Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

J P P Journal of Pharmacognosy and Phytochemistry

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2014; 3(5): 73-78
Received: 27-11-2014
Accepted: 07-12-2014

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A new biologically active ecdysteroid from the aerial parts of *Sida glutinosa*

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Abstract
The aerial parts of *Sida glutinosa* have been analyzed for their chemical constituents, resulting in the isolation of a new ecdysone, named glutinosterone (**1**). The structure of glutinosterone was determined on the basis of extensive spectroscopic analysis, including 2D NMR data and the results of hydrolytic cleavage. The structure of glutinosterone was elucidated as 1β-hydroxy 24(28)-dehydromakisterone A. The *in vitro* biochemical analysis of compound **1** showed a significant effect on different enzymes involved in human liver function along with lipid metabolic enzymes. The compound **1** also exhibits moderate to significant anti-bacterial property.

Keywords: *Sida glutinosa*, Malvaceae, Ecdysterone, Glutinosterone.

1. Introduction
Sida glutinosa Roxb.syn *S. mysorensis* Wt. & Arn. is an annual herb belonging to the family Malvaceae and is distributed in wastelands of South and Eastern India, Burma to South east Asia. The roots and aerial parts of this plant and its sister species were used in traditional system in the treatment of pulmonary tuberculosis and rheumatism [1, 2]. Literature survey indicated that no considerable research on phytochemical characterizations have been carried out to explore the significance of this plant for humankind. However, an extensive research is indeed important to screen naturally occurring bioactive compounds from *Sida glutinosa* and also to decipher their bioactivity. In this context, we have already isolated and characterized nine different phytochemicals from this plant and also established the antioxidant property of three among them [3, 4]. We have also reported earlier among the isolated nine compounds, two compounds showed significant reduction of enzyme activity and also antibacterial activity [5].
The present investigation on the *n*-BuOH fraction of MeOH extract of aerial part of *S. glutinosa* has resulted in the isolation of a new Ecdysterone, named glutinosterone (**1**). In addition, *in vitro* biological activity of the isolated compound **1** was studied to elucidate its role in the modulation of enzymatic activity. The enzymes studied in the present work were the markers for hepatic function like aminotransferases, alkaline phosphatases, lipid metabolic enzyme activity. The antibacterial function of compound **1** was also studied to characterize its bioactivity. In brief, the present study deals with the structure elucidation of compound **1** on the basis of extensive spectroscopic analysis, including 2D NMR data and the results of hydrolytic cleavage and also the evaluation of *in vitro* liver function in terms of serum enzyme activity, lipid metabolic enzyme and antibacterial activity.

2. Materials and Methods
2.1. Experimental
Melting points were determined by the use of Kofler type electrical melting point apparatus and are uncorrected. UV spectra were recorded on a Perkin Elmer Lambda 25 spectrometer on MeOH and IR spectra on a Shimadzu 8100 FT-IR spectrometer from KBr discs. ^{1}H and ^{13}C-NMR spectra were measured on a varian 600 spectrometer. Chemical shifts are given in δ values (ppm) relative to tetramethylsilane (TMS) as internal standard. HR-FAB-MS and FAB-MS were obtained by JEOL JMS 700 spectrometer. For column chromatography, silica gel (mesh 60-120, Merck, India), Diaion HP-20 (Mitsubishi-Chemical, Tokyo, Japan) and basic alumina (Al_2O_3) (Merck, India) were used. Silica gel G (Merck, India) was used for TLC.

2.2. Plant material
The aerial parts of *Sida glutinosa* were collected from Kalsi (Jolaibari), South Tripura in April 2008. The plant was identified by Prof. B. K. Datta, Taxonomist, Department of Botany, Tripura University. A voucher specimen (No. BD/01/08) has been deposited in the National Herbarium, Shibpur Botanical Garden, Howrah-711 103, India.

2.3. Extraction and isolation

Air dried, powdered aerial parts of *S. glutinosa* (3.3 kg) were extracted with MeOH (6.0 L each) at room temperature (three times) during a 1 week. The MeOH extract was concentrated under reduced pressure *in vacuo* to a gummy mass (106 g). The residue (90 g) was suspended in 125 mL water and extracted three times with CH_2Cl_2, $CHCl_3$ and *n*-BuOH, successively.

The *n*-BuOH soluble fraction (12 g) was subjected to column chromatography (CC) through Diaion HP-20 and eluted stepwise with H_2O/MeOH (100:0, 75:25, 50:50, 25:75, 0:100) and 150 mL fraction being collected. The fraction obtained from H_2O/MeOH (25:75 and 0:100) were mixed together with a semi-solid mass (4.2 g) and then subjected to Silica gel CC with a stepwise gradient of $CHCl_3$/MeOH (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60 and 0:100) and 100 mL fraction being collected. The fractions eluted with $CHCl_3$/MeOH (90:10, 80:20 and 70:30) were mixed together and were further subjected to basic alumina CC with a stepwise gradient of EtOAc/MeOH (100:0, 95:5, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60 and 0:100; each 100 mL). The fraction eluted with EtOAc/MeOH (80:20) gave a light yellow residue, which on repeated crystallization from $CHCl_3$/MeOH (90:10) afforded light yellow crystals of glutinosterone (**1**, 30 mg).

2.4. Glutinosterone [(20*R*, 22*R*)-1*β*, 2*β*, 3*β*, 14*α*, 20, 22, 25-heptahydroxy-5*β*-cholest-7, 24 dien-6-one] (1)

A light yellow crystals, mp 230°C(dec), UV(MeOH) λ_{max} nm (log ε): 248(3.61); IR(KBr) max cm^{-1}: 3389, 1658, 1642, 1464, 1445, 1381, 1150, 1059, 874; HR-FAB-MS: *m/z* $[M+Na]^+$ 531.2922 (calcd for $C_{28}H_{44}O_8Na$: 531.2933); ^1H and ^{13}C-NMR (600 MHz and 150 MHz, CD_3OD) : see Table 1; FAB-MS : *m/z* 531$[M+Na]^+$ (4), 491 $[MH-H_2O]^+$ (33), 473 $[MH-2H_2O]^+$ (38), 455 $[MH-3H_2O]^+$ (67), 379 (21), 361 (21), 343 (25), 301 (25), 173 (29), 165 (29), 147 (21), 75 (100).

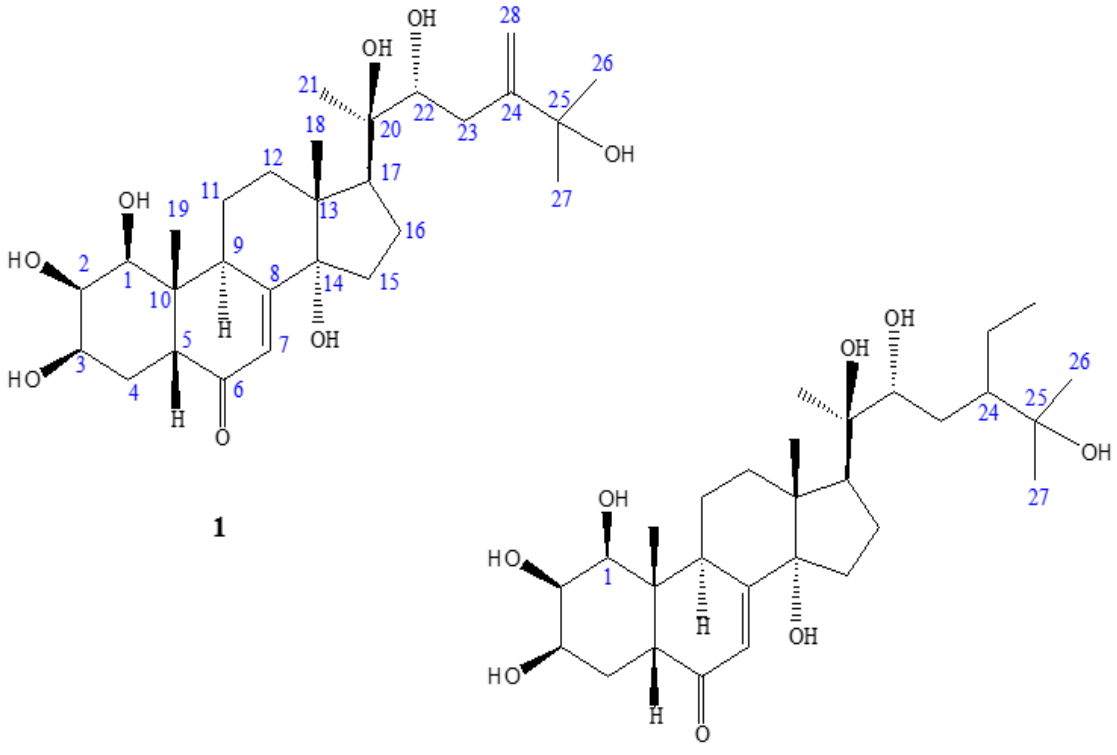
2.5. Biochemical study of compound 1

Serum was separated from human blood sample and the activity of compound **1** was studied for identifying their role on enzymes that generally used as marker for hepatic function like aminotransferases & alkaline phosphatases, lipid metabolic enzyme activity and anti-bacterial function at different concentration (Table 2, Table 3 and Table 4).

3. Results and discussion

3.1. Structure Elucidation of Compound 1

Air dried, powder aerial parts of *S. glutinosa* were extracted with MeOH at room temperature for 1 week. The resulting MeOH extract was successively extracted with $CHCl_2$, $CHCl_3$, EtOAc and *n*-BuOH. The *n*-BuOH soluble fraction was subjected to column chromatography (CC) through Diaion HP-20, Silica gel and basic Alumina afforded the new compound, glutinosterone (**1**) (Fig. 1), as light yellow crystals, mp 230 °C (dec.). Its molecular formula was deduced as $C_{28}H_{44}O_8$ from the quasi-molecular ions at *m/z* 509.2852 $[M+H]^+$ (calcd for $C_{28}H_{45}O_8$: 509.2862) and 531.2922 $[M+Na]^+$ in positive mode HR-FAB-MS as well as analysis of ^{13}C-NMR including DEPT data. It show UV absorption maxima in MeOH 248 (logε 3.61) characteristic of cyclic enone system. Its IR spectrum in KBr exhibited the bands for hydroxyl (3389 cm^{-1}), *α,β* unsaturated carbonyl (1658 cm^{-1}) and exomethylene olefinic (1642 and 874 cm^{-1}) functions. The ^1H and ^{13}C-NMR analyses were performed with the aid of 2D-NMR (^1H-^1H-COSY, HMQC, HMBC and NOESY).



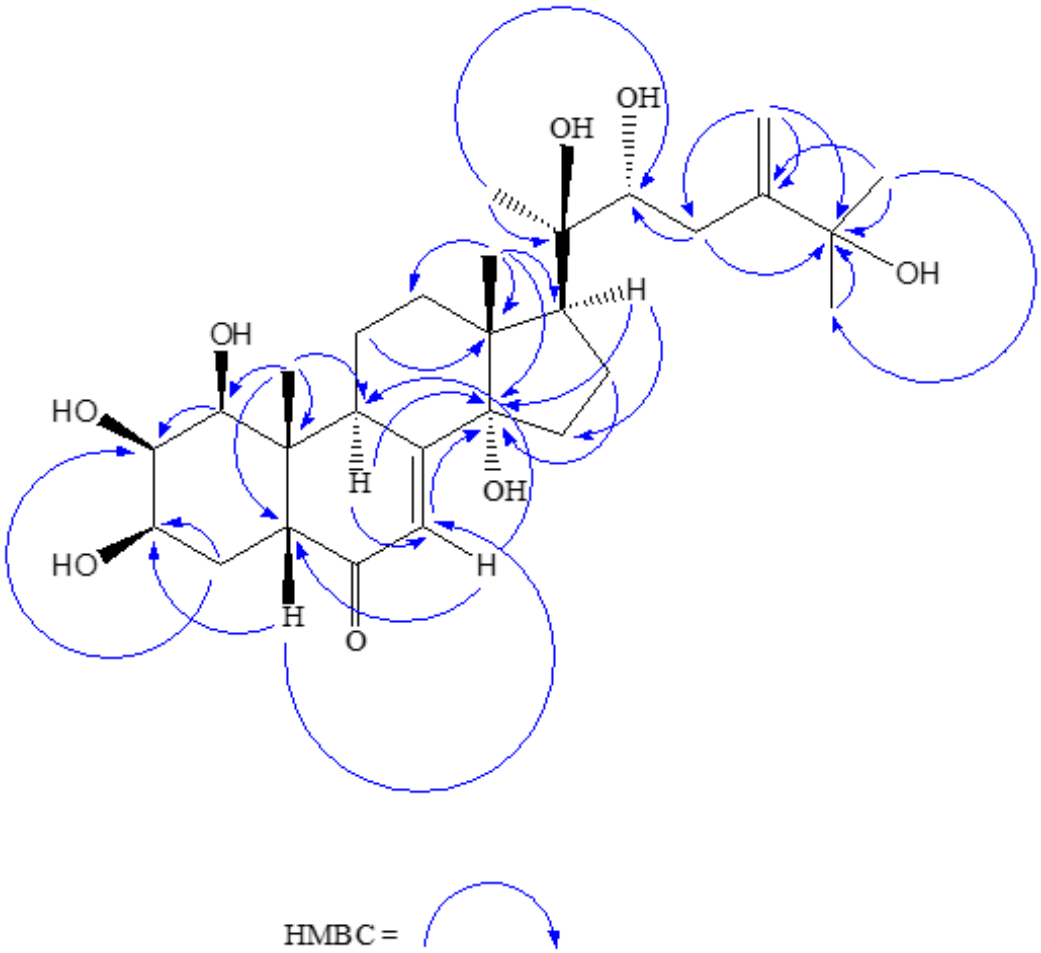
1 **1a**

Fig 1: Structures of compound **1**and **1a**

Its 600 MHz ^{1}H-NMR spectrum in CD_3OD (Table 1) showed five tertiary methyl groups at [δ_H 0.91 (3H, s, H_3-18), 0.98 (3H, s, H_3-19), 1.25 (3H, s, H_3-21), 1.39 (3H, s, H_3-26) and 1.33 (3H, s, H_3-27)], four carbinol methines at [δ_H 3.82 (1H, br d, J = 10.2 Hz, H-1), 3.87 (1H, ddd, J = 11.5, 4.0, 3.0 Hz, H-2), 3.97 (1H, br q, J = 2.5 Hz, H-3) and 3.61 (1H, br d, J = 10.5 Hz, H-22)], one enone proton [δ_H 5.83 (1H, br s, H-7)] and two protons of an exomethylene olefinic group [δ_H 5.15 and 4.97 (each 1H, s, H_2-28)], suggesting its 20-hydroxy ecdysone like structure with an exomethylene group [6]. The ^{13}C/DEPT-NMR data in CD_3OD recorded 28 carbon signals (Table 1), including 5 methyl, 7 methylene, 8 methine and 8 quaternary carbons. The ^{1}H and ^{13}C-NMR data were very similar to that of 1β-hydroxymakisterone C (**1a**) except C-24 and C-28 positions [7]. FAB-MS of the compound recorded mass ions at *m/z* 491, 473 and 455 resulting from the loss of one, two and three molecules of water from the mass ion at *m/z* 509 $[M+H]^+$ ion. The mass spectrum also recorded mass ions at *m/z* 379, 361 and 343, the formation of which could be rationalized by the fission of C-20/22 bond in the steroid side chain and followed by the loss of one and two molecules of water, characteristic of 20-hydroxy ecdysone like structure with 1β-OH group. In HMBC correlations the observed cross peaks at H-28 (δ_H 5.15 and 4.97) with C-24 (δ_C 155.3), C-25 (δ_C 73.6) and C-23 (δ_C 34.6) confirm the presence of an exomethylene group at C-24 position (Fig. 2). The H_α-9/H_α-1, H_α-9/H_α-2 and Me-19/ H_β-5 correlations in the NEO spectra of compound 1 established a cis-type junction of ring A and B. The OH-1β configuration was established from a medium NOE observed between H-1 and H-9 and the strong NOE between H-1 and H-11. Therefore, on the basis of the above evidence, the structure of glutinosterone was estabilished as 1β-hydroxy 24(28)-dehydromakisterone A.

3.2. Biochemical assay of Compound 1

In vitro biochemical analysis of compound **1** exhibited a significant inhibition in activities of four human serum enzymes, i.e. SGOT, SGPT, ALP and glycerol kinase in a dose dependent manner [8, 9, 10] (Fig. 3 and Table 2). At the concentration of 10 µg/mL, it reduced the levels of SGOT, SGPT, ALP and glycerol kinase to 9.0 U/mL, 4.2 U/mL, 8.0 U/mL and 107.2 U/mL in comparison to the control (without drug treatment) of 30 U/mL, 25 U/mL, 9 U/mL and 140 U/mL respectively wherein at the same concentration the standard drug, atorvastatin reduced the levels to 25.5 U/mL, 21.2 U/mL, 8.1 U/mL and 123.6 U/mL. Moreover, at the concentration of 25 µg/mL, compound **1** inhibited the activity of SGOT above 55% and at the concentration of 10 µg/mL it inhibited the activity of SGPT above 80%. Besides, at the concentration of 15 µg/mL, it reduced the activity of ALP above 30%.



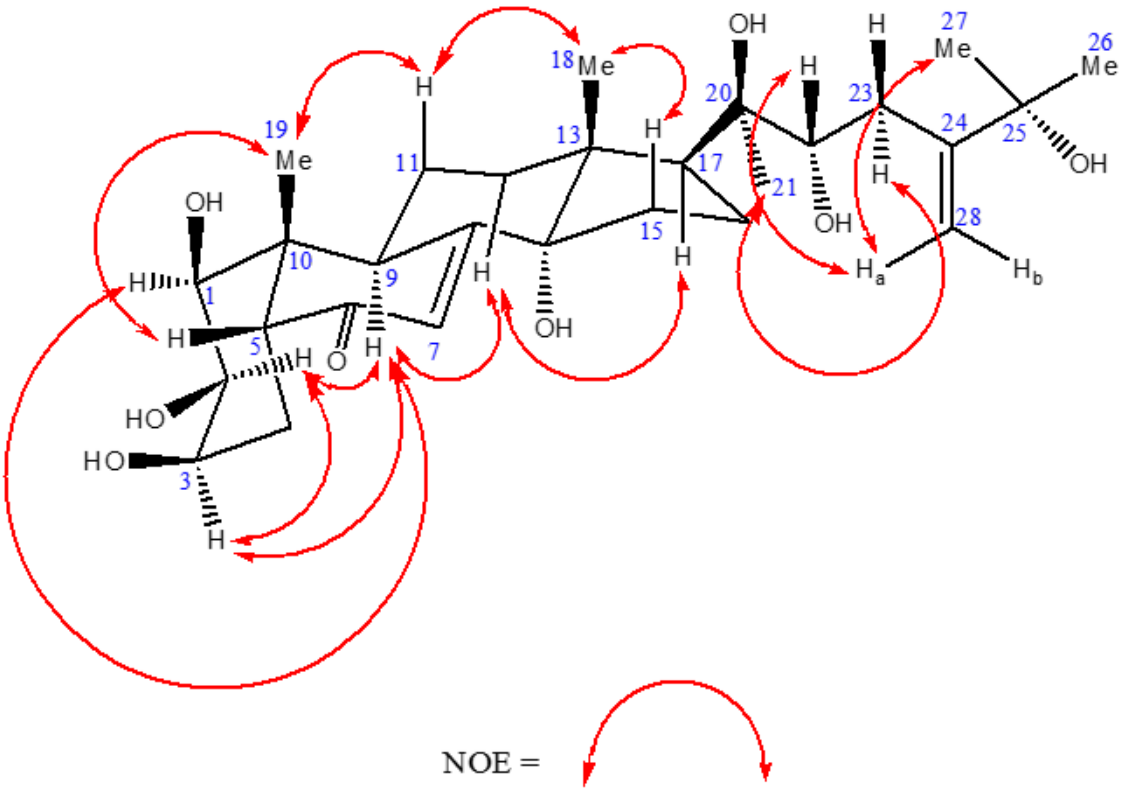


Fig 2: Significant HMBC and NOE correlations of compounds 1

In continuation with the aforesaid facts, the compound **1** also showed maximum inhibition of the activity of glycerol kinase by about 30% at the concentration of 15 µg/mL (Fig. 3). Enzymological profile of blood is commonly used as an indicator to determine the functional status of health [11]. Modulations in activities of liver enzymes like SGOT, SGPT, and ALP suggest liver dysfunction in the experimental animal model. It was found that the elevation in the liver enzymes and their activity are associated with inflammation or damage to the cells in the liver. This study exhibited decreases in the activity of serum enzymes ALP, SGOT, and SGPT as well as glycerol kinase in serum (Table 2). Since, SGOT, SGPT and ALP are the diagnostic markers of liver damage, reduction in their activity in the presence of compound **1** suggested that it might reduce the hepatotoxicity.

3.3. Antimicrobial assay of Compound 1

Towards exploring the antimicrobial activity of this isolated compound, two different bacterial species like *Escherichia coli* (Gram negative) and *Bacillus subtilis* (Gram positive) were selected and the experiment was carried out by disc diffusion method using the Kirby-Bauer technique [12] (Fig. 4 and Table 3). The compound **1** shows high antibacterial sensitivity ≥ 255 µg as compared to the standard drug, Gentamicin (Table 4). Antimicrobial properties of active constituents of medicinal plants are being increasingly reported from different parts of the world. As per the reports from the World Health Organization the plant extract and their active constituents are used in traditional therapies as folk medicine in almost 80% of the world's population [13]. In the present work, the compound **1** shows moderated to significant activity against the tested bacterial strains. The results were compared with standard antibiotic drug Gentamicin.

Table 1: ^{1}H (300 MHz) and ^{13}C-NMR (75 MHz) Data of Glutinosterone (**1**) in CD_3OD

No	$^{1}H^{a}$	$^{13}C^{b}$
1	3.82 br d (10.2)	76.7 (d)
2	3.87 ddd (11.5, 4.0, 3.0)	68.7 (d)
3	3.97 br q (2.5)	71.0 (d)
4	1.78 dd (15.6,12.9), 1.64 dd (11.1,7.5)	32.9 (t)
5	2.60 br d (13.5)	46.8 (d)
6	—	206.0 (s)
7	5.83 br s	122.2 (d)
8	—	167.5 (d)
9	3.17 dd	35.5 (d)
10	—	43.6 (s)
11	1.64 dd (11.1,7.5)	21.5 (t)
12	2.10 dd (11.4,11.4)	32.9 (t)
13	—	48.7 (s)
14	—	85.1 (s)
15	1.80 m	31.8 (t)
16	2.02 m	21.5 (t)
17	2.40 dd (9.5,8.5)	50.5 (d)
18	0.91 s	18.1 (q)
19	0.98 s	20.1 (q)
20	—	77.9 (s)
21	1.25 s	21.0 (q)
22	3.61 brd (10.5)	77.5 (d)
23	2.39 br d (13.5), 2.14 dd (13.5,8.5)	34.5 (t)
24	—	155.3 (s)
25	—	73.6 (s)
26	1.39 s	32.0 (t)
27	1.34 s	29.2 (q)
28	5.15 (a)br s , 4.97 (b) br s	110.4 (t)

Table 2: Enzyme activity study with compound **1**.

Tested Compound-1	SGOT level (U/mL)	SGPT Level (U/mL)	ALP Level (KA/mL)	Glycerol kinase (mg/dL)
Control (Serum without drug treatment)	30±2.32	25±3.06	9±1.8	140±11.7
Standard (Serum treatment with Atorvastatin*, 10 µg/mL)	25.5±3.41	21.2±4.9	8.1±0.74	123.6±17.5
Compound-1, 5 µg/mL	14.6±3.26	5.1±0.76	9±0.44	123.3±9.28
Compound-1, 10 µg/mL	9.2±0.96	4.6±1.06	8.2±1.08	109.4±10.8
Compound-1, 15 µg/mL	7.7±1.06	5.7±0.51	6.2±0.84	98.6±8.72
Compound-1, 20 µg/mL	14.6±2.8	6.3±1.2	8.3±0.87	100.4±16.7
Compound-1, 25 µg/mL	12.3±1.6	6±0.86	8.5±0.79	100±5.3

*Trade name, Storvas-10 by Ranbaxy, India.

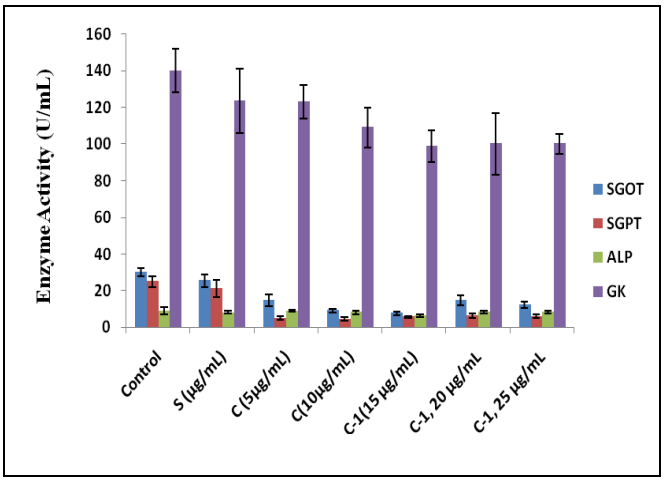


Fig 3: Enzyme activity study with compound **1.** Data represented as SD.

Table 3: MIC of Compound **1** & Compound **2**.

Name of drug Test compounds	*E. coli*	*S. aureus*
Compound **1**	105±17.48 µg/ml	140± 13.7 µg/ml
Gentamicin (Standard Drug)	125± 9.72 µg/ml	125± 16.3µg/ml

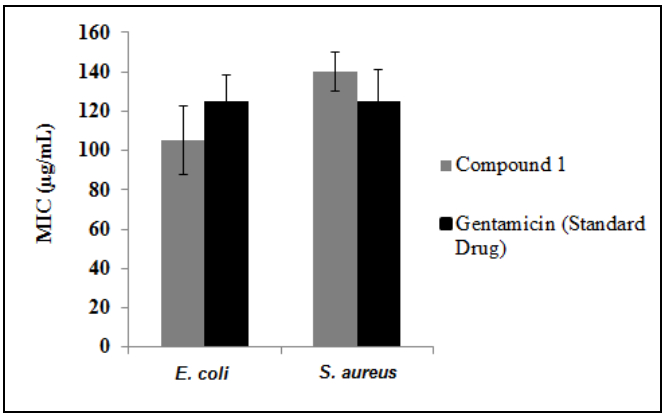


Fig 4: MIC of compound **1** against *E. coli* and *S. aureus.*

Table 4: Anti-bacterial susceptibility test.

Name of drug (10 µg/ µL)	Resistant		Highly Sensitive	
	E.coli	S. aureus	E.coli	S. aureus
Compound **1**	≤200 µg	≤240 µg	≥ 205 µg	≥ 255 µg

4. Conclusion

In the present investigation, the Compound **1** isolated from the methanolic extract of aerial parts of *S. glutinosa* shown to possess inhibitory effect on enzymes like SGOT, SGPT and ALP, which are known as a marker for hepatotoxicity. Compound **1** also shown to inhibit the activity of glycerol kinase, which is an enzyme involved in lipid metabolism. The compound **1** was found to be active on both gram positive and gram negative microorganism as compare with standard drugs. However, further studies are needed to characterize the possible mechanism of bioactivity shown by compound **1**. To this end, it can be suggested that Glutinosterone (**1**) may be an effective drug for the treatment of human hepatic disorders and also have moderate to significant anti-bacterial property.

5. Acknowledgments

The authors are thankful to IICB, Kolkata for NMR and FAB-MS spectral facility, Mr. Manash Chandra Das, Department of Medical Lab Technology, Women's Polytechnic, Hapania, West Tripura for providing laboratory facility to study bio-activity and Prof. B. K. Datta, Department of Botany, Tripura University and Joint Director, National Herbarium, Shibpur, Howrah for authentication of the plant.

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