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MOLLUSCICIDAL SPIROSTANOL SAPONINS FROM *AGAVE HETERACANTHA*

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أمكن فصل أربعة مركبات صابونينية جلوكوسيدية من المستخلص الميثانولى لنبات أجاف هيتراكنثا (عائلة الأجافيسى) وقد تم التعرف على التركيب الكيميائى لكل من الاجليكونات وهى التايجوجنين والجايتوجنين بالإضافة إلى السكريات المصاحبة.
ووضح أن الصابونينات الأربعة المفصولة لها فاعلية عالية ضد قواقع بيموفلاريا الكسندرينا (LC_{90} = 10, 15, 7 and 9 ppm) وليمنيا كايودى (LC_{90}= 8, 13, 5 and 6 ppm) العوائل الوسيطة لطفيل البلهارسيا المعوية والدودة الكبدية فى مصر.

Four spirostanol saponin glycosides were isolated from the butanol fraction of the methanolic extract of the leaves of Agave heteracantha (Agavaceae). The structures of these saponins were elucidated by a combination of spectroscopic analysis and hydrolysis followed by identification of both aglycones as tigogenin and gitogenin as well as the sugar moieties. The four saponins exhibited strong molluscicidal activities against Biomphalaria alexandrina (LC_{90}= 10, 15, 7 and 9 ppm) and Lymnaea cailliaudi (LC_{90}= 8, 13, 5 and 6 ppm), the intermediate hosts of Schistosoma mansoni and Fasciola gigantica in Egypt respectively.

INTRODUCTION

Schistosomiasis and Fascioliasis represent two public health problems in Egypt and other countries. Control of both diseases can be achieved by eradication of their snail vectors.[1,2]

Molluscicides are agents toxic to the snails (Molluscs). The interest in studying plant material containing molluscicidal compounds is based on the idea of a local supply of molluscicides which can be sufficiently produced at low costs by simple technologies.[3-8]

Agave species (Agavaceae) are reported to have anticancer, pisticidal, molluscicidal and other biological properties.[9-13] Steroidal saponins and sapogenins have been isolated from the leaves, fruits and rhizomes of *Agave* species.[14-16]

In a previous study, it was found that *Agave heteracantha* leaves dry powder showed molluscicidal activity.[17] However no published work has been traced concerning the saponin content of this plant. Therefore, the present study describes the isolation and characterization of some molluscicidal saponins from *A. heteracantha* and test of the isolated saponins against *Biomphalaria alexandrina* and *Lymnaea cailliaudi*, the intermediate hosts of *Schistosoma mansoni* and *Fasciola gigantica* in Egypt.

EXPERIMENTAL

Melting points were uncorrected. IR spectra were measured using KBr as discs on a Perkin-Elmer 1650 Spectrophotometer. ^{1}H-NMR were recorded in DMSO-d_6 on Jeol GIMEX-FT 270 MHz with TMS as internal standard and chemical shifts on a (ppm) scale. Mass spectra were recorded on a Varian Mat 311 A spectrometer with direct techniques at 70 ev. Column chromatography was performed on silica gel 60 G (Merck) using a glass column (5x120 cm). TLC were carried out on silica gel (Merck) GF_{254}, visualized by spraying with 10% alcoholic H_2SO_4 or Ehrlich reagent followed by heating at 120°C for 5 min. Paper chromatography was performed on Whatman No. 1 using descending technique and aniline hydrogen phthalate as spray reagent.

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Plant material

The fresh leaves of *Agave heteracantha* (Agavaceae) were collected from the Botanical Garden of Orman, Giza, Egypt. The plant was kindly authenticated by Eng. Badia H. Diwan (Agriculture Engineer at Orman Garden). The collection of the plant was done in May 1994. A voucher specimen was deposited in our Laboratory. The plant was dried in shade and then powdered by electric mill.

Extraction and isolation

The powdered leaves of *A. heteracantha* (2.5 kg) were extracted with methanol. The methanolic extract (125 g) was dried under reduced pressure, defatted with petroleum ether (60-80°C). The defatted portion was suspended in water and partitioned with chloroform, ethyl acetate and n-butanol. The butanolic layer was concentrated to yield a residue (36 g). This residue was subjected to column chromatography using gradient elution with $CHCl_3$, $CHCl_3$-MeOH mixture and finally pure methanol. Four major fractions (A-D) were obtained after monitoring by TLC.

Fraction A ($CHCl_3$-MeOH; 90:10) yielded compound 1 after purification on TLC using solvent $CHCl_3$:MeOH:H_2O (65:35:5) and by repeated recrystallization from methanol. Fraction B ($CHCl_3$-MeOH; 85:15) provided impure compound. Further purification on prep. TLC using solvent system $CHCl_3$:MeOH:H_2O (65:35:5) gave compound 2. Products of fractions C and D ($CHCl_3$-MeOH; 60:40 and 20:80) were combined and rechromatographed over silica gel column. Elution with $CHCl_3$:MeOH:H_2O (7:3:1) furnished compound 3 whereas elution with $CHCl_3$:MeOH:H_2O (6:4:1) gave impure compound. Further purification on prep. TLC using solvent system $CHCl_3$:MeOH:H_2O (65:35:10) gave compound 4.

Compound 1: Colourless needles, crystallized from methanol, mp. 269-270°C; R_f 0.38 ($CHCl_3$:MeOH:H_2O; 65:35:5). IR ν_{max}^{KBr} cm^{-1} 3400 (OH); 2935 (CH); 1450, 1370, 1065, 1045 (C-O-C); 982, 920, 895 and 870 (intensity 895 > 920; 25R-spiroketal). ^{1}H-NMR δ 0.67 (3H, s, 18-Me), 0.70 (3H, d, 27-Me), 0.80 (3H, s, 19-Me), 1.13 (3H, d, 21-Me) and anomeric protons at δ 4.82, 5.12, 5.16 and 5.52. CI/CH_4-MS; m/z 1063 (M^+ + 29), 1035 (M^+ + H), 903 (M^+ + H - xylose), 873 (M^+ + H - glucose), 741 (M^+ + H - glucose - xylose), 579 (M^+ + H - 2x glucose - xylose) and 417 (M^+ + H - galactose - 2x glucose - xylose).

Compound 2: Colourless needles, crystallized from methanol, mp 280-283°C, R_f 0.33 ($CHCl_3$:MeOH:H_2O; 65:35:5). IR ν_{max}^{KBr} cm^{-1} 3420 (OH); 2940 (CH); 1450, 1360, 1165, 1070 (C-O-C), 986, 925, 900 and 875 (intensity 900 > 925; 25R-spiroketal). ^{1}H-NMR δ 0.68 (3H, s, 18-Me), 0.70 (3H, d, 27-Me), 0.84 (3H, s, 19-Me), 1.13 (3H, d, 21-Me) and 1.64 (3H, d, 6-Me of rhamnose). The anomeric protons at δ 4.68, 4.81, 5.16 and 5.45. CI/CH_4-MS: m/z 1079 (M^+ + 29), 1050 (M^+), 903 (M^+ - rhamnose), 726 (M^+ + H - 2x glucose), 579 (M^+ - 2x glucose - rhamnose), 417 (M^+ - galactose - 2x glucose - rhamnose).

Compound 3: Colourless powder, crystallized from methanol, mp. 240-242°C; R_f 0.34 ($CHCl_3$:MeOH:H_2O; 65:35:10). IR ν_{max}^{KBr} cm^{-1} 3420 (OH); 2940 (CH); 1450, 1370, 1240, 1157, 1075, 1040 (C-O-C); 980, 920, 900 and 865 (intensity 900 > 920; 25R-spiroketal). ^{1}H-NMR δ 0.69 (3H, s, 18-Me), 0.71 (3H, d, 27-Me), 0.81 (3H, s, 19-Me), 1.13 (3H, d, 21-Me) and anomeric protons at δ 4.87, 4.96, 5.12, 5.20, 5.56. CI/CH_4-MS; m/z 1241 (M^+ + 29), 1212 (M^+), 1080 (M^+ - xylose), 1050 (M^+ - glucose), 756 (M^+ - 2x glucose - xylose), 594 (M^+ - 3x glucose - xylose) and 431 (M^+ - 4x glucose - xylose).

Compound 4: Colourless needles, crystallized from methanol, mp. 255-257°C; R_f 0.23 ($CHCl_3$:MeOH:H_2O; 65:35:10). IR ν_{max}^{KBr} cm^{-1} 3400 (OH); 2945 (CH); 1375, 1070 (C-O-C); 985, 925, 900 and 870 (intensity 900 > 925; 25R-spiroketal). ^{1}H-NMR δ 0.66 (3H, s, 18-Me), 0.70 (3H, d, 27-Me), 0.82 (3H, s, 19-

Me), 1.14 (3H, d, 21-Me), 1.65 (Me of Rha) and anomeric protons at δ 4.89, 5.09, 5.14, 5.26, 5.32 and 5.92. CI/CH_4-MS; m/z 1387 (M^+ + 29), 1359 (M^+ + H), 1227 (M^+ + H - xylose), 1212 (M^+ + H - rhamnose), 1065 (M^+ + H - glucose - xylose), 1049 (M^+ + H - glucose - rhamnose), 756 (M^+ + H - 2x glucose - xylose - rhamnose), 594 (M^+ + H - 3x glucose - xylose - rhamnose) and 432 (M^+ + H - 4x glucose - rhamnose - xylose).

Acid hydrolysis of saponins 1-4

50 mg of each compound was refluxed with 2N HCl-EtOH (1:1; 15 ml) on a steam bath for 4 hours. The reaction mixture was diluted with water and extracted with $CHCl_3$ (3x250 ml). The chloroformic extract was concentrated under reduced pressure. Each aglycone was purified by recrystallization from $CHCl_3$ containing few drops of methanol. Their structures were established by spectroscopic analysis and by direct comparison with an authentic samples on TLC using solvent system C_6H_6:EtOH (9:1).

Compounds 1 and 2 afforded tigogenin, mp. 204-205°C (Lit. 202-204°C).[18] EI/MS; m/z (rel.int.) 416 (M^+, 36), 400 (16), 357 (9), 302 (28), 287 (47), 274 (52), 139 (100), 115 (25). IR ν_{max}^{KBr} cm^{-1} 3400, 980, 920, 900, 860 (intensity 900>920; 25R-spiroketal). This aglycone was identified by direct comparison with an authentic sample on TLC and using solvent system C_6H_6:EtOH (9:1).

Compounds 3 and 4 provided gitogenin, mp. 269-271°C (Lit. 268-270°C).[19] EI/MS; m/z (rel. int.) 432 (18), 417 (25), 318 (29), 303 (42), 139 (100), 115 (51). IR ν_{max}^{KBr} cm^{-1} 3400, 990, 960, 925, 900 (intensity 900>925; 25R-spiroketal). It was identified by direct comparison with an authentic sample on TLC using solvent system C_6H_6:EtOH (9:1).

The aqueous hydrolysate was neutralized, filtered and concentrated. The residue was extracted with pyridine and filtered. The pyridine extract was concentrated and dissolved in 10% isopropanol and examined by PC (n-BuOH:AcOH:H_2O; 4:1:5) against sugar samples.

The sugars resulted from compound 1 were D-galactose, D-glucose and D-xylose but from compound 2 were D-galactose, D-glucose and L-rhamnose. Compound 3 gave D-glucose and D-xylose whereas 4 yielded D-glucose, D-xylsoe and L-rhamnose.

Molluscicidal assay

Molluscicidal activity of methanol extract and the isolated saponins were tested against *Biomphalaria alexandrina* and *Lymnaea cailliaudi* according to established procedures.[20,21] Testes were done in duplicate with ten snails for each test in 1000 ml dechlorinated water with known concentrations of the extract or saponin under test. Control groups of snails were placed in dechlorinated water only. The exposure time was 24 hours followed by 24 hours recovery period. Statistical analysis of the data was carried out according to Litchfield and Wilcoxon methods.[22]

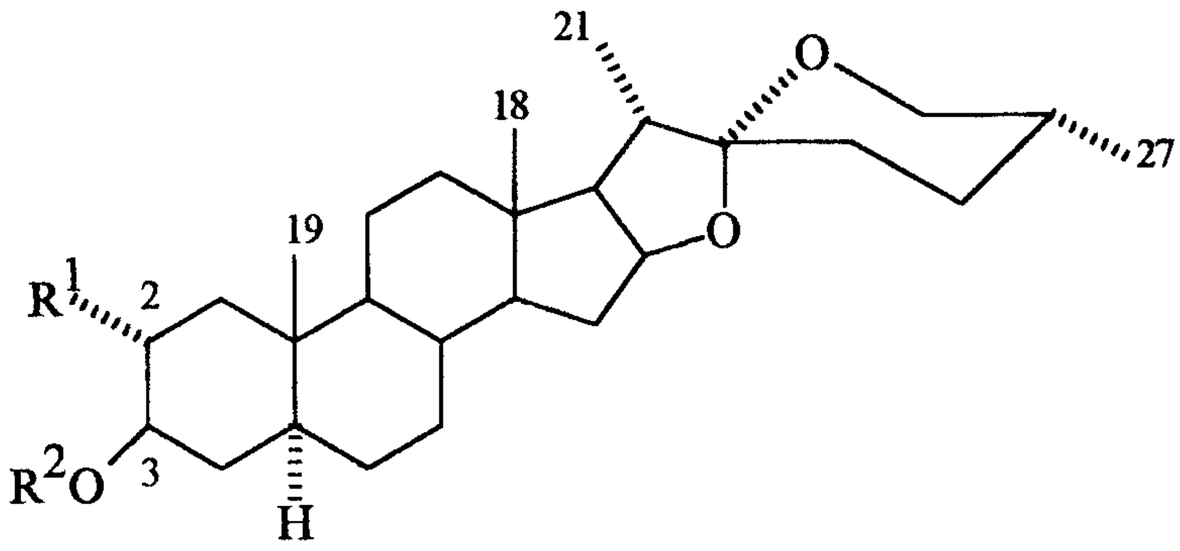
RESULTS AND DISCUSSION

The results of biological testing as shown in Table 1 revealed that the methanol extract of *Agave heteracantha* leaves killed *B. alexandrina* and *L. cailliaudi* snails within 24 hours at concentration 71 and 68 ppm. Therefore, this methanolic extract was defatted with petroleum ether then suspended in water and partitioned with $CHCl_3$, EtOAc and n-BuOH. The butanolic fraction was subjected to chromatographic separation using silica gel column followed by prep. TLC. All the isolated compounds exhibited high molluscicidal activities (Table 1) against *B. alexandrina* (LC_{90}= 10, 15, 7 and 9 ppm) and *L. cailliaudi* (LC_{90}= 8,13,5 and 6 ppm respectively). Also, these compounds responded positively to the Liebermann-Burchard and Molish tests and formed a soapy lather when shaken with water but they did not respond to Ehrlich reagent. Their IR spectrum showed well-defined spiroketal absorption bands. This suggested that the four compounds have monodesmosidic spirostanol saponin structure.[23-31]

Saponin 1 was predicated to have a (25R)-spirostanol skeleton based on the appearance of the characteristic bands at 920, 895 and 870 with the absorption band at 895 cm^{-1} being of greater intensity than that at 920 cm^{-1} in its IR

Table 1: Comparative susceptibility of *Biomphalaria alexandrina* and *Lymnaea cailliaudi* towards the action of the methanol extract and isolated saponins from *Agave heteracantha* after 24 hours exposure time.

Material	*Biomphalaria alexandrina*			*Lymnaea cailliaudi*		
	LC_{50}	LC_{90}	S	LC_{50}	LC_{90}	S
Methanol extract	50 (42.37-57)	71	1.31	48 (40.74-55.21)	68	1.30
Saponin 1	7.5 (6.62-8.49)	10	1.27	5.8 (4.17-6.49)	8	1.27
Saponin 2	11 (9.82-12.37)	15	1.25	9.8 (8.59-11.72)	13	1.25
Saponin 3	5.2 (4.56-6.32)	7	1.29	3 (2.32-5.42)	5	1.34
Saponin 4	6.5 (5.78-7.52)	9	1.26	4.3 (3.58-5.16)	6	1.35



Saponin	R_1	R_2
1	H	–Gal—Glc(Xyl, Glc)
2	H	–Gal—Glc(Rha, Glc)
3	OH	–Glc—Glc(Glc-Xyl, Glc)
4	OH	–Glc—Glc(Glc-Xyl, Glc-Rha)

Gal= galactose, Glc = glucose, Xyl = xylose, Rha = rhamnose

spectrum. The glycosidic nature of this saponin was suggested by the strong absorption bands at 3400 and 1045 cm^{-1} in its IR spectrum.[30-34] In the ^{1}H-NMR spectrum, four methyl proton signals were observed at δ 0.67-1.13. Also, the signals of anomeric protons appeared at δ 4.82, 5.12, 5.16 and 5.52.[30-36] From the CI/CH_4-MS spectrum, compound 1 has a molecular weight m/z 1034. The fragments at m/z 903 (M^+ + H - xylose), 873 (M^+ + H - glucose) and 741 (M^+ + H - glucose - xylose) corresponding to the loss of one terminal pentose (xylose) unit and one terminal hexose (glucose) unit. Another fragment at m/z 579 (M^+ + H - 2x glucose - xylose) indicated the loss of another glucose unit. The fragment at m/z 417 (M^+ + H - galactose - 2x glucose - xylose) was attributed to the loss of a tetrasaccharide unit and exhibited that the aglycone of this saponin was directly attached to hexose (galactose) unit.[32-36] Acid hydrolysis of saponin 1 furnished D-galactose, D-glucose and D-xylose as sugar moiety as well as aglycone. This aglycone was established as tigogenin through its mass fragmentation m/z 416, 400, 302, 287 and 139 (base peak) and by direct comparison with an authentic sample on TLC using solvent system C_6H_6:EtOH (9:1). On the basis of the above data, saponin 1 was proposed to be monodesmosidic spirostanol saponin having tigogenin as aglycone and one tetrasaccharide containing one galactose unit, two glucose units and one xylose unit (tigogenin 3-O-tetrasaccharide).

Saponin 2 was obtained as a colourless needles, mp. 280-283°C. Its IR spectrum indicated the presence of hydroxyl groups at 3420 cm^{-1} and 25R-sprioketal moiety at 986, 925, 900 and 875 (intensity 900 > 925).[30-36] The ^{1}H-NMR spectrum displayed four methyl proton signals at δ 0.68-1.13 and the signals at δ 1.64 due to the methyl group of rhamnose unit. The anomeric proton signals were observed at δ 4.68, 4.81, 5.16 and 5.45.[30-35] The CI/CH_4-mass spectrum of this saponin yielded fragment at m/z 1079 (M^+ + 29) suggesting its molecular weight as 1050. The fragment at m/z 903 (M^+ - rhamnose) and 726 (M^+ - 2x glucose) were attributed to loss of one rhamnose unit and two glucose units at the terminal positions. Fragments at m/z 579 (M^+ - 2x glucose - rhamnose) confirmed that the rhamnose and glucose units are branched with an inner glucose and suggested that the aglycone of this saponin was attached to hexose (galactose) unit and this suggestion was supported by presence of fragment at m/z 417 (M^+ - galactose - 2 glucose - rhamnose).[30-37] On the other hand, acid hydrolysis of saponin 2 gave D-galactose, D-glucose and L-rhamnose as well as tigogenin as aglycone which was identified by comparison with authentic sample and by its mas fragmentation m/z 416, 400, 357, 302, 287, 274, 139 and 115. From the above data, saponin 2 was suggested to be tigogenin-3-O-tetrasaccharide. The sugar chain contain one galactose unit, two glucose units and one rhamnose unit.

Saponin 3 was predicated to be a glycoside of a (25R)-spirostanol steroid from its IR spectrum 980, 920, 900 and 865 (intensity 900 > 920). The ^{1}H-NMR spectrum exhibited the characteristic signals of the methyl protons at δ 0.69-1.13 as well as anomeric proton signals of the sugar moiety at δ 4.87, 4.96, 5.12, 5.20 and 5.56.[38-41] When saponin 3 was submitted to acid hydrolysis, D-glucose and D-xylose were obtained as well as gitogenin as the aglycone. This aglycone was identified through its mass fragmentation m/z 432, 417, 318, 303, 139 and 115 and by comparison with authentic sample. CI/CH_4-MS of this saponin gave m/z 1241 (M^+ + 29) and 1212 (M^+) indicating that it is gitogenin pentaglycoside. Fragments at m/z 1080 (M^+ - xylose) and m/z 1050 (M^+ - glucose) were assignated to the loss of one xylose unit and one glucose unit from the terminal positions.[39-41] Fragments at m/z 756 (M^+ - 2x glucose - xylose) indicated that each xylose and glucose units are attached to the inner glucose unit. Fragment at m/z 594 (M^+ - 3x glucose - xylose) indicating the aglycone of this saponin is attached to hexose (glucose) unit and this confirmed by presence of fragment at m/z 431 (M^+ - 4x glucose - xylose).[38-41] Thus the structure of saponin 3 was suggested to be monodesmosidic spirostanol having gitognin as aglycone and one pentasaccharide containing four glucose units and one xylose unit (gitogenin-3-O-pentasaccharide).

Saponin 4 was predicated to be a glycoside of a (25R)-spirostanol skeleton based on the characteristic absorption bands 985, 925, 900, 870 with the absorption band at 900 cm^{-1} being of greater intensity than at 925 cm^{-1} in the IR spectrum.[30,31] The ^{1}H-NMR spectrum exhibited four methyl proton signals at δ 0.66-1.14 and a signal of methyl group of rhamnose units at δ 1.65 and the six anomeric proton signals at δ 4.89, 5.09, 5.14, 5.26, 5.32 and 5.92. Acid hydrolysis of saponin 4 yielded gitogenin as well as D-glucose, D-xylose and L-rhamnose. CI/CH_4-MS showed (M^+ + 29) at 1387 and (M^+ + H) at m/z 1359 suggesting the molecular weight as 1358. Fragments at m/z 1227 (M^+ + H - xylose) and 1212 (M^+ + H - rhamnose) were ascribed to the loss of terminal xylose unit and one terminal rhamnose unit. Another two fragments at 1065 (M^+ + H - glucose - xylose) and 1049 (M^+ + H - glucose - rhamnose) indicated that each two of disaccharide (glucose - xylose) and (glucose - rhamnose) units are branched from an inner glucose. Fragments at m/z 594 (M^+ + H - 3x glucose - rhamnose - xylose) suggested that the aglycone was attached to hexose (glucose) unit and this confirmed by appearance of fragment at m/z 432 (M^+ + H - 4x glucose - xylose - rhamnose).[36-41] Based on the above data, the structure of saponin 4 has been suggested to be gitogenin hexasaccharide. The sugar moiety composed of four glucose units, one xylose and one rhamnose unit.

REFERENCES

1- K. Hostettmann, Helv. Chim. Acta, 63, 606-609 (1980).
2- A. A. Magdoub, M. F. El-Sawy, H. K. Bassiony and M. E. Hasaballa, J. Egypt Socity Parasitology, 12, 153 (1982).
3- K. Hostettmann, K. Kizu and T. Tomimori, Planta Medica, 44, 34-35 (1982).
4- H. Kloos, F. S. McCullough, Planta Medica, 46, 195 (1982).
5- A. Lemma, Eth. Med. J., 3, 187 (1965).
6- M. M. El-Sayed, H. A. El-Nahas and L. A. Refay, Egypt. J. Bilh., 12, 207 (1990).
7- M. M. El-Sayed, M. Abdel Hadi, H. A. El-Nahas, Arch. Pharm. Res., 14, 81 (1991).
8- M. M. El-Sayed and H. A. El-Nahas, Egypt J. Pharm. Sci., 34, 539-553 (1993).
9- B. S. Aswal, D. S. Bhakuni, A. K. Goel, B. Kar, B. N. Mehrotra and K. C. Mukherjee, Indian J. Exp. Biol., 22, 312 (1984).
10- S. P. Grover and D. C. Bhatkoti, Indian J. For., 5, 71 (1982).
11- K. R. Kirtikar and B. D. Basu (1981) Indian Medicinal Plants, p. 2466, M/S Periodical Experts Delhi.
12- H. A. Shoeb, G. H. Tammam, S. M. El-Amin and M. Abd El-Motagally, Az. J. Nat. Prod., 8, 236-246 (1992).
13- H. A. Sheob, S. M. El-Amin, G. H. Tammam and L. A. Refahy, 8, 247-253 (1992).
14- G. C. Uniyal, P. K. Agrawal, R. S. Thakar and O. P. Sati, Phytochemistry, 29, 973-940 (1990).
15- G. C. Uniyal, P. K. Agrawal, O. P. Santi and R. S. Thakur, Phytochemistry, 30, 1336-1339 (1991).
16- B. Wilkomirski, V. A. Bobeyko and P. K. Kintia, Phytochemistry, 14, 2657-2659 (1975).
17- H. A. Shoeb, M. M. El-Sayed, M. A. El-Askalany, H. A. El-Nahas and L. A. Refai, Assiut Vet. Med. J., 23, 39-47 (1990).
18- S. C. Sharma, R. Chand, O. P. Sati and A. K. Sharma, Phytochemistry 22, 241 (1983).
19- K. Nakano, E. Matsuda, K. Tsurumi, T. Yamasaki, K. Murakami, Y. Takaishi and T. Tomimotsu, Phytochemistry, 27, 3235-3239 (1988).
20- WHO (1953), Export Committee on Bilharziasis Control Methods WHO 65, 33.
21- WHO (1965), WHO Monograph. Ser. 50, 124-138.
22- J. T. Litchfield and F. Wilcoxon, Exp. Therap., 96, 99-113 (1949).
23- S. B. Mahato, A. N. Ganguly and N. P. Saha, Phytochemistry, 21, 959 (1982).
24- A. Marston and K. Hostettmann, Phytochemistry, 24, 639-652 (1985).

25- S. Kiyosawa, M. Hutoh, T. Komori, T. Nohara, I. Hossokawa and T.Kawasaki, Chem. Pharm. Bull., 16, 1162 (1968).

26- T. Konishi, S. Kiyosawa and J. Shoji, Chem. Pharm. Bull., 33, 591 (1985).

27- G. Liebermann, Ber. Deut. Chem. Ges., 18, 1804 (1885).

28- R. S. Chandel and R. P. Rastogi, Phytochemistry, 19, 1889-1908 (1980).

29- I. P. Dragalin and P. K. Kintya, Phytochemistry, 14, 1817 (1975).

30- M. E. Wall, C. R. Eddy, M. L. McChemnan and M. E. Klumpp, Analyt. Chem., 24, 1337 (1952).

31- C. R. Eddy, M. E. Wall and M. K. Scott, Analyt. Chem., 25, 266 (1953).

32- V. Ahmed, T. Baqai and R. Ahmed, Phytochemistry, 34, 511-515 (1993).

33- O. P. Santi, U. Rana and D. C. Chankiyal, J. Nat. Prod., 50, 263-265 (1987).

34- G. Pant, O. P. Sant, K. Miyahara and T. Tawaski, Phytochemistry, 25, 2895-2896 (1986).

35- W. Yan, K. Ohtani, R. Kasai and K. Yamasaki, Phytochemistry, 42, 1417-1422 (1996).

36- J. Yong, Z. J. Jan, Phytochemistry, 33, 1193-1195 (1993).

37- F. Ferreira, S. Soule, V. Vazquez, P. Moyna and L. Kenne, Phytochemistry, 42, 1409-1416 (1996).

38- Y. Mimaki, O. Nakamura, Y. Sashida, T. Nikaido and T. Ohmato, Phytochemistry, 1279-1286 (1995).

39- K. Nakano, E. Matsuda, K. Tsurumi, T. Yamasaki, K. Murakami, Y. Takaishi and T. Tomimartsu, Phytochemistry, 27, 3235-3239 (1988).

40- V. Ahmad, F. T. Buquai, T. Fatima and R. Ahmad, Phytochemistry, 30, 3057 (1991).

41- S. Yahara, T. Ura, C. Sakamoto and T. Nohara, Phytochemsitry, 37, 831 (1994).