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Phytochemical and biological studies on *Nephelium longan*

[Estudios fitoquímicos y biológicos sobre *Nephelium longan*]

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Abstract

Extensive chromatographic separation and purification of the organic solvent extracts of *Nephelium longan* (Sapindaceae) stem bark afforded two compounds; scopoletin and stigmasterol. The structures of these compounds were determined by spectroscopic analyses, including ^{1}H and ^{13}C NMR. Different crude extracts (*n*-hexane, carbon tetrachloride, chloroform and methanol) were tested for antimicrobial activity by standard disc diffusion method known as the Kirby-Bauer method and cytotoxicity was measured by brine shrimp lethality bio-assay. In the brine shrimp lethality bio-assay, the plant extracts showed some promising results as compared to the standard vincristine sulphate, and the test results showed statistical validity. The chloroform and carbon tetrachloride extracts were subjected to antimicrobial and antifungal study and with some exceptions the results are insignificant compared to the standard antibiotic ampicillin.

Key words: Nephelium longan, Sapindaceae, scopoletin, stigmasterol, antimicrobial, cytotoxic activity, Artemia salina.

Resumen

Tras extensivo uso de técnicas cromatográficas, la separación y purificación de los extractos orgánicos de las cortezas de *Nephelium longan* (Sapindaceae) hemos aislado los compuestos escopoletina y estigmasterol. Las estructuras de estos compuestos se determinaron por métodos espectroscópicos usando ^{1}H y ^{13}C RMN. La actividad antimicrobiana de los extractos crudos (*n*-hexano, tetracloruro de carbono, cloroformo y metanol) fueron ensayados usando la difusión en disco estándar (método Kirby-Bauer) y la activadad citotóxica se midió con el ensayo de la *Artemia salina*. En la prueba de citotoxidad, los extractos mostraron efectos significativos comparados con vincristina. Para los estudios antimicrobianos solo se probaron los extractos clorofórmico y tetracloruro de carbono pero los resultados fueron insignificantes comparados con el antibiótico de referencia ampicilina.

Palabras clave: Nephelium longan, Sapindaceae, escopoletina, estigmasterol, actividad antimicrobiana, citotoxicidad, Artemia salina.

INTRODUCTION

Nephelium longan (Fam. - Sapindaceae; Bengali name – *Kathlichu*) is a tree of 30 or 40 ft in height and 45 ft in width, with rough-barked trunk to 2 1/2 ft thick and long, spreading, slightly drooping, heavily foliaged branches. The longan is native to China and India, and is cultivated in Bangladesh, Thailand, Cambodia, Laos, Vietnam and Taiwan (Hooker, 1897). Botanical synonyms for this species include *Dimocarpus longa*n Lour., *Euphoria longan* Steud., *Euphoria longana* Lam., and *Nephelium longana* Cambess. Closely allied to the glamorous lychee, in the family Sapindaceae, the longan, or lungan, also known as dragon's eye or eyeball, and as *mamoncillo chino* in Cuba, has been referred to as the "little brother of the lychee" (Morton, 1987).

The extract of the plant is anxiolytic (Okuyama *et al*.,1999) and anti-mutagenic (Minakata *et al*., 1985). No extensive work has been recorded previously on this plant. It has been reported to contain gallic acid, corilagin (an ellagitannin), ellagic acid (Rangkadilok *et al*., 2005), soyacerebrosides I and II, 1-O-β-D-glucopyranosyl-(2*S*,3*R*,4*E*,8*E*)-2-(2'-lignoceroyl amino)-4,8-octadecadiene-1,3-diol (longan cerebroside I) and its 8*Z* isomer (longan cerebroside II), momor-cerebroside I, and phytolacca cerebroside (Ryu *et al*., 2003).

MATERIALS AND METHODS

General experimental procedures

^{1}H- and ^{13}C- NMR spectra were obtained from BCSIR (400 MHz Bruker NMR spectrometer with TMS as the internal reference). Silica gel (kieselgel G 60, mesh 70-230, particle size 0.043-0.063 mm) was used for column chromatography. PTLC was done on coated glass plates (kieselgel 60 PF_{254}, Merck). All solvents used in the study were purchased from Merck.

Plant material

The stems of *Nephelium longan* were collected in the surroundings of Comilla, Comilla district, Bangladesh in August 2004 and were taxonomically identified by Mrs. Mahbuba Begum (Chief Scientific Officer, Bangladesh National Herbarium) and a voucher specimen has been deposited there (DACB 21369).

Extraction and isolation

The air dried and pulverized plant material (200.0 g) was cold extracted with methanol and was successively partitioned with *n*-hexane, carbon tetrachloride and chloroform using modified Kupchan partitioning method. Evaporation under reduced pressure at 40ºC using a Buchii Rotary Evaporator provided 2.5, 1.1, 3.0, and 4.5 g of *n*-hexane, carbon tetrachloride, chloroform and methanol soluble materials, respectively. The *n*-hexane solubles were fractionated by column chromatography (CC) over silica gel (60-120 mesh) eluting with *n*-hexane, EtOAc and MeOH in order of increasing polarity to obtain a total of 30 fractions (each 50 ml). The eluates were combined together on the basis of TLC analysis. The fraction eluted with 10% EtOAc in *n*-hexane was subjected to PTLC (mobile phase, 20% EtOAc in toluene with few drops of acetic acid, multiple development) to obtain compound **1** and fraction eluted with 15% EtOAc in *n*-hexane in were subjected to PTLC (mobile phase, 25% EtOAc in toluene with few drops of acetic acid, multiple development) to obtain compound **2**.

Compound 1: ^{1}H- NMR (300 MHz, $CDCl_3$): δ 6.25 (1H, *d*, *J*=9.5 Hz, H-3), 7.57 (1H, *d*, *J*=9.5 Hz, H-4), 6.90 (1H, *s*, H-5), 3.94 (3H, *s*, OMe-6), 6.09 (1H, *s*, OH-7), 6.83 (1H, *s*, H-8); ^{13}C- NMR (125 MHz, $CDCl_3$): 161.5 (C-2), 103.3 (C-3), 114.0 (C-4), 113.5 (C-5), 143.3 (C-6), 144.1 (C-7), 107.6 (C-8), 149.8 (C-9), 111.6 (C-10), 56.5 (OMe-7).

Compound 2: ^{1}H- NMR (400 MHz, $CDCl_3$): δ 3.55 (1H, *m*, H-3), 5.37 (1H, *m*, H-6), 0.90 (1H, H-20), 5.16 (1H, *dd*, *J*=15.0, 6.5 Hz, H-22), 5.03 (1H, *dd*, *J*=15.0, 9.0 Hz, H-23), 0.70 (3H, *s*, Me-18), 1.03 (3H, *s*, Me-19), 0.94 (3H, *d*, Me-21), 0.84 (3H, *d*, Me-26), 0.86 (3H, *d*, Me-27), 0.82 (3H, *t*, Me-29).

Antimicrobial Screening

The microorganisms were obtained from the Institute of Nutrition and Food Sciences (INFS), University of Dhaka, Bangladesh. The antibacterial activity of the test samples was measured by standard disc diffusion method following the protocols described by Bauer *et al*. (1966). Standard ampicillin disc and blank sterile filter paper disc (BBL, Cocksville USA, 6 mm in diameter) were used as positive and negative controls, respectively. A total of 16 microorganisms were used for the experiment. They are listed in tables 1 and 2.

Cytotoxicity Activities

All the tested extractives were dissolved in DMSO; the final concentrations were achieved by serial dilution from 50 to 0.39 μg/ml and cytotoxicity was evaluated by the Brine shrimp lethality bioassay. The assay was performed using three replicates and the results were compared with the standard, vincristine sulfate. DMSO was used as a negative control. For hatching, eggs were kept in brine with a constant oxygen supply for 48 h; the mature nauplii were then used in the experiment (Meyer *et al.*, 1982; Persoone, 1988).

For the statistical validity of the results in the cytotoxicty analysis, the LC_{50}'s obtained from triplicate experiments and corresponding 95% confidence limits were calculated for the acute tests utilizing the computer program CT-TOX that uses the Binomial, Moving Average Angle, Probit, Spearman-Karber analyses (CTDEP, 1990; Vanhaecke *et al*., 1981).The statistical analysis used was dependent on the dose response of the test organisms. When multiple methods produced valid LC_{50} values, the method that produced the narrowest 95% confidence limits was chosen. The Chi-square statistic for heterogeneity of variance was calculated for every set of data and compared with the tabular (critical) value to indicate how well the data fit the model.

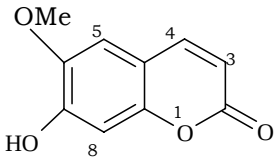
RESULTS AND DISCUSSION

Repetitive chromatography of the *n*-hexane soluble of a methanol extract of *N. longan* stems afforded two

compounds **1** and **2**. Compound **1** was obtained as a white gum, which appeared as a blue spot on TLC plate under UV light at 254 nm. It also exhibited a blue fluorescence under UV light at 366 nm. The compound was identified as scopoletin by comparing the ^{1}H NMR data with those published for this compound (Aldrich, 1992).

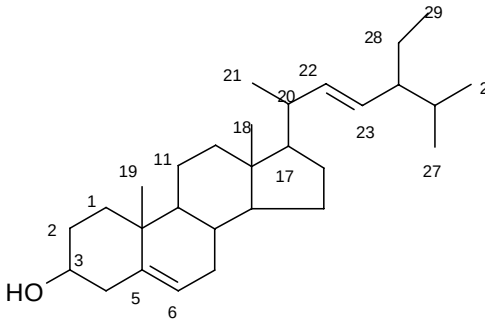
The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound **1** displayed signals characteristic of a 6,7-dioxygenated coumarin. The spectrum revealed two doublets at δ 6.28 and δ 7.60 characteristic of H-3 and H-4 protons respectively of the pyrone ring of a coumarin. The presence of two aromatic proton singlets at δ 6.92 and δ 6.85 were attributable to H-5 and H-8 respectively. In this spectrum a three-proton singlet at δ 3.95 was assigned for a methoxy group. Besides, a singlet at δ 6.09 could be attributable for a hydroxyl group. On this basis it was identified as scopoletin.

Figure 1. Scopoletin (**1**)



Compound **2** was obtained as white needle shaped crystal, which gave purple colour with vanillin-sulfuric acid spray reagent on TLC plate. The compound was identified as stigmasterol by comparing the ^{1}H NMR data with those published for the compound (Aldrich, 1992). The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) revealed a one-proton multiplet at δ 3.55, the position and multiplicity of which is indicative of H-3 of the steroidal nucleus. The typical signal for H-6 of the steroidal skeleton was evident from a multiplet at δ 5.37 integrating one proton. The olefinic protons H-22 and H-23 appeared as characteristic downfield signals at δ 5.16 and 5.03 respectively in the ^{1}H NMR spectrum. Each of the signals were observed as double doublets (*J*=15.0 Hz, 8.3 Hz) which is indicative of *trans* coupling with the olefinic proton and vicinal coupling with neighbouring methine proton. The spectrum further revealed signals at δ 0.70 and δ 1.03 (3H each) assignable to the protons of two tertiary methyl groups at C- 18 and C - 19 respectively. In addition, two doublets (each 3H) centered at δ 0.82 (*J* = 6.0 Hz) and δ 0.84 (*J* = 6.0 Hz) could be ascribed to the methyl groups at C - 29 and C-26 respectively. The doublet at δ 0.94 integrating three-proton was demonstrative of a methyl group at C-21. These ^{1}H-NMR spectral features are characteristics of a steroidal carbon skeleton of stigmasterol and these are in close agreement to those data published for stigmasterol. Therefore, it was identified as stigmasterol.

Figure 2. Stigmasterol (**2**)



In our preliminary antimicrobial screening, the chloroform and carbon tetrachloride showed moderate activity against *Vibrio mimicus* and carbon tetrachloride extract showed mild to moderate activity against *Staphylococcus aureus* and *Vibrio parahaemolyticus*, as compared to the standard ampicillin (Table 1). Both chloroform and carbon tetrachloride extracts showed moderate antifungal activity against *Candida albicans* and *Aspergillus* niger (Table 2). The toxicological study showed some promising results for carbon tetrachloride extract which yielded LC_{50} of 3.13 μg/ml. Chloroform and methanol extracts showed moderate cytotoxic activity, LC_{50} 17.17 μg/ml and 13.63 μg/ml respectively, whereas the positive control vincristine sulphate demonstrated an LC_{50} of 0.44 μg/ml.

CONCLUSIONS

The phytochemical study on the *n*-hexane soluble fraction yielded two pure compounds, scopoletin and stigmasterol, whose structures were established through comparison with published results. In the brine shrimp lethality bio-assay, the plant extracts showed some promising results as compared to the standard vincristine sulphate, and the test results showed statistical validity. The chloroform and carbon tetrachloride extracts were subjected to antimicrobial and antifungal study and with some exceptions the results are insignificant compared to the standard antibiotic ampicillin.

Table 1. Antibacterial activity of extracts of *Nephelium longan*

Species	Diameter of Zone of Inhibition (mm)		
	$CHCl_3$ extract (100 µg/disc)	CCl_4 extract (100 µg/disc)	Ampicillin (30 µg/disc)
Gram Positive			
Bacillus megaterium	7	7	14
Bacillus subtilis	-	-	8
Sarcina lutea	-	-	17
Staphylococcus aureus	-	7	10
Bacillus cereus	7	-	10
Gram Negative			
Pseudomonas aeruginosa	7	-	15
Escherichia coli	-	-	9
Salmonella typhi	-	7	15
Shigella boydii	-	-	11
Shigella dysenteriae	-	-	6
Vibrio mimicus	7	7	8
Salmonella paratyphi	-	-	9
Vibrio parahaemolyticus	-	7	12

'-' indicates no sensitivity

Table 2: Antifungal activity of extracts of *Nephelium longan*

Species	Diameter of Zone of Inhibition (mm)		
	$CHCl_3$ extract (100 µg/disc)	CCl_4 extract (100 µg/disc)	Ampicillin (30 µg/disc)
Saccharromyces cerevaceae	-	-	7
Candida albicans	7	7	10
Asperigillus niger	7	7	9

'-' indicates no sensitivity

Table 3. Cytotoxicity of extracts of *Nephelium longan* on brine shrimps

Sample	LC_{50} (µg/ml)	95% Confidence Limit	Regression equation	χ^2	
				Calculated	Tabular
Vincristine	0.44	0.20-0.98	y=3.1817+0.407x	1.125	15.507
Hexane extract	29.93	17.78-49.71	y=0.238+0.7131x	1.766	15.507
CCl_4 extract	3.13	1.08-9.04	y=4.048+0.3411x	1.604	15.507
$CHCl_3$ extract	17.17	8.59-34.34	y=3.1507+0.3828x	1.943	15.507
MeOH extract	13.63	8.04-23.12	y=1.6987+0.5892x	2.703	15.507

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