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Phytochemical and Biological Activity Studies of *Tinospora crispa* Stem

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Abstract

Three compounds namely, tinosporol A, 8-methoxy palmatine and callecdysterol C were isolated from the methanol extract of the stem of *Tinospora crispa*. Biological activities of different partitionates of the parent extract was also evaluated. Antimicrobial screening of different partitionates was carried out against sixteen different microorganisms and only n-hexane fraction exhibited significant zone of inhibition against *Staphylococcus aureus* (9 mm), *Shigella boydii* (9 mm), *Shigella dysenteriae* (9 mm), *Candida albicans* (10 mm) and *Aspergillus niger* (10 mm).The crude methanol extract showed the highest general toxicity with LC_{50} values of 57.14 µg/mL against brine shrimp lethality bioassay.The total antioxidant capacity of aqueous fraction was found to be 61.61 mg as ascorbic acid equivalentper gram of plant extract.The antioxidant activity of DCM fraction revealed the highest activity having IC_{50} values 54.74 µg/mL. No significant cytotoxicity was observed on both HeLa and Vero cell lines.

Keywords: Secondary metabolites, Antimicrobial, Lethality bioassay, Antioxidant, Cytotoxicity.

I. Introduction

Plants produce a wide range of secondary metabolites, making them an excellent source of various assorted kinds of medicines[1]. *T. crispa* (Family: Menispermaceae) is a well-known medicinal plant for its versatile pharmaceutical significances due to the presence of various phytoactive compounds[2,3]. Phytochemical analyses of *T. crispa* revealed the presence of alkaloids[4], flavonoids, and flavone glycosides, triterpenes, diterpenes and diterpene glycosides, *cis*-clerodane-type furanoditerpenoids[5], lactones, sterols, lignans, and nucleosides[6]. It was reproted that the crude extracts and isolated compounds of *T. crispa* possessed a broad range of pharmacological activities such as anti-inflammatory[7], antioxidant[8], immune-modulatory[9], cytotoxic[10], antimalarial[11], cardio-protective[12] and anti-diabetic activities[13].

The paper reports the isolation of three known compounds and biological activities of different extracts of the stems of *T. crispa* of the variety from Bangladesh.

II. Experimental

Collection of sample

The stems of the plant were collected from Sylhet, Bangladesh and taxonomic identification was made by the renowned plant taxonomist Professor Dr. Mohammad Zashim Uddin, Department of Botany, University of Dhaka. The collected stems were dried and crushed into powder.

Phytochemical screening

Phytochemical screening was carried out using standard procedure[14] for identifying the chemical constituents. The presence of phytochemicals such as tannins, phlabotannins, saponins, flavonoids, steroids, terpenoids and alkaloids were detected.

Extraction and Isolation

The dried powder (500 g) of *T. crispa* stems were extracted with methanol and partitioned with n-hexane and fractionation of methanol extract was done with dichloromethane (DCM) and ethyl acetate (EtOAc).The extracts were separately concentrated to dry mass using rotary vacuum evaporator at 40°C under reduced pressure. DCM extract (3.25 g) and EtOAc extract (4.65g) were subjected to column chromatography and 15 fractions (P-1 to P-15) were obtained from the TLC study. The fraction P-5 (~40 mg) showed single spot with tailing and was subjected to preparative thin layer chromatography (PTLC) and a white crystalline compound was obtained which was labeled as **3** (~4.5mg). The fraction P-7 appeared to contain two spots and was subjected to sub column. One of the fraction was found with single spot under TLC and yielded white powdered compound after washing with solvent (**1**~5mg). The fraction P-9 gave with two spots on TLC and was subjected to sub-column, and finally a single yellow crystalline compound was obtained **2**(~4.5mg).

Biological activity screening

Freshly prepared stem powder was extracted successively with n-hexane (HEX), dichloromethane (DCM), ethyl acetate (EA) and methanol (ME) at room temperature. All the extracts were evaporated to dryness and used for antimicrobial activity screening, brine shrimp lethality bioassay, cytotoxicity assay on cancer cell lines and determination of total antioxidant capacity using their individual standard procedures[15-19].

Cytotoxicity assay was examined against HeLa cell line (a human cervical carcinoma cell) and Vero cell line (kidney epithelial cells extracted from an African green monkey) in Center for Advanced Research (CARS), University of Dhaka[17].

III. Results and Discussion

Three compounds (**1-3**) were isolated from the methanol extract of the stem of *T. crispa* by column and preparative thin layer chromatography. The compound-**1** (~5mg) was a white powdered solid having R_f value 0.80 (50%DCM: 50% EtOAc). It was soluble in DCM. The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of compound-**1** showed peaks at δ 1.94, 2.30, 7.06, 5.57, 2.23, 2.95, 1.45, 1.63, 4.85, 6.07, 6.25, 1.34, 1.26,

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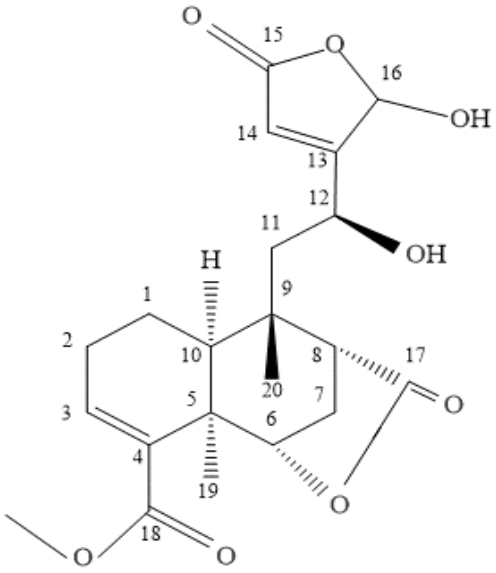
3.74 ppm. The presence of one sharp triplet signal at δ 7.04 is for olefinic bond which might be for the proton at C-3. The signals at δ 5.94 and 6.25 ppm for protons of C-14, C-16, respectively indicated the existence of furan ring (C-13–C-16)[20]. Again there was carbonyl groups (C-18 and C-15), and two angular methyl groups (C-19 and C-20). The spectra also suggested the presence of two sp^3-hybridized quaternary carbons (C-5 and C-9), two sp^3-hybridized methines (C-6, C-8). The spectra showed signals attributed to four sp^3-hybridized methylenes (C-1, C-2, C-7, and C-11) and two oxygenated methine groups (C-6 and C-12 and C-16). The 13C-NMR spectrum (100 MHz, $CDCl_3$) of compound-**1** showed main chemical shift at δ 177.4, 173.5, 170.50, 166.5, 142.6, 134.0,117.5, 97.3, 83.3 , 68.2, 51.8, 51.0, 43.8, 42.5, 38.7, 36.2, 28.1 26.9, 23.4, 19.3 and 17.0 ppm. The ^{1}H and ^{13}C-NMR spectra data of the compound-**1** was compared with reported and comparing the reported value and found identical with tinosporol A[20].

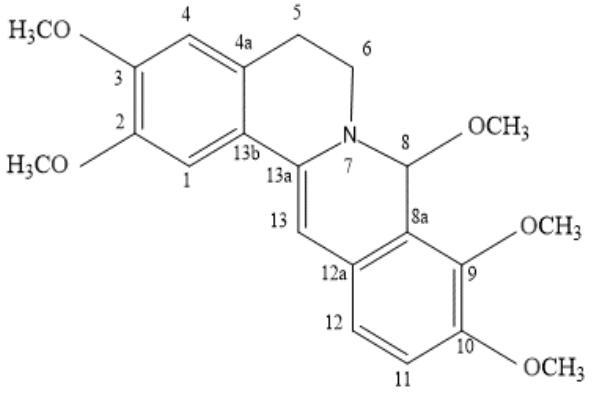
The compound-**2** (~4.5mg) was a light yellow crystalline solid having R_f value 0.60 (in 60% DCM: 40% EtOAc) and soluble in dichloromethane. The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of compound-**2** showed signals at δ 7.23, 6.96, 6.87, 6.57, 6.17, 6.14, 3.94, 3.90, 3.88, 3.60, 3.50, 2.99and 2.88ppm. Here peaks at δ 7.23, 6.65, 6.96, 6.87 ppm due to presence of hydrogens attached to aromatic ring at position H-1, H-4, H-11, H-12 and at δ 6.14 ppm for H-13 due to presence of double bond between C-13 and C-13a. The signals at δ 3.94, 3.90, 2.99, 3.94, 3.88 ppm was observed due to presence of five -OMe groups. The 13C-NMR spectrum (100 MHz, $CDCl_3$) of compound-**2** showed main chemical shift at δ 150.2,147.8,147.4, 146.7, 136.2, 134.2,128.8,126.9, 120.1, 118.0,114.7, 109.6, 106.6, 94.7,83.9, 60.4, 56.4, 55.9, 55.6 54.7, 51.9 and 29.6 ppm. Compound-**2** showed the presence of 22 carbons. The signals at δ 150.2,147.8,147.4, 146.7, 134.2,128.8,126.9, 120.1, 118.0,114.7, 109.6 and 106.6 ppm for aromatic carbon atoms. The signals at δ 94.7 and 136.2 ppm for C-13 and C-13a was due to the presence of C=C bond between C-13 and C-13a. Five signals at δ 60.4, 56.4, 55.9, 55.6 and 54.7 ppm revealed the presence of five -OMe groups and at δ51.9 and 29.6 ppm were due to the presence of two methylene carbons at C-6 and C-5 respectively. The signal at δ 83.94 showed the presence of an oxymethine carbon at C-8.From the spectral analysis (^{1}H-NMR and ^{13}C-NMR) data of the compound-**2** and comparing the reported value of 8-methoxy palmatine[21], the structure of compound was confirmed as 8-Methoxy palmatine.

The compound-**3** (~4.5mg) was a colorless crystalline solid having R_f value 0.80 (in 90% DCM: 10% EtOAc). It was soluble in dichloromethane. The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of compound-**3** showed signals at δ 0.86, 1.24, 1.75, 1.85, 2.22, 2.24, 2.35, 3.85, 3.86, 4.17, 5.67, 6.27 ppm. The compound-**3** had two singlet signals at δ 0.85 and 1.24 ppm typical for the presence of methyl protons at C-18 and C-19 respectively. The spectrum had multiplet at δ 3.85, 3.86 and 4.15 ppm indicative of the presence of oxymethine protons at C-2, C-3 and C-17 respectively. Two signals appeared at δ 5.67 and 6.27 ppm for olefinic protons at C-7 and C-11 respectively. The other signals of the spectrum between δ 1.50 to 2.35 due to presence different methylene (-CH_2-) and methine (>CH-) protons.The 13C-NMR spectrum(100 MHz, $CDCl_3$) of compound-**3** gave signals at δ 206.1, 155.7, 136.2, 134.1, 118.0, 83.9, 79.0, 68.7, 67.2, 51.6, 48.3, 42.3, 38.2, 35.8, 35.1, 29.7, 31.8, 29.5, 16.1 ppm. The spectrum showed the presence of 19 carbons. Among them 5 signals were assignable to sp^2 hybridized carbons, 2 signals to methyl carbons, 5 signals to methylene carbons, 5 signals to methine carbons and 2 signals to quaternary carbons. The signalsat δ 118.0, 155.7, 136.2, 134.1 ppm were due to presence of olefinic carbons. The signals at δ 42.3, 48.3 ppm were due to presence of two quaternary carbons. The signals at δ 16.1, 31.8 ppm were due to presence of two methyl carbons and signals at δ 38.2, 35.8, 35.1, 29.7, 29.5 ppm were due to presence of five methylene carbons. Signals at δ 68.7, 67.2, 51.6 ppm were due to presence of methine carbons.

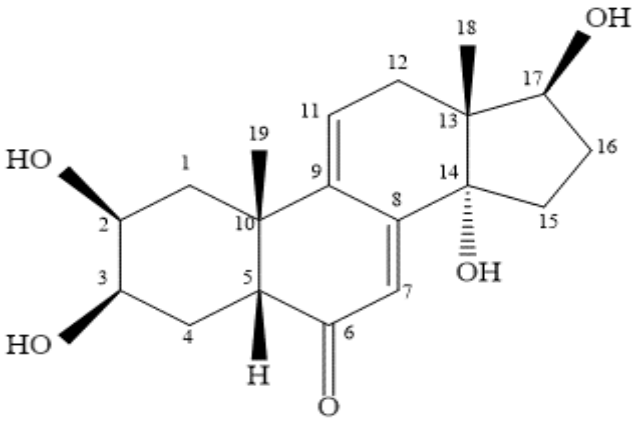
The ^{1}H and ^{13}C-NMR spectra data of the compound-**3** was compared with reported value and found identical with spectral data of callecdysterol C[22]**.**



Tinosporol A (1)



8-Methoxy palmatine (2)



Callecdysterol C (3)

Fig. 1. Structure of isolated compounds.

Antimicrobial Activity Screening

Antimicrobial activity of crude methanol extract (ME), n-hexane (HEX), dichloromethane (DCM), ethyl acetate (EA) and aqueous (AQ) extracts of *T. crispa* was evaluated by disc diffusion method[15] using 400 µg sample per disc in each case. The HEX extract showed significant zone of inhibition against gram positive *S. aureus (9mm), S. boydii (9mm)* and gram negative *S. dysenteriae (9mm), V. mimicus (8mm) and C. albicans (10mm)* and also *A. niger (10 mm)* was observed.

Brine shrimp lethality bio assay

The LC_{50} values of ME, HEX, DCM, EA and AQ extracts were found to be 57.13, 73.82, 391.21, 424.47, 441.37 and 737.34 µg/mL respectively. ME and HEX extracts demonstrated moderate lethality whereas DCM, EA and AQ extracts revealed very low activity.

Cytotoxicity assay on cancer and non-cancer cell line

Cytotoxicity assay of the different extracts of *T. crispa* was carried out against HeLa cell line (a human cervical carcinoma cell) and Vero cell line (kidney epithelial cells extracted from an African green monkey). 1 mg/ mL sample of different extracts were applied on both HeLa and Vero cell but none of the extracts were found to be cytotoxic against HeLa cell and Vero cell lines.

Total antioxidant capacity (TAC)

The TAC of different extracts of *T. crispa* extracts was evaluated by the phosphomolybdenum assay method[18]. The TAC was determined and expressed as mg ascorbic acid equivalents per gram of dry extract using the equation obtained from a standard ascorbic acid calibration curve.The highest TAC was demonstrated by AQ extract (61.61 mg ascorbic acid equivalents/g sample) comparing with other fractions. On the other hand, the lowest TAC was shown by HEX extract (12.11 mg ascorbic acid equivalents/g sample).

Antioxidant Activity

The antioxidant activity was evaluated in terms of free radical scavenging activity. Among HEX, DCM, ME partitions, DCM fraction showed the highest antioxidant activity (IC_{50}= 54.74 µg/mL).

IV. Conclusion

Tinsporol A, 8-Methoxy palmatine and callecdysterol C were isolated from the stems of *T. crispa* using different chromatographic separation techniques. The isolated compounds were identified by extensive analysis of their high resolution ^{1}H-NMR (400 MHz), ^{13}C-NMR (100 MHz) spectroscopic data. Different types of tests were carried out to evaluate the biological activities of the stems of *T. crispa*. The HEX extract revealed a significant antibacterial activity against gram negative bacteria, *C. albicans* and antifungal activity against *A. niger*. ME extract demonstrated highest lethality with LC_{50} value of 57.13 µg/mL. Different extracts of *T. crispa* stem showed moderate antioxidant activity.

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