. K., I. Rahman and T. K. Das. 1998. Antifungal activity of Indian plant extracts. *Mycoses* ***41***, *535-536*.

5. Cheng, H. Y., T. C. Lin, K. H. Yu, C. M. Yang and C. C. Lin. 2003. Antioxidant and free radical scavenging activities of *Terminalia chebula*. *Biol. Pharm. Bull*. ***26***, *1331-1335*.

6. Arora, S., K. Kaw and S. Kaur. 2003. Indian Medicinal plants as reserver of protective phytochemicals. *Tetragenesis, Carcinogenesis and Mutagenesis* ***23***, *295-300*.

7. Perasamy, C. S. K., A. Palanisams, S. K. Durairaj and P. S. Sovimuthu. 2006. Antidiabetic activity of *Terminalia chebula* on streptozotocin-induced diabetic rats. *J. Health Sci*. ***52***, *283-291*.

8. (a) Ahmed, Z. M. and F. Mohammad. 1998. Screening of some Indian medicinal plants for their antimicrobial properties. *J. Ethnopharmacol*. ***62***, *183-193*.

   (b) Phadke, S. A. and S. D. Kulkarni, 1989. Screening of in vitro antibacterial activity of *Terminalia chebula, Eclapta alba and Ocimum sanctum*. *Indian J. Med. Sci*. ***43***, *113-117*.

9. Jaung, L. J., S. J. Sheu and T. C. Lin, 2004. Determination of hydrolizable tannis in the fruit of Terminalia chebula by high performance liquid chromatography and capillary electrophoresis. *J. Sep. Sci*. ***27***, *718-724*.

10. Evans, W., 1996. Trease and Evan's Pharmacology. 14th Edn. W. B. Saunders Co. Pvt. Ltd. Singapore.

11. VanWagenen, B. C., R. Larsen, J. H. Cardellina II, D. Randazzo, Z. C. Lidert and C. Swithenbank, 1993. Ulosantoin, a potent insecticide from the sponge Ulosa ruetzleri. *J. Org. Chem.* ***58***, *335-337*.

12. Bauer, A. W., W. M. M. Kibry, J. C. Sheris and M. Truck, 1966. Antibiotic susceptibility testing by a standarded single disc method. *Am*. *J. Clin*. *Pathol*. ***45***, *493-496*.

13. Meyer, B. N., N. R. Ferringni, J. E. Puam, L. B. Lacobsen, D. E. Nichols and J. L. McLaughlin, 1982. Brine shrimp: a convenient general bioassay for active constituents. *Planta Med.* ***45***, *31-32*.

14. Anonymous, 2001. *Dictionary of natural products* (DNP) CD-ROM Ver. **9:2**, Chapman & Hall/CRC, Florida.

15. White powder; mp 223-225 °C; ^{1}H NMR ($CDCl_3$) δ 0.73 (3H, s, position-24), 0.78 (3H, s, position-26), 0.85 (3H, s, position-29), 0.87 (3H, s, position-25), 0.96 (3H, s, position-30), 1.03 (3H, s, position-23), 1.08 (3H, s, position-27), 2.38 (3H, s, position-32), 2.79 (1H, t, *J* 6.2, position-18), 3.30 (1H, q, *J* 4.3, position-2), 3.35 (1H, s, –OH, position-2), 3.53 (1H, d, *J* 6.6, position-3), 5.23 (1H, t, *J* 5.8, position-12), 11.76 (1H, s, –COOH, position-28).