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Article *in* Food and chemical toxicology: an international journal published for the British Industrial Biological Research Association · November 2009

DOI: 10.1016/j.fct.2009.11.030 · Source: PubMed

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Antimicrobial and cytotoxic constituents from leaves of *Sapium baccatum*

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ARTICLE INFO

Article history:
Received 3 September 2009
Accepted 9 November 2009

Keywords:
Bioactive products
Cytotoxicity
Antimicrobial activity
Sapium baccatum

ABSTRACT

Six compounds, namely, Lupeol (**1**), Betulin (**2**), β-Taraxerol (**3**), Taraxerone (**4**), Stigmasterol (**5**) and β-Sitosterol (**6**) were isolated from the petroleum ether extract of the leaves of *Sapium baccatum* based on spectroscopic evidence. Lupeol (**1**), Betulin (**2**) and Stigmasterol (**5**) were isolated for the first time from this plant. The cytotoxic potential of the different solvent extracts (methanol, petroleum ether, carbon-tetrachloride and dichloromethane); six column fractions (F-4, F-7, F-10, F-12, F-18 and F-22) of petroleum ether extract and three pure compounds **1**, **4** and **6** were determined by using brine shrimp lethality bioassay. The LC_{50} of all the tested samples were showed to be lethal to brine shrimp nauplii. However, petroleum ether, carbon-tetrachloride extract, column fractions F-4 and F-18 of petroleum ether extract and pure compound **6** showed quite potent activity in brine shrimp lethality bioassay with LC_{50} 1.33, 1.35, 1.40, 1.58 and 1.58 μg/ml, respectively. These result suggested that they might be contain antitumor or pesticidal activity. Further, the methanol extract and four column fractions (F-7, F-12, F-18 and F-22) of petroleum ether showed significant activity against the tested microorganisms.

1. Introduction

A general bioassay that appears capable of detecting a board spectrum of bioactivity present in crude extracts is the brine shrimp lethality bioassay (BSLT). The technique is easily mastered, costs little, and utilizes small amount of test material. The aim of this method is to provide a front-line screen that can be backed up by more specific and more expensive bioassays once the active compounds have been isolated. It appears that BSLT is predictive of cytotoxicity and pesticidal activity (Ghisalberti, 1993). Since its introduction in 1982 (Meyer et al., 1982), this *in vivo* lethality test has been successively employed for bioassay-guide fractionation of active cytotoxic and antitumor agents such as trilobacin from the bark of *Asimina triloba* (Zhao et al., 1992), *cis*-annonacin from *Annona muricata* (Rieser et al., 1996) and ent-kaur-16-en-19-oic acid from *Elaeoselinum foetidum* (Mongelli et al., 2002).

Sapium baccatum (Common name – Koilan, Kala-boil, Chhoto mel, Chamfata, Kalagota, Bolos, Boloch, Billa, mousedeer's rubber tree, salee nok, pho bai, budi, banai, ludai, ludai pelandok, memaya; Synonyms – *Excoecaria affinis*, *Balakata baccata*; Family – Euphorbiaceae) is a tree: native to tropical South Asia, but also found in Eastern Himalaya, India (Sikkim) and Bangladesh to Indochina and South-West China (only known from Yunnan), Andamans, and in West Malaysia: Malay Peninsula (excl. Singapore), Sumatra, Borneo (Kalimantan) (Esser, 1999). This plant used as a timber tree and as a wayside plant. The wood is not very durable. The fruits are mealy and sweet and in Sumatra sometimes used for a flavouring (Heyne, 1950). In Bangladesh, *S. baccatum* is widely distributed in the district of Dhaka, Chtiagong and Sylhet. Previous phytochemical investigations resulted in the isolation of taraxerone, taraxerol, 3-acetoxy aleuritolic acid, 1-hexacosanol, β-sitosterol (Khastigir et al., 1969), baccatin (Saha et al., 1977) and (−)-bukittinggine (Arbain et al., 1990). So far no details biological studies have been carried out on this plant.

Therefore, the present study was undertaken to isolate and elucidate structure of some pure compounds from the petroleum ether extract of the leaves of *S. baccatum* by using spectroscopic techniques and the preliminary antimicrobial and cytotoxic activities of the crude extracts and pure compounds.

2. Materials and methods

2.1. General experimental procedure

The ^{1}H NMR spectra was recorded using a Bruker DPX-400 (400 MHz) instrument (Bruker BioSpin GmbH, Silberstreifen 4, 76287 Rheinstetten, Germany). For NMR studies deuterated chloroform was used and the δ values for ^{1}H spectra were referred to the residual nondeuterated solvent signals.

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doi:10.1016/j.fct.2009.11.030

2.2. Plant material

Fresh leaves of *S. baccatum* were collected from Botanical garden, Mirpur in the month of February, 2008. It was identified by a senior scientific officer Boshra Khan, Bangladesh National Herbarium, Dhaka, where a voucher specimen (DACB 32760) has been deposited.

2.3. Extraction and Isolation

The air-dried powdered leaves (1.4 kg) of *S. baccatum* were soaked in 5 L of methanol for 7 days at room temperature and the solvent was evaporated under reduced pressure at (45–50) °C using a Buchii Rotary Evaporator (BÜCHI Labortechnik AG in Flawil, Switzerland) to provide 100 g of a gummy concentrate of the crude extract. A portion of the methanol extract (10.5 g) was dissolved in 90% methanol. It was partitioned with petroleum ether, then with carbon-tetrachloride (CCl_4) and finally with dichloromethane (DCM). All the extracts were filtered through a cotton plug followed by Whatman filter paper No. 1 and then concentrated by using a rotary vacuum evaporator to provide petroleum ether (5.5 g), carbon-tetrachloride (1.5 g) and dichloromethane (0.8 g) extracts. Solvents (analytical grade) for extraction were obtained from commercial sources (Sigma–Aldrich, St. Louis, MO, USA).

The concentrated crude petroleum ether extract (3.5 g) was subjected to column chromatography by using gradients of petroleum ether-dichloromethane, then dichloromethane, followed by a gradient of dichloromethane-methanol, and finally with methanol to afford a total of 30 fractions (each 100 ml). Fractions 13, 15, 17, 19, 20 and 23 upon repeated washing with petroleum ether gave compounds **1** (15 mg), **2** (13 mg), **3** (10 mg), **4** (12 mg), **5** (15 mg) and **6** (15 mg), respectively.

*Lupeol (**1**):* White crystals; ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.68 (1H, m, Hb-29), 4.56 (1H, m, Ha-29), 3.18 (1H, dd, J = 11.4, 4.6 Hz, Hα-3), 1.67 (3H, s, CH_3-30), 1.02 (3H, s, CH_3-26), 0.96 (3H, s, CH_3-23), 0.94 (3H, s, CH_3-27), 0.82 (3H, s, CH_3-25), 0.78 (3H, s, CH_3-28), 0.75 (3H, s, CH_3-24). ^{13}C NMR: δ_C: 38.0 (C-1), 27.4 (C-2), 79.0 (C-3), 38.7 (C-4), 55.3 (C-5), 18.3 (C-6), 34.2 (C-7), 40.1 (C-8), 50.4 (C-9), 37.7 (C-10), 20.9 (C-11), 25.1 (C-12), 38.0 (C-13), 42.8 (C-14), 27.4 (C-15), 35.6 (C-16), 48.2 (C-17), 48.2 (C-18), 48.0 (C-19), 150.9 (C-20), 28.5 (C-21), 40.0 (C-22), 28.1 (C-23), 15.4 (C-24), 16.1 (C-25), 15.9 (C-26), 14.6 (C-27), 18.0 (C-28), 109.5 (C-29), 19.4 (C-30).

*Betulin (**2**):* White crystals; ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.70 (1H, m, Hb-29), 4.60 (1H, m, Ha-29), 3.75 (1H, d, J = 11.8, H-28), 3.30 (1H, d, J = 11.8, H-28), 3.18 (1H, dd, J = 11.3, 4.6 Hz, Hα-3), 1.67 (3H, s, CH_3-30), 1.00 (3H, s, CH_3-27), 0.96 (3H, s, CH_3-26), 0.91 (3H, s, CH_3-23), 0.82 (3H, s, CH_3-25), 0.75 (3H, s, CH_3-24). ^{13}C NMR: δ_C: 38.1 (C-1), 27.4 (C-2), 79.0 (C-3), 38.8 (C-4), 55.3 (C-5), 18.3 (C-6), 34.2 (C-7), 40.2 (C-8), 50.4 (C-9), 37.8 (C-10), 20.8 (C-11), 25.2 (C-12), 37.5 (C-13), 42.7 (C-14), 27.0 (C-15), 29.4 (C-16), 48.2 (C-17), 48.6 (C-18), 48.0 (C-19), 150.5 (C-20), 28.5 (C-21), 34.3 (C-22), 28.1 (C-23), 15.4 (C-24), 16.1 (C-25), 15.9 (C-26), 14.7 (C-27), 60.5 (C-28), 109.5 (C-29), 19.2 (C-30).

*β-Taraxerol (**3**):* Colorless crystal; ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.52 (1H, dd, J = 8.0, 3.2 Hz, H-15), δ 3.19 (1H, dd, J = 9.2 Hz, H-3), 1.08 (3H, s, Me-8), 0.97 (3H, s, Me-10), 0.94 (3H, s, Me-20α), 0.92 (3H, s, Me-4β), 0.90 (6H, s, Me-4α, Me-20 β), 0.82 (3H, s, Me-13), 0.80 (3H, s, Me-17).

*Taraxerone (**4**):* White crystal; ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.55 (1H dd, J = 8.0, 3.2 Hz, , H-15), 2.56 (m, H-2a), 2.32 (m, H-2b), 1.13 (3H, s, CH_3), 1.08 (3H, s, CH_3), 1.07 (3H, s, CH_3), 1.06 (3H, s, CH_3), 0.95 (3H, s, CH_3), 0.91 (3H, s, CH_3), 0.90 (3H, s, CH_3), 0.82 (3H, s, CH_3).

*Stigmasterol (**5**):* Colorless needles; ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.35 (1H, m, H-6), 5.13 (1H, dd, J = 14.4, 8.4 Hz, H-22), 5.03 (1H, dd, J = 14.4, 8.4 Hz, H-23), 3.51 (1H, m, H-3), 1.0 (3H, s, CH_3-10), 0.91 (3H, d, J = 6.4 Hz, CH_3-20), 0.85 (3H, d, J = 7.4 Hz, CH_3-27), 0.81 (3H, d, J = 7.4 Hz, CH_3-26), 0.67 (3H, s, CH_3-13).

*β-Sitosterol (**6**):* White crystals; ^{1}H NMR (400 MHz,$CDCl_3$): δ 3.51(1H, m, H-3), 5.34 (1H, m, H-6), 1.00 (3H, s, 10-CH_3), 0.67 (3H, s, 13-CH_3), 0.91 (3H, d, J = 6.8, 20-CH_3), 0.81 (3H, d, J = 7.6, 25-CH_3), 0.82(3H, d, J = 7.6, 25-CH_3).

2.4. Bioassays

2.4.1. Cytotoxicity bioassays

The cytotoxic activity was performed as described previously (Persoone, 1980; Meyer et al., 1982; McLughilin and Rogers, 1998). The test samples for crude extracts were dissolved in DMSO and serial dilution were made as 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781 μg/ml. On the other hand, the pure compounds were dissolved in DMSO and serial dilution were made as 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781, 0.3906 μg/ml. Then each of these test solutions was added to test tubes containing 10 shrimps in simulated brine water (5 ml) and incubated at room temperature for 24 h. After 24 h, the median lethal concentration (LC_{50}) of the test samples was obtained by a plot of percentage the shrimps killed against the logarithm of the sample concentration. Vincristine sulphate was used as positive control in this assay to compare the cytotoxicity of the test samples.

2.4.2. Antimicrobial bioassays

The test samples were dissolved separately in specific volume of chloroform because most of the extracts and fractions dissolve in this solvent. The antimicrobial activities were then carried out by the disc diffusion method (Bauer et al., 1966; Barry, 1980). The diluted samples were applied onto sterile filter paper discs at a concentration of 500 μg/disc for the antimicrobial assays.

3. Results and discussion

Repeated chromatographic separation and purification of the petroleum ether extracts of the leaves of *S. baccatum* provided six pure compounds (Fig. 1), the structure of which were determined by spectroscopic analysis as well as by comparison of their spectral data with previously reported values.

Compound **1** was obtained as white crystals. Its ^{1}H NMR spectrum exhibited seven tertiary methyl singlets at [δ_H: 0.75, 0.78, 0.82, 0.94, 0.96, 1.02 and 1.67], a secondary carbinol group at [δ_H: 3.18 (dd, J = 11.4 and 4.6 Hz)] and an exomethylene group at [δ_H: 4.56 (1H, m) and δ_H: 4.68 (1H, m)]. Further, the ^{13}C NMR spectrum of this compound showed seven methyl groups at [δ_C: 28.1 (C-23), 19.4 (C-30), 18.0 (C-28), 16.1 (C-25), 15.9 (C-26), 15.4 (C-24), 14.6 (C-27)], an exomethylene group at [δ_C: 150.9 (C-20), 109.5 (C-29)] and a secondary hydroxyl bearing carbon at [δ_C: 79.0 (C-3)], in addition to ten methylene, five methine and five quaternary carbons. The shielding of C-23 methyl of compound **1** could be due to the influence of the adjacent C-3 hydroxyl group. These data were in close agreement with those reported for Lupeol (Sholichin et al., 1980; Wenkert et al., 1978). On the basis of the above evidence; compound **1** was determined to be Lupeol.

Compound **2** was obtained as white crystals. Its ^{1}H NMR spectrum revealed AB doublets at δ 3.75 and 3.30 (J = 11.8 Hz) due to the C-28 methylene protons. A signal at δ 3.18 (dd, J = 11.3 and 4.6 Hz) was assigned to H-3 geminal to a hydroxyl group. The tertiary methyl signals at δ 0.75, 0.82, 0.91, 0.96, 1.00 and 1.67 were assigned to CH_3-24, CH_3-25, CH_3-23, CH_3-26, CH_3-27 and CH_3-30, respectively. A pair of board singlets at δ 4.60 and 4.70 (1H each) was indicative of an exomethylene group. The ^{13}C NMR spectrum of **2** was similar to that of **1**, suggesting it has the similar carbon skeleton. The main difference was C-28 chemical shift. The ^{13}C NMR spectrum of **2** exhibited the presence of 30 carbon signals and also showed a carbonyl signal, two olefinic signals at δ 150.5 and 109.5, and an oxygenated carbon signal of C-28 at δ 60.5. From these results, compound **2** indicated to be a 3-oxo lupane type triterpene. Besides the above evidences, by the direct comparison of its spectral data with those of the reported literature (Siddiqui et al., 1988; Kim et al., 2002), the structure of **2** was determined to be 3,28-dihydroxylup-20(29)-ene (Betulin), which has been isolated from *Ilex macropod* (Kim et al., 2002).

Compound **3** was obtained as colorless crystal. The ^{1}H NMR spectrum of compound **3** showed eight three proton singlets at δ 0.80, 0.82, 0.90, 0.90, 0.92, 0.94, 0.97, and 1.08, these were attributed to the methyl group protons at C-17, C-13, C-4α, Me-4β, C-20α, C-10 and C-8 respectively. The double doublet (J = 3.2 Hz, 8.0 Hz) centered at δ 5.52 attributable to the olefinic proton at C-15. The broad doublet (J = 9.2 Hz) centered at δ 3.19 could be assigned to the oxymethine proton at C-3. The large coupling of this proton (H-3) with the vicinal methylene protons suggested a β (beta) orientation of the hydroxyl group at C-3. The above ^{1}H NMR signals suggested the presence of a typical of pentacyclic triterpene skeleton. This was identified as β-taraxerol by comparison these data with those reported previously for taraxerol (Laphookhieo et al., 2004; Corbett et al., 1972).

Compound **4** was obtained as white crystals. The structure of compound **4** was elucidated by direct comparison of its spectral data with that of compound **3**. Although, the ^{1}H NMR spectral data of **4** was in close agreement to that of **3**, except the resonance at δ

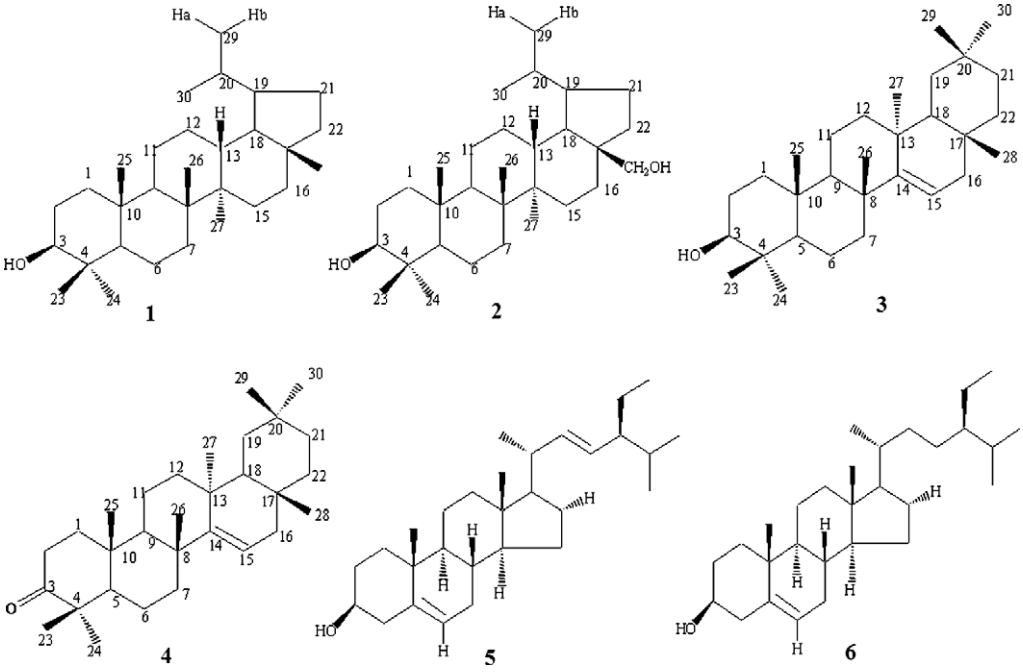

Fig. 1. The structures of compounds **1**–**6**.

3.19 for the oxymethine proton at C-3 in the spectrum of **3** was absent in **4**. This suggested that the hydroxyl group at C-3 in compound **3** was replaced by a carbonyl function. On the basis of the above spectral features, compound **4** was characterized as taraxerone, the identity of which was further substantiated by comparison of its spectroscopic data with published values (Sakurai et al., 1987; Kiem et al., 2004).

Compound **5** was obtained as colorless needles. The ^{1}H NMR spectra of compound **5** revealed a one-proton multiplet at δ 3.51, the position and multiplicity of which was indicative of H-3 of the steroidal nucleus. The typical signal for the olefinic H-6 of the steroidal skeleton was evident from a multiplet at δ 5.33 integrating for one-proton. The olefinic protons (H-22 and H-23) appeared as characteristics downfield signals at δ 5.13 and δ 5.03 respectively in the ^{1}H NMR spectrum. Each of the signal was observed as double doublets (J = 14.4, 8.4 Hz) which indicated coupling with the neighboring olefinic and methine protons. The spectrum further revealed signals at δ 0.67 and δ 1.00 (three-proton each) assignable to two tertiary methyl groups at C-13 and C-10, respectively. The ^{1}H NMR spectrum also showed two doublets centered at δ 0.81 (J = 7.4 Hz) and 0.85 (J = 7.4 Hz) which could be attributed to the two methyl groups at C-25. The doublet at δ 0.91 (J = 6.4 Hz) was demonstrative of a methyl group at C-20. These spectral features are in close agreement to those observed for stigmasterol (Khan, 1991; Chowdhury et al., 2008). On the basis of the above evidence; compound **5** was confirmed as stigmasterol.

Compound **6** was obtained as white crystals. The ^{1}H NMR spectrum of compound **6** showed two single-proton multiplet at δ 3.51 and δ 5.33 ppm typical for H-3 and H-6 of a steroidal nucleus. The spectrum further revealed two singlets at δ 0.67 and δ 1.00 each integrating for three protons, assignable to two tertiary methyl groups at C-13 and C-10 respectively and two doublets centered at δ 0.81 ppm (J = 7.6 Hz) and δ 0.83 ppm (J = 8.0 Hz) which could be attributed to two methyl group at C-25. The doublet at δ 0.92 ppm (J = 6.0 Hz) was demonstrative of a methyl group at C-20. These NMR spectral features are characteristics of a steroidal carbon skeleton of β-sitosterol (Morales et al., 2003). On the basis of the above spectroscopic data; compound **6** was confirmed as β-sitosterol.

The cytotoxic activity of the solvent extracts (methanol, petroleum ether, carbon-tetrachloride and dichloromethane); six column fractions (F-4, F-7, F-10, F-12, F-18 and F-22) of petroleum ether extract and three pure compounds **1**, **4** and **6** were determined by using brine shrimp lethality bioassay. The LC_{50} for crude petroleum ether, carbon-tetrachloride, dichloromethane, methanol extract and six column fractions (F-4, F-7, F-10, F-12, F-18 and F-22) of petroleum ether extract and three pure compounds **1**, **4** and **6** obtained from the best-fit line slope were found to be 1.33, 1.35, 1.86, 4.47, 1.40, 2.99, 2.51, 4.22, 1.58, 3.55, 4.22, 2.99 and 1.58 μg/ml, respectively (Table 1). In comparison with the positive control (vincristine sulphate), it is evident that all the test samples were lethal to brine shrimp nauplii. However, petroleum ether, carbon-tetrachloride extract, column fractions F-4 and F-18 of petroleum ether extract and pure compound **6** showed quite potent activity in brine shrimp lethality bioassay. These positive results suggested that they may contain antitumor or pesticidal activity.

Further, the antimicrobial activity of solvent extracts (methanol, petroleum ether, carbon-tetrachloride and dichloromethane) and six column fractions (F-4, F-7, F-10, F-12, F-18 and F-22) of petroleum ether extract of the *S. baccatum* were subjected to screening at 500 μg/disc. The average zone of inhibition produced

Table 1
LC_{50} values of solvent extracts, some column fractions of petroleum ether extract and pure compounds from *S. baccatum*.

Samples	LC_{50} (μg/ml)	Samples	LC_{50} (μg/ml)
VS	0.33	F-10	2.51
PE	1.33	F-12	4.22
CE	1.35	F-18	1.58
DCME	1.86	F-22	3.55
ME	4.47	SB-1	4.22
F-4	1.40	SB-4	2.99
F-7	2.99	SB-6	1.58

VS: vincristine sulphate (Std.); PE: petroleum ether extract; CE: carbon tetrachloride extract; DCME: dichloromethane extract; ME: methanol extract. Column fractions F-4, F-7, F-10, F-12, F-18 and F-22 of petroleum ether extract, SB-1 SB-4, SB-6 for pure compounds **1**, **4**, **6**, respectively.

Table 2
Antimicrobial activity of methanol extracts and some column fractions of petroleum ether extract (500 μg/disc) of *S. baccatum*.

Microorganisms	ME	Diameter of zone of inhibition (mm)				
		F-7	F-12	F-18	F-22	Kanamycin
Gram-positive bacteria						
B. cereus	11	10	10	13	10	33
B. megaterium	10	10	10	12	10	33
B. subtilis	10	10	NA	11	10	32
S. aureus	11	NA	NA	12	9	33
Sarcina lutea	11	NA	NA	15	11	35
Gram-negative bacteria						
E. coli	11	10	10	15	11	35
S. paratyphi	13	NA	10	13	10	32
S. typhi	11	10	10	15	10	31
S. boydii	12	NA	NA	13	10	33
S. dysenteriae	11	10	10	13	11	34
V. mimicus	11	10	10	10	10	32
V. parahemolyticus	10	NA	NA	10	10	32
Fungi						
C. albicans	11	10	10	14	11	33
A. Niger	11	10	10	13	10	34
S. cerevacae	12	10	10	15	11	34

ME: methanol extract; column fractions F-7, F-12, F-18 and F-22 of petroleum ether extract; NA: no activity; Kanamycin (Std.) (30 μg/disc).

by the crude methanol extract and column fractions F-7, F-12, F-18 and F-22 of petroleum ether extract showed mild to moderate inhibitory activity to microbial growth and having the zone of inhibition of 9–15 mm each (Table 2). On the other hand, petroleum ether extract, carbon-tetrachloride, dichloromethane extract and column fractions F-4 and F-10 of petroleum ether extract did not show any activity against the tested microorganisms.

4. Conclusions

It is concluded that six compounds were isolated from the petroleum ether extract of the leaves of *S. baccatum* based on spectroscopic evidence among them three compounds namely, Lupeol (**1**), Betulin (**2**) and Stigmasterol (**5**) were isolated for the first time from this plant. The antimicrobial and cytotoxicity screening of the different solvent extracts, six column fractions (F-4, F-7, F-10, F-12, F-18 and F-22) of petroleum ether extract and three pure compounds (**1**, **4** and **6**) were found to be consistent with the folk uses of *S. baccatum* by local people.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

We are grateful to the Bangladesh Council of Scientific and Industrial Research under Ministry of Science and Information & Communication Technology, Government of the Peoples' Republic of Bangladesh.

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