AMERICAN JOURNAL OF PHARMTECH RESEARCH

Journal home page: http://www.ajptr.com/

Phytochemical and biological investigation on *Ipomoea pescaprae*(L.)R.Br.

S. Sharmin[1*], F.T. Zohora[1], F.S. Tareq[2], S.N.Islam[3], C.M. Hasan[1], M. Ahsan[1]

1.Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Dhaka, Dhaka-1000, Bangladesh.

2.Department of Applied Chemistry, University of Dhaka, Dhaka-1000, Bangladesh.

3.Institute of Nutrition and food science, University of Dhaka, Dhaka-1000, Bangladesh

ABSTRACT

A total of five compounds eicosanyl-*trans*-p-coumarate (**1**), stigmasterol(**2**), β-sitosterol (**3**), β-amyrin(4), 6-hydroxy stigmasta-4,22-dien-3-one (5) were isolated from the methanolic extract of the stem bark of *Ipomoea pescaprae*(L.) R.Br.(Family:Convolvulaceae). The petroleum ether, carbon tetrachloride; chloroform and aqueous soluble Kupchan fractions of crude methanol extract were studied for antioxidant, antimicrobial and cytotoxic activities. Among the different fractions tested for antioxidant activity, the aqueous soluble partitionate was the most potent with IC_{50} value of 7.50µg/ml as compared to *tert*butyl-1-hydroxytoluene (IC_{50}=20.96µg/ml). Antimicrobial screening of the different extractives was conducted by the disc diffusion method and the crude methanol extract as well as aqueous soluble fractions exhibited moderate antimicrobial activity with zone of inhibition ranging from 10-12 mm. In brine shrimp lethality bioassay, the aqueous soluble materials demonstrated the highest toxicity with LC_{50} of 1.01µg/ml. Compounds eicosanyl-*trans*-p-coumarate, stigmasterol, β-sitosterol, 6-hydroxy stigmasta-4,22-dien-3-one are the first report of isolation of compounds from *Ipomoea pescaprae*.

Key words: *Ipomoea pescaprae,* chemical constituents, antioxidant, antimicrobial, cytotoxicity.

*Corresponding Author Email: sharmin.shanjida@gmail.com

Received 15 March 2013, Accepted 29 March 2013

Please cite this article in press as: Sharmin S. *et al.*, Phytochemical and biological investigation on *Ipomoea pescaprae*(L.)R.Br.American Journal of PharmTech Research 2013.

INTRODUCTION

Ipomoea pescaprae Roxb (Syn:*I. Biloba* Forsk.) is a member of the family Convolvulaceae and is most commonly seen in the Hawaiian Islands. It is one of the most widely distributed beach plant species which is found in most of the tropical and subtropical regions of the world. It is called Goat's Foot Creeper in English and in Ayurveda it is called Chhagalaantri, Maryaada-valli. Extract of *I.pescaprae* has antispasmodic activity as evidenced by its ability to inhibit contractions induced by spasmogen – histamine, acetylcholine, bradykinin and barium chloride. It was postulated that this action is a direct one on the smooth muscles of the ileum. A subsequent study lead to the isolation of two compounds which showed antispasmodic activity i.e. beta-damascenone and E-phytol.[2,3] It was postulated that this action is a direct one on the smooth muscles of the ileum. An extract of *Ipomoea pescaprae* was found to inhibit actions of all jelly fish venom.[4] In the crude extract of *I. pescaprae* there were four compounds identified as having inhibitory effects on prostaglandin synthesis in vitro. These four compounds are 2-hydroxy-4,4,7-trimethyl-1(4H)-naphthalenone (1), (-)-mellein (2), eugenol (3) 4-vinyl-guaiacol (4) and of these compound 3 and 4 seems to be the most active. This substantiate the anti-inflammatory activity of the plant.[5] It was found that the hydroalcoholic extract of *I.pescaprae* has pronounced antinociceptive properties and the compounds responsible includes glochidone, betulinic acid, alpha- and beta-amyrin acetate, isoquercitrin.[6] A screening of 18 plants for their antiplatelet aggregation and [14C]5-hydroxytryptamine activity showed that extract of *I.pescaprae* showed significant anti-platelet aggregation activity.[7] The hexane-soluble extract of aerial parts of *I.pescaprae* yielded six lipophilic glycosides jalapinolic acid, pescaproside A (1) and pescapreins I-IV [2-5], as well as the known stoloniferin III[6]. All six showed weak cytotoxic activity to a series of cancer cell lines.[8] Three Brazilian medicinal plants methanol extracts were evaluated for in vitro proliferation of human mononuclear cells. It was found that the extract of *I.pescaprae* showed immune stimulatory activity.[9] The whole plant is used in a medicinal bath to treat fatigue, strains, arthritis and rheumatism. In Myanmar, the people use an infusion of the plant with rusted iron as a cure for menorrhagia. The tuberous roots have diuretic properties and are used to treat bladder problems, strangury, dysuria and oedema.[10, 11, 12, 13]

MATERIALS AND METHOD

General experimental procedure

The ^{1}H NMR spectra were obtained using a Varian Unity 500 spectrometer (500 MHz) instrument in $CDCl_3$. For NMR studies deuterated chloroform was used and the δ values for ^{1}H

spectra were referenced to the residual non deuterated solvent signals.

Plant materials

The whole plant of *Ipomoeapescaprae* (Family: Covolvulaceae**)** was collected from St. Martin Island, Bangladesh in the month of June, 2012. A voucher specimen had been maintained in the National herbarium of Bangladesh under the accession number DACB-37535. The samples were cut into small pieces and sun dried for several days followed by oven drying for 24 hours at 40°C to facilitate grinding.

Extraction and isolation

The air-dried and powdered whole plant (1.4 kg) of *Ipomoea pescaprae* was soaked in 3L of methanol for 10 days at room temperature and then filtered through a cotton plug, followed by Whatman filter paper number 1. The extract was concentrated with a rotary evaporator. The solvent was evaporated to obtain a solid residue of around 29 gm. Then this was subjected to Vacuum liquid chromatography[14] for the initial rapid fractionation of the crude extract. The column was first eluted with 100% Petroleum ether. Then the mobile phases with progressively increasing polarity were passed through the column until it reached to 100% ethyl acetate. Thin layer chromatographic technique[16] was used for the initial screening of the VLC fraction extracts and the compounds – **1** (8 mg), **2** and **3**(9 mg), **4** (5 mg), **5** (5 mg) were isolated from the VLC extracts of different percentage of EtOAc in petroleum ether followed by TLC using Merck pre-coated TLC plates (Silica gel 60, F_{254}), Eluted with petroleum ether and chloroform.

An aliquot (5.0 g) of the concentrated methanolic extract was fractionated by the modified Kupchan partitioning method into Petroleum ether (2.0 g), carbon tetrachloride (0.5 g), chloroform(0.75 g) and aqueous (1.75 g) soluble fractions for biological activity.

DPPH free radical scavenging activity:

The free radical scavenging activity of the extractives were determined by the method developed by Brand-Williams *et al.*[17] based on the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. In short, 2.0 ml of a methanol solution of the extract at different concentrations were mixed with 3.0 ml of a DPPH methanol solution (20µg/ml) and the mixture was kept in dark for 20 minutes for reaction to occur. The absorbance of the resultant solution was determined at 517 nm and the percent inhibition was calculated from [(Ao–A1)/Ao] × 100, where Ao is the absorbance of the control and A1 is the absorbance of the test sample.

Antimicrobial screening:

The disc diffusion method[18] was used to evaluate the antimicrobial activity of the extractives against 15 bacteria (Table-2) collected as pure cultures from the Institute of Nutrition and Food

Sciences (INFS),University of Dhaka, Bangladesh. Solutions of known concentration (400µg/ml) of the test samples were made by dissolving measured amount of the samples in calculated volume of solvents, $CHCl_3$ or CH_3OH. Dried and sterilized filter paper discs (6 mm diameter) were then impregnated with known amount of the test substance using micropipette and the residual solvents were completely evaporated. Standard disc of ciprofloxacin (30µg/disc) and blank discs (impregnated with solvents followed by evaporation) were used as positive and negative control, respectively. These plates were then kept at low temperature (4º C) for 24 hours to allow maximum diffusion of the test materials and ciprofloxacin. The plates were finally incubated at 37º C for 24 hours to allow maximum growth or inhibition of growth of the organisms. The antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition expressed in mm. The experiments were carried out in triplicate and the mean values were taken.

Evaluation of cytotoxicity:

Brine shrimp lethality bioassay[19,20] technique was applied for the determination of general toxic property of the plant extractives. DMSO solutions of the samples were applied against *Artemia salina* in a 1-day assay. For the experiment, 4 mg of each of the extractive was dissolved in DMSO and solutions of varying concentrations (400, 200, 100, 50, 25, 12.50, 6.25, 3.125, 1.563 and 0.781µg/ml) were obtained by serial dilution using DMSO. Vincristine sulphate (10, 5, 2.5, 1.25, 0.625, 0.3125, 0.15625, 0.078125 and 0.0390µg/ml) was used as positive control.

Compound **1**:

^{13}C NMR (125 MHz, $CDCl_3$): δ127.5(C-1), 129.9(C-2), 115.9 (C-3), 157.5(C-4), 115.9(C-5), 129.9(C-6), 144.1(C-7), 115.8(C-8), 167.5(-CO-), 64.7(C-1′), 28.8(C-2′), 26(C-3′), 29.3(C-4′), 29.6-29.7(C-5′-16′), 29.3(C-17′), 31.9(C-18′), 22.7(C-19′), 14.1(C-20′). ^{1}H NMR (500 MHz, $CDCl_3$): δ7.43 (d, J=8.8 Hz, H-2,6), 6.84(d, J=8.8 Hz, H-3,5), 7.62 (1H, d, J=16 Hz, H-7), 6.30 (1H, d, J=16 Hz, H-8), 4.18 (2H, dd, J=6.8, 6.4 Hz, H-1′), 1.69 (2H, m, H-2′), 1.24 (br, s, H-3′-19′),0.88 (3H, t, J= 6.8 Hz, H-20′)

Compound **2**:

^{13}C NMR (125 MHz, $CDCl_3$): δ37.5(C-1), 31.9(C-2), 72.0(C-3), 42.5(C-4), 141.0(C-5), 121.9(C-6), 32.1(C-7), 32.1(C-8), 51.5(C-9), 36.7(C-10), 21.3(C-11), 39.8(C-12), 42.5(C-13), 57.1(C-14), 24.6(C-15), 29.1(C-16), 56.3(C-17), 11.8(C-18), 21.4(C-19), 40.7(C-20), 21.3(C-21), 138.5(C-22), 129.5(C-23), 50.3(C-24), 32.1(C-25), 21.3(C-26), 19.6(C-27), 25.6(C-28), 12.2(C-29). ^{1}H NMR (500 MHz, $CDCl_3$): δ3.51(m, H-3), 5.33 (br s, H-6), 0.69 (3H, s, H-18), 1.00 (3H, s, H-

19), 1.02 (d, J= 7.5 Hz, H-21), 5.00 (1H, m,H-22), 5.14 (1H, m, H-23), 0.79 (d, J=6.5 Hz, H-26), 0.83 (d, , J=5.5 Hz, H-27), 0.81 (t, , J=7.7 Hz, H-29)

Compound **3**:

^{13}C NMR (125 MHz, $CDCl_3$): δ37.5(C-1), 31.9(C-2), 72.0(C-3), 42.4(C-4), 141.0(C-5), 121.9(C-6), 32.1(C-7), 32.1(C-8), 50.3(C-9), 36.7(C-10), 21.3(C-11), 39.9(C-12), 42.5(C-13), 57.0(C-14), 24.5(C-15), 28.5(C-16), 56.2(C-17), 12.0(C-18), 19.4(C-19), 36.4(C-20), 18.9(C-21), 34.2(C-22), 26.3(C-23), 46.0(C-24), 29.4(C-25), 19.8(C-26), 19.6(C-27), 23.3(C-28), 12.2(C-29). ^{1}H NMR (500 MHz, $CDCl_3$):δ3.51**(**m, H-3**)**, 5.34 **(**br s, H-6**),** 0.67 (s, 3H, H-18), 1.00 (s, 3H, H-19), 0.91 (d, J=6.5 Hz, H-21), 0.80 (d, J=6.1 Hz, H-26), 0.83 (d, 3H, J=6.0 Hz, H-27),0.84 (t, J=7.1 Hz, H-29).

Compound **4**:

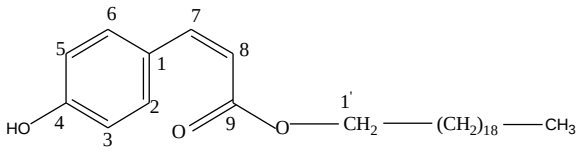
^{1}H NMR (500 MHz, $CDCl_3$): δ3.55 (1H, dd, J= 3.5 Hz, 3.0 Hz, H-3), 5.30 (1H, dd, J = 3.5 Hz, H-12), 0.90 (s, Me-23), 0.76 (s, Me-24), 0.98 (s, Me-25),0.98 (s, Me-26),1.02 (s, Me-27),0.84 (s,Me-28), 0.86 (s, Me-29), 0.86 (s, Me-30).

Compound **5**:

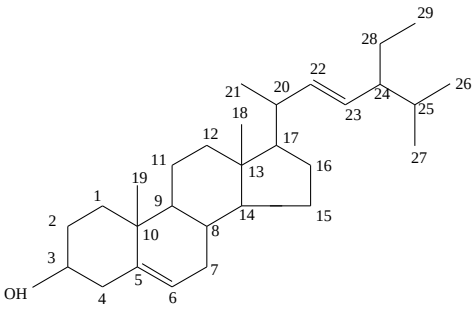
^{1}H NMR (500 MHz, $CDCl_3$): δ5.68 (1H,br s, H-4), 0.67(3H, s, H-18), 1.02(3H, s, H-19), 0.92(3H, d, J = 5.5 Hz, H-21),5.18(1H, m, H-22), 5.03 (1H,m, H-23), 0.84 (3H, d, J = 7.5 Hz, H-26), 0.81 (3H, d, J = 4.0 Hz, H-27), 0.89(3H, t, J = 7.5 Hz, H-29).

RESULTS AND DISCUSSION

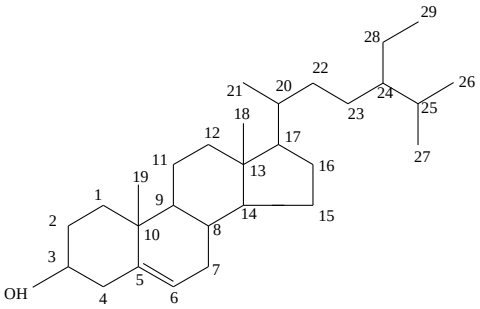
Repeated chromatographic separation and purification over silica gel of the methanol extract of *Ipomoea pescaprae* provided a total of five compounds (**1-5**). The structures of isolated compounds were solved by extensive NMR data analysis, comparison with published values as well as co-TLC with authentic samples.



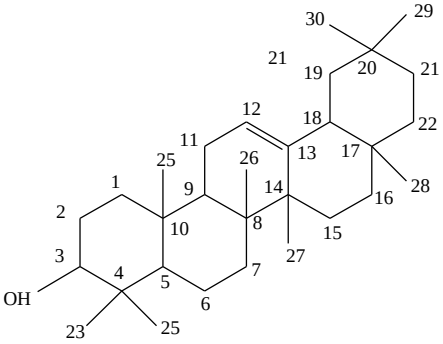
(1)



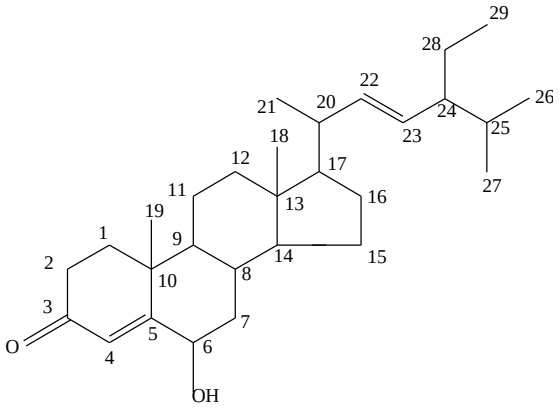
(2)



(3)



(4)



(5)

Compound **1**(8 mg): was obtained as white powder. It appeared as a purple spot on TLC (silica gel PF_{254}) under short wavelength of UV (254 nm). From the ^{1}H NMR spectrum, compound **1** was characterized as eicosanyl-*trans*-p-coumarate. The ^{1}H NMR spectrum showed two doublets centered at δ 6.84 (*J*=8.8 Hz) and δ 7.43 (*J*=8.8 Hz) integrating two protons each which indicated the presence of a di-substituted benzene ring. Two doublets at δ7.62 and δ 6.30 with a coupling constant of 16.0 Hz each revealed the presence of two *trans* coupled olefinic protons. A triplet at δ 4.18 (2H, *J*=6.6 Hz) indicated the presence of methylene group attached with an electronegative atom. The spectrum also shows a two proton multiplet at δ 1.69 (2H, m) indicated the presence of $–OCH_2-CH_2-CH_2-$ group. The presence of a broad signal at δ1.29 integrated for 34 protons which revealed the presence of $–(CH_2)_{17}-$ group. A three proton triplet at δ 0.88 (*J* = 6.8 Hz) indicated the presence of terminal primary methyl group of the esterified alkyl chain. The above spectral features were similar to the ones reported for eicosanyl-*trans*-p-coumarate. On this basis, the identity of compound **1** was confirmed as eicosanyl-*trans*-p-coumarate.[21]This is the first report of this compound from *I. pescaprae*.

Compound **2**(9 mg): was obtained as needle shaped white crystals. It appeared as a purple spot on TLC (silica gel PF_{254}) when the developed plate was sprayed with vanillin sulphuric acid followed by heating at 110^0 C for 5-10 minutes. The ^{1}H NMR spectrum (500 MHz, $CDCl_3$)

showed the presence of two methyl singlets at δ 0.69, and 1.00; three methyl doublets at δ 0.79, 0.83, and 1.02; and a methyl triplets at δ 0.81. This compound also showed protons at δ 5.00 (m), 5.14 (m), and 5.33 (s) suggesting the presence of three protons corresponding to that of a disubstituted and a trisubstituted olefinic bond. A multipletat δ 3.51 indicated the proton corresponding to the H-3 of a sterol moiety. The above spectral features were similar to the ones reported for stigmasterol. On this basis, the identity of compound **2** was confirmed as stigmasterol.[18, 19].This is the first report of this compound from *I.pescaprae*.

Compound **3** (9 mg): was obtained as needle shaped white crystals. It appeared as a purple spot on TLC (silica gel PF_{254}) when the developed plate was sprayed with vanillin sulphuric acid followed by heating at 110^0C for 5-10 minutes. The ^{1}H NMR spectrum (500 MHz, $CDCl_3$) showed the presence of two methyl singlets at δ 0.67, and δ 1.00; three methyl doublets at δ 0.80, δ 0.83, δ 0.91andδ 0.91; and a methyl triplet at δ 0.84. This compound also showed proton at 5.34 (s) suggesting the presence of a proton corresponding to that of a trisubstituted olefinic bond. A multipletat δ 3.51 indicated the proton corresponding to the H-3 of a sterol moiety. The above spectral features were similar to the ones reported for β-sitosterol. On this basis, the identity of compound **3**was confirmed as β-sitosterol.[18, 19].This is the first report of this compound from *I.pescaprae*.

Compound **4**(5 mg): The compound was obtained as white powder .It was visualized as a spot under long wavelength of UV light (366 nm) on TLC plate and showed an intense blue colure. The ^{1}H NMR spectrum showed the presence of eight methyl singlets at 0.90 (s, Me-23), 0.76 (s, Me-24), 0.98 (s, Me-25),0.98 (s, Me-26),1.02 (s, Me-27),0.84 (s,Me-28), 0.86 (s, Me-29), 0.86 (s, Me-30)of an oleanane skeleton i.e. the compound must be a pentacyclic compound. The above spectral features were similar to the ones reported for β-amyrin. On this basis, the identity of compound **4** was confirmed as β-amyrin.[18, 19]

Compound **5**(5 mg): was obtained as needle shaped white crystals. It appeared as a purple spot on TLC (silica gel PF_{254}) when the developed plate was sprayed with vanillin sulphuric acid followed by heating at 110^0 C for 5-10 minutes. The ^{1}H NMR spectrum showed two methyl singlets at δ 0.67 and δ 1.17, three methyl doublets at δ 0.91, δ 0.83 and δ0.81 and a methyl triplet at δ 0.83 indicating the presence of two tertiary methyls, tree secondary methyls and a primary methyl group. The spectrum showed a broad singlet at δ 5.68 suggesting the presence of a trisubstitutedolefinic bond. The multiplets at δ 5.03 and δ 5.18 are characteristic for H-22 and H-23 protons of a stigmasta type sterol. In addition the spectrum exhibited a multiplet at δ 4.33 which indicated a proton attached to a carbinol carbon. The above spectral features were similar

to the ones reported for 4-hydroxy stigmasta-4, 22-dien-3-one. On this basis, the identity of compound **5** was confirmed as 4-hydroxy stigmasta-4, 22-dien-3-one. This is the first report isolation of this compound from *I.pescaprae*.

Table 1: IC_{50} values of the standard partitionates of *Ipomoea pescaprae*in DPPH assay

Test Samples	IC_{50} (µg/ml)
BHT	20.96
PESF	72.25
CTSF	27.09
CSF	10.85
AQSF	7.50

BHT = *Tert*- butyl-1-hydroxytoluene

Among the different fractions tested for antioxidant activity (Table 1), the aqueous soluble partitionate and carbon tetrachloride soluble fractions demonstrated maximum free radical scavenging activity with IC_{50} value of 7.50 µg/ml and 10.85µg/ml followed by the other fraction chloroform (CTF, IC_{50}=27.09 µg/ml) soluble fraction exhibiting significant antioxidant activity as well.

In the antimicrobial screening, the methanolic crude extract as well as its aqueous soluble fractions exhibited moderate antimicrobial activity with average zone of inhibition ranging from 8-12 mm each as compared to standard (40-42 mm) (Table 2) exhibited by ciprofloxacin. However, the chloroform soluble fraction, pet-ether soluble fraction and the negative control disc showed no inhibition of microbial growth (data not shown in the table).

Table 2:` Antimicrobial activity of *Ipomoea pescaprae* extractives and ciprofloxacin

Test organisms	Diameter of the zone of inhibition (mm)				
	PESF	CTCSF	CSF	AQSF	Ciprofloxacin
Gram positive bacteria					
Bacillus aureus	-	11	10	-	37
Bacillus megaterium	-	7	7	-	35
Bacillus subtilis	-	9	8	-	45
Staphylococcus aureus	-	7	9	-	40
Sarcinalutea	-	7		-	20
Bacillus polymyxa	-	-	-	-	40
Gram negative bacteria					
Escherichia coli	-	-	-	-	46
Pseudomonas aureus	-	12	-	-	-
Salmonella paratyphi	-	-	-	-	-
Salmonella typhi	-	-	-	-	-
Shigellaboydii	-	-	-	-	27
Shigelladysenteriae	-	-	-	-	45
Klebsiellapneumonia	-	-	-	-	17
Shigellaflexi	-	-	-	-	-
Proteusvulgaris	-	-	-	-	40

Table 3 shows the results of brine shrimp lethality testing after 24 hours of exposure to the samples and the positive control, vincristine sulphate. The LC50 values were found to be 1.01µg/ml for Petroleum ether soluble fractions of the methanol extract revealed its toxicity to a significant degree.

Table 3. LC50 values of the standard and partitionate of *Ipomoea pescaprae*in brine shrimp leothalics assay.

Test samples	LC_{50} (µg/ml)
VS	0.451
PESF	1.01
CTCSF	1.23
CSF	3.10
AQSF	1.62

VS = Vincristine sulphate, PEF = Petroleum ether soluble fraction of the methanolic extract of the whole plant, CTF = Carbon tetrachloride soluble fraction of the methanolic extract of the whole plant, CLF =Chloroform soluble fraction of the methanolic extract of the whole Plant, AQ= Aqueous soluble fraction of the methanolic extract of the whole Plant.

CONCLUSION:

Medicinal plants used in the folk medicine may be an interesting and unexplored source for the development of potential new compounds. It was our attempt to identify new compounds in this plant that revealed five compounds and four of them are the first reports of this plant. The aqueous soluble fraction as well as chloroform soluble fractions of the plant exhibited good antimicrobial activity; petroleum ether soluble fraction demonstrated the highest toxicity. This is only a preliminary study and a more detailed study is under progress.

REFERENCES:

1. http://www.springerreference.com/docs/html/chapterdbid/68738.html
2. Pongprayoon U, Bohlin L, Sandberg F, Wasuwat S. Inhibitory effect of extract of Ipomoea pes-caprae on guinea-pig ileal smooth muscle. Acta Pharm Nord. 1989;1(1):41-4.
3. Pongprayoon U, Baeckström P, Jacobsson U, Lindström M, Bohlin L. Antispasmodic activity of beta-damascenone and E-phytol isolated from Ipomoea pes-caprae. Planta Med. 1992 Feb;58(1):19-21.
4. Pongprayoon U, Bohlin L, Wasuwat S. Neutralization of toxic effects of different crude jellyfish venoms by an extract of Ipomoea pes-caprae (L.) R. Br J Ethnopharmacol. 1991 Oct;35(1):65-9.

5. Pongprayoon U, Baeckström P, Jacobsson U, Lindström M, Bohlin L. Compounds inhibiting prostaglandin synthesis isolated from Ipomoea pes-caprae. Planta Med 1991 Dec;57(6):515-8
6. Kroth R, Berti C, Madeira AO, Souza MM, Cechinel-Filho V, Delle-Monache F, Yunes RA, Isolation and identification of compounds with antinociceptive action from Ipomoea pes-caprae (L.) R. Br. Pubmed, Die Pharmazie.1999;54(6):464-6.
7. Rogers KL, Grice ID, Griffiths LR. Inhibition of platelet aggregation and 5-HT release by extracts of Australian plants used traditionally as headache treatments. Eur J Pharm Sci. 2000;9(4):355-63.
8. Pereda-Miranda R, Escalante-Sánchez E, Escobedo-Martínez C. Characterization of lipophilic pentasaccharides from beach morning glory (Ipomoea pes-caprae). J Nat Prod. 2005 ;68(2):226-30.
9. Philippi ME, Duarte BM, Da Silva CV, De Souza MT, Niero R, CechinelFilho V, Bueno EC. Immuno stimulatory activity of Calophyllumbrasiliense, Ipomoea pes-caprae and Mataybaelaeagnoides demonstrated by human peripheral blood mononuclear cells proliferation. Acta Pol Pharm. 2010;67(1):69-73.
10. Hwee Ling Koh, Chua Tung Kian, ChayHoon Tan, A guide to medicinal plants: an illustrated, scientific and medicinal approach, World Scientific Publishing Co. Pte. Ltd., Singapore, 2009. Pg77-78.
11. Prof. HM, Hembing Wijayakusuma, Atasi ASAM URAT & REMATIK ala Hembing, PuspaSwara, Indonesia, 2007:46-47.
12. Francis E. Putz, Harold A. Mooney, The Biology of vines, Cambridge University Press, Cambridge, 1991:442.
13. Kamarudin Mat-Salleh, A. Latiff, Tumbuhan Ubatan Malaysia, Pusat Pengurusan Penyelidikan Universiti Kebangsaan Malaysia, Selangor, 2002:582-583.
14. Pelletier, SW, Chokshi HP, Desai HK. Separation of diterpenoid alkaloid mixtures using vacuum liquid chromatography. J Nat Prod 1986;49:892-900.
15. Liu QT, Kinderlerer JL. Preparative thin-layer chromatographic separation and subsequent gas chromatographic-mass spectrometric analysis of monoacylglycerols derived from butter oil by fungal degradation. J Chromatography A; 1999. 855 (2):617-624.

16. Vanwagenen, BC, Larsen R, Cardellina II, JH, Randazzo, D, Lidert ZC, Swithenbank, C. Ulosantoin, a potent insecticide from the sponge *Ulosaruetzleri*. J Org Chem 1993;58:335-337.
17. Brand-Williams, W., Cuvelier M.E. and Berset C. 1995. Use of free radical method to evaluate antioxidant activity, *Lebensm. Wiss. Technol.* 28, 25-30.
18. Bauer, A. W., Kirby, W. M. M., Sherris J. C. and Turck, M.. Antibiotic susceptibility testing by a standardized single disc method. Am. J. Clin. Pathol 1966;45:493-496.
19. McLaughlin JL, Rogers, LL.T he use of biological assays to evaluate botanicals. *Drug Info. J*. 1998; 32, 513-524.
20. Meyer BN, Ferrigni NR, Putnam JE, Jacobsen JB, Nicholsand D.E. McLaughlin, JL. Brine shrimp: a convenient general bioassay for active plant constituents, Planta Med.1982; 45:31-34.
21. Umar Mahmooda, Vijay K. Kaula, RuchiAcharyaa, Leopold Jirovetzb, p-Coumaric acid esters from Tanacetumlongifolium, Phytochemistry 2003;64:851–853.
22. Pateh UU, Haruna AK, Garba M, Iliya I, Sule IM, Abubakar MS, Ambi AA. Nigerian J Pharma Sci 2009;8(1):19 – 25
23. Venkata Sai Prakash Chaturvedula, Indra Prakash. Isolation of Stigmasterol and β-Sitosterol from the dichloromethane extract of *Rubussuavissimus*, Chaturvedula and Prakash. Int Current Pharma J 2012;1(9):239-242
24. Tetsuo Kushiro, Masaaki Shibuya,Yutaka Ebizuk, â-Amyrin synthase Cloning of oxido squalenecyclase that catalyzes the formation of the most popular triterpene among higher plants. Eur J Biochem 1998; 256:2382244.
25. Kamrun Nahar Asha, Rasheduzzaman chowdhury, Choudhury M. Hasan, M.A. Rashid. Acta Pharma.2004;54, 57-63.