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Research Article

# Enzyme Inhibitor & Antimicrobial Phytochemicals from Aerial Parts of *Sida glutinosa*

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**ABSTRACT**

Glutinoside (compound **1**) & 24(28)-dehydromakisterone A (compound **2**) were isolated from an annual herb called *Sida glutinosa* Roxb. syn *S. mysorensis* Wight and Arn. (Malvaceae) through phytochemical investigation which were later on characterised and structural elucidation were done through NMR, MS & other spectroscopic methods. Biological activity (*In vitro*) of these compounds were studied for identifying their role on enzymes that generally used as marker for hepatic function like aminotransferases & alkaline phosphatases, lipid metabolic enzyme activity and antibacterial function. In all enzyme expression profile study optimum concentration of these isolated molecules on enzyme were found out along with the pattern of enzyme kinetics through statistical tools. *In vitro* biochemical analysis shows significant reduction in enzyme expression in their optimum inhibitory concentration, both compound **1** & **2** also shows antibacterial property both against positive & negative bacteria as compared with standard drug Gentamicin.

**Keywords:** Glutinoside; 24(28)-dehydromakisterone A; aminotransferases (AST, ALT); non-competitive inhibition

## INTRODUCTION

*Sida glutinosa* Roxb. syn *S. mysorensis* Wight and Arn. (Malvaceae) is an annual herb mostly distributed in waste places of south and eastern India, Burma to southeast Asia. In India, the roots and aerial parts of this plant and its sister species are used in traditional medicine for the treatment of pulmonary tuberculosis and rheumatism [1, 2]. A literature survey indicated that no phytochemical work as well as biological activity has been reported on this plant. We have reported earlier the isolation and characterization of eight compounds [3] and *in vitro* antioxidant activity of isolated three compounds from this plant [4]. The present paper deals with the studies of biological activity of Glutinoside (1) and 24(28)-Dehydromakisterone A (2) for identifying role on enzymes that generally used as marker for hepatic function like aminotransferases and alkaline phosphatises, lipid metabolic enzyme activity and also antibacterial function. Liver performs numerous metabolic, secretary, excretory, storage and detoxifying functions. Liver posses enormous functional reserves and regenerative capacity. Activity of hepatic enzymes in serum may be affected by their increased or decreased synthesis, release from damaged cells, extrahepatic tissue and their disappearance rates from plasma. These enzymes include (i) aminotransferases i.e. Aspartate Aminotransferases (AST) or Serum Glutamic-oxaloacetic Transaminase (SGOT) and Alanine Aminotransferases (ALT) or Serum Glutamic-pyruvic Transaminase (SGPT), (ii) Alkaline Phosphatase (ALP) as very important marker enzyme. Aminotransferases are a group of enzyme that catalyse the transfer of an amino group from a $\alpha$–amino acid to an $\alpha$–oxo acid which is a very important step in metabolism of amino acids. Glycerol kinase is an enzyme that in combination with lipoprotein lipase and glycerol-3-phosphate oxidase act as central molecule in metabolism of fatty acid ester called triglyceride. The isolated compound 1 & 2 were checked for their efficacy in controlling particular enzyme activity either by activating or by inhibiting enzyme kinetics. These isolated molecules were also used to find their antibacterial property against gram positive and negative bacterial strains.

## MATERIALS AND METHODS

General**:** Melting points were determined on a kofler type melting point apparatus and are uncorrected. Optical rotation was measured on a JASCO D/P-1400 digital polarimeter. UV spectrums were recorded in MeOH on a Perkin Elmer Lambda 25 spectrophotometer and IR spectra in KBr disk on a Shimadzu 8100 FT-IR spectrophotometer. $^{1}H$ and $^{13}C$-NMR spectra were measured on a Varian 300 and 600 spectrometers. Chemical shifts were expressed in $\delta$ values (ppm) with tetramethylsilane (TMS) as an internal standard and coupling constants were in hertz (Hz). EI- and FAB-MS were taken using a JEOL JMS 700 mass spectrometer. For column chromatography (CC), silica gel (mesh 60-120, Merck), Diaion HP-20 (Mitsubishi-Chemical, Tokyo, Japan) and basic alumina ($Al_2O_3$) (Merck) were

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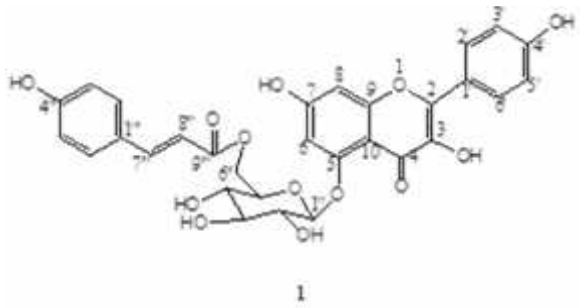

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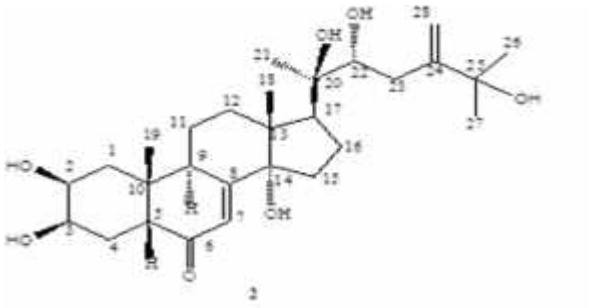

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Table1: Enzyme activity study with compound 1.

| 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 | 25 | 9 | 140 |
| Standard (Serum treatment with Atorvastatin, 10 µg/mL) | 25.5 | 21.2 | 8.1 | 123.6 |
| Compound-1, 5 µg/mL | 7.6 | 2.1 | 6.6 | 154.17 |
| Compound-1, 10 µg/mL | 7.3 | 2.4 | 6.6 | 154.17 |
| Compound-1, 15 µg/mL | 7.6 | 2.1 | 7.0 | 154.17 |
| Compound-1, 20 µg/mL | 7.1 | 2.9 | 7.2 | 100.00 |
| Compound-1, 25 µg/mL | 6.9 | 3.2 | 7.1 | 29.17 |

Table 2: Enzyme activity study with compound 2.

| Tested Compound-2 | SGOT level (U/mL) | SGPT Level (U/mL) | ALP Level (KA/mL) | Glycerol kinase (mg/dL) |
|---|---|---|---|---|
| Control (Serum without drug treatment) | 30 | 25 | 9 | 140 |
| Standard (Serum treatment with Atorvastatin, 10µg/mL) | 25.5 | 21.2 | 8.1 | 123.6 |
| Compound-2, 5 µg/mL | 14.6 | 5.1 | 9 | 123.3 |
| Compound-2, 10 µg/mL | 9.2 | 4.6 | 8.2 | 109.4 |
| Compound-2, 15 µg/mL | 7.7 | 5.7 | 6.2 | 98.6 |
| Compound-2, 20 µg/mL | 14.6 | 6.3 | 8.3 | 100.4 |
| Compound-2, 25 µg/mL | 12.3 | 6 | 8.5 | 100 |

Table 3: Optimum enzyme inhibitory concentration of compound 1 & 2.

| Inhibition with drug | Optimum enzyme inhibitory conc$^n$ of drug SGOT level (U/mL) | SGPT Level (U/mL) | ALP Level (KA/mL) | Glycerol kinase (mg/dL) |
|---|---|---|---|---|
| Compound 1 (µg/mL) | 25 | 15 | 10 | 25 |
| Compound 2 (µg/mL) | 15 | 10 | 15 | 15 |

used. Silica gel G (Merck) was used for thin layer chromatography (TLC). The spots were visualized either by spraying mixture of acetic anhydride and conc. $H_2SO_4$ or in iodine chamber.

Plant material: The whole aerial parts of *Sida glutinosa* were collected from Kalsi (Jolaibari), South Tripura in March 2008 and identified by Prof. B. K. Datta, taxonomist, Department of Botany, Tripura University. A Voucher specimen (#BD/01/08) has been deposited in the National Herbarium, Botanical Survey of India, Botanical Garden, Howrah 711 103.

Extraction and isolation: Fresh air-dried aerial parts of *S. glutinosa* were dried in shaded floor and crushed into coarse powder. Dried coarse powders (3.3 kg) were extracted with MeOH (6L × 3) at room temperature for 1 week. The MeOH extract was concentrated under reduced pressure in *vacuo* to a gummy mass (106 g). It was divided into two parts. One parts of extract (70 g) was suspended in water (100 mL) and extracted three times with $CH_2Cl_2$, $CHCl_3$ and *n*-BuOH (A) (each 200 mL), successively. Another part of extract (30 g) was churned with 5% aq. citric acid (250 mL) for 6 h and filtered. The filtrate was basified with dilute $NH_4OH$ (~2 N) and extracted three times with $CH_2Cl_2$, $CHCl_3$ and *n*-BuOH (B) (each 100 mL), successively. The *n*-BuOH fraction (A) (8.2 g) of the crude extract was column chromatographed through Diaion HP-20 and eluted with MeOH gave a residue (3.5 g), which was divided into two parts. One part (2.0 g) on repeatedly column chromatographed through silica gel gave glutinoside (1,

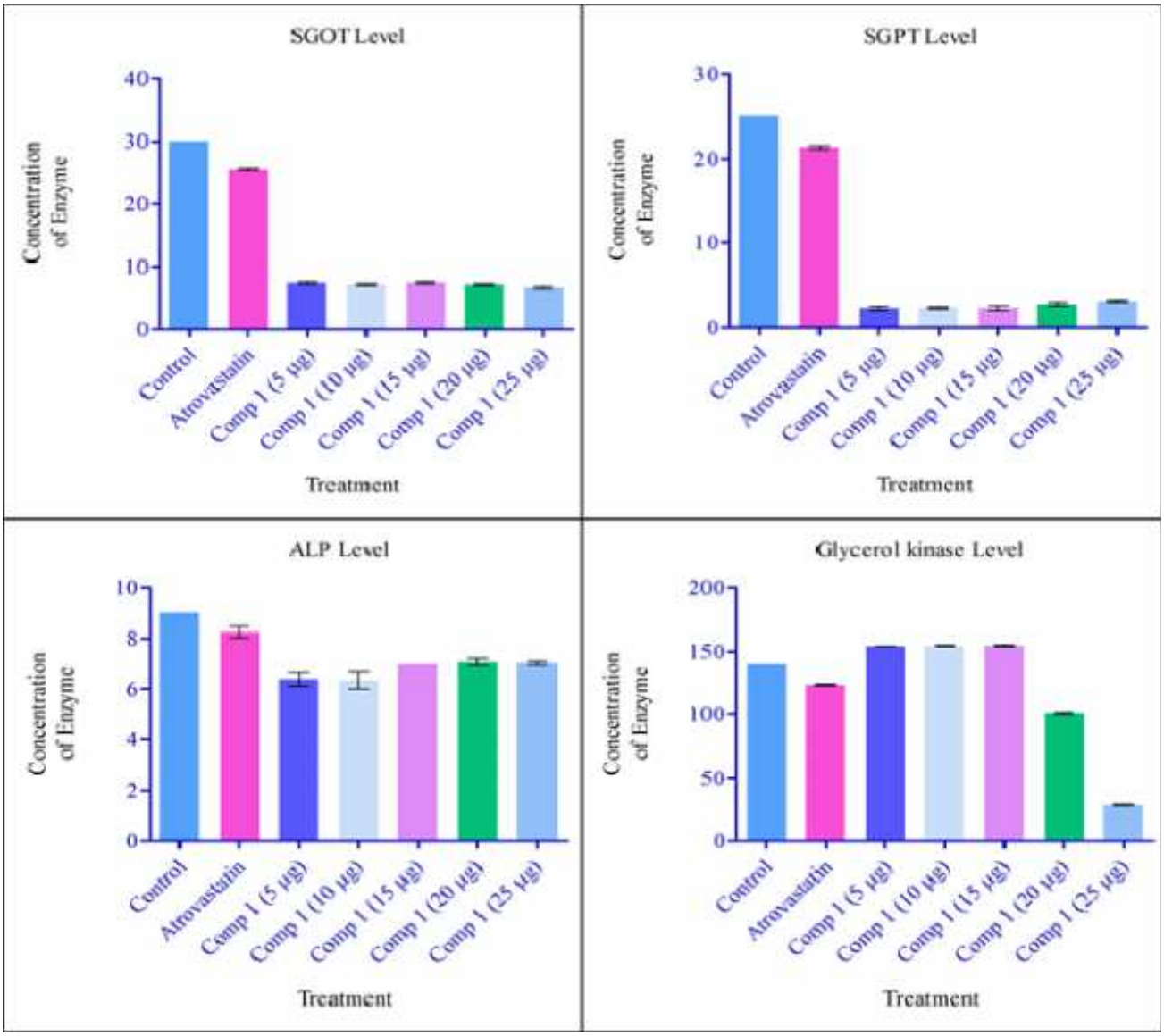


*Figure 2. Study of enzyme inhibition profile of compound 1 using SD/SEM*

35 mg). Another part of MeOH eluted residue (1.2 g) was successively column chromatographed through silica gel and basic $Al_2O_3$. The fraction eluted from $Al_2O_3$ column with EtOAc-MeOH (90:10) afforded a residue, which on repeated crystallization from $CHCl_3$-MeOH gave 24(28)-dehydromakisterone A (2, 45 mg).

*2.3.1 Glutinoside (1)*

Yellow crystals, mp 225°C, $[\alpha]_D^{24}$ -19.5 (C = 0.32 MeOH). UV (MeOH) $\lambda_{max}$ nm (log$\epsilon$) : 255 (4.08), 322 sh (3.68), 372 (4.18). IR (KBr) $\upsilon_{max}$ $cm^{-1}$: 3460, 3252, 1684, 1655, 1628, 1607, 1589, 1501, 1360, 1294, 1182, 1067, 827. HR-FAB-MS (positive-ion mode) *m/z* (%): 617.1261 $[M+Na]^+$ (Calcd for $C_{30}H_{26}O_{13}Na$ : 617.1266). FAB-MS *m/z* (%): 617 $[M+Na]^+$ (38), 595 $[M+H]^+$ (13), 287 $[aglycone+H]^+$ (100), 286 $[aglycone]^+$ (72), 259 (16), 165 (28), 147 (75), 107 (50), 77 (59), 65 (22).

*2.3.2 24(28)-Dehydromakisterone A (2)*

Light yellow crystals, mp 220°C (dec). UV (MeOH) $\lambda_{max}$ nm (log$\epsilon$) : 248 (3.61). IR (KBr) $\upsilon_{max}$ $cm^{-1}$: 3389, 1642, 1464, 1445, 1381, 1150, 1059, 874. HR-FAB-MS (positive-ion mode) *m/z* (%) : 515.2998 $[M+Na]^+$ (Calcd for $C_{28}H_{44}O_7Na$ : 515.2984). FAB-MS (positive-ion mode) *m/z* (%) : 515 $[M+Na]^+$ (60), 493 $[M+H]^+$ (53), 475 (60), 457 (100), 439 (40), 363 (33), 345 (35), 327 (13), 301 (33), 191 (27), 173 (47), 165 (47), 147 (33), 129 (40).

Serum preparation for determination of enzyme inhibition: EDTA anticoagulated blood sample were collected from clinical biochemistry laboratory which was mixed with different concentration of compound 1 & 2 separately taking 2 mL aliquot in different test tubes keeping one tube as control without drug treatment and one tube as standard. After gentle mixing of these molecules with blood all perforated blood culture tubes were incubated in $37^0$C for 4-6 hrs maintaining sterile condition. After that stipulated time period all tubes were centrifuged at 3000 rpm for 10 minute where plasma will separate as straw yellow coloured fluid above cell palette. Use that plasma immediately without delay.

Determination of serum hepatic enzyme activity: Allow L-aspertate and $\alpha$-ketoglutarate to react with SGOT of different concentration drug treated sample along with a

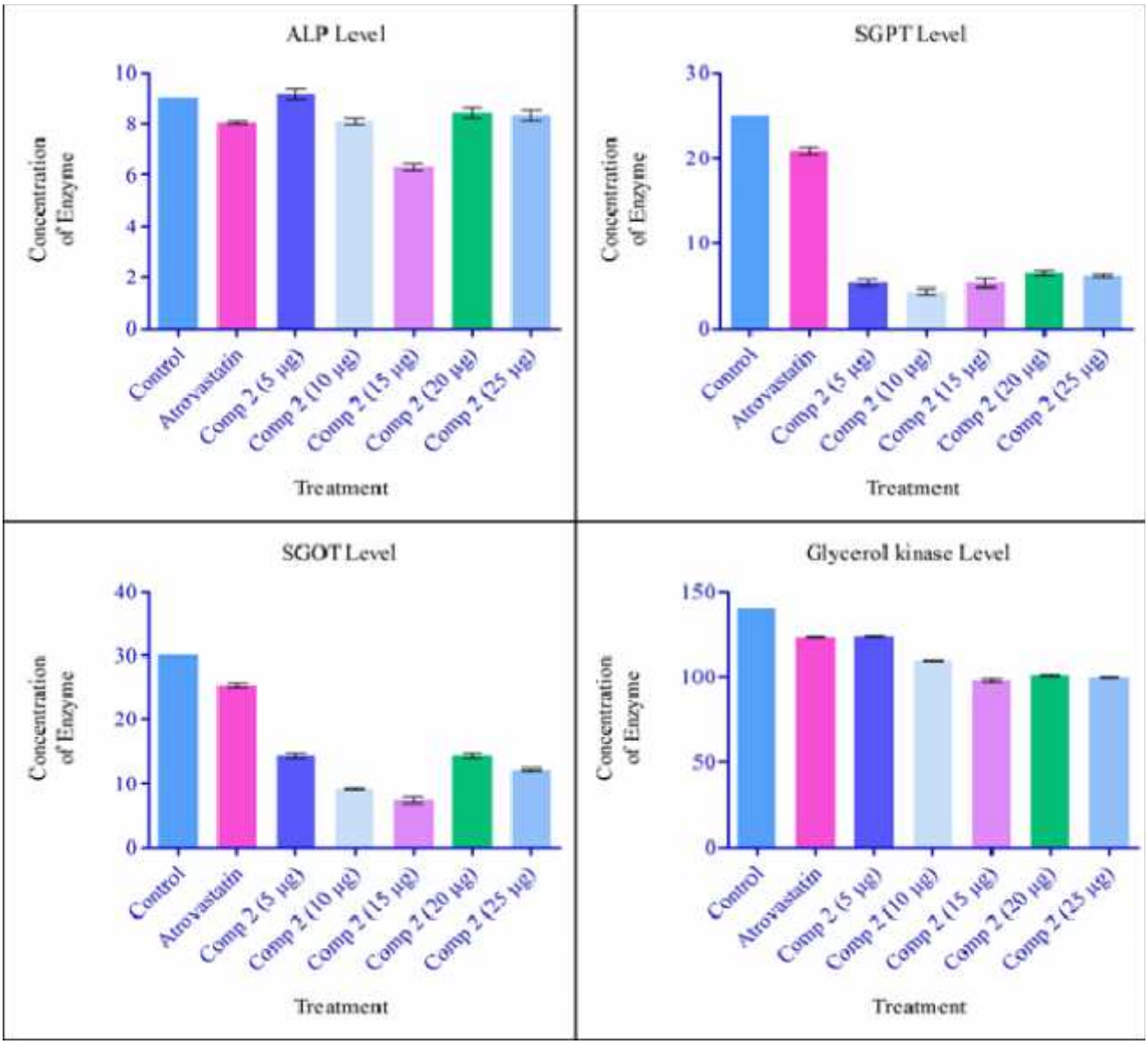


*Figure 3. Study of enzyme inhibition profile of compound **2** using SD/SEM*

Effect on enzyme kinetics

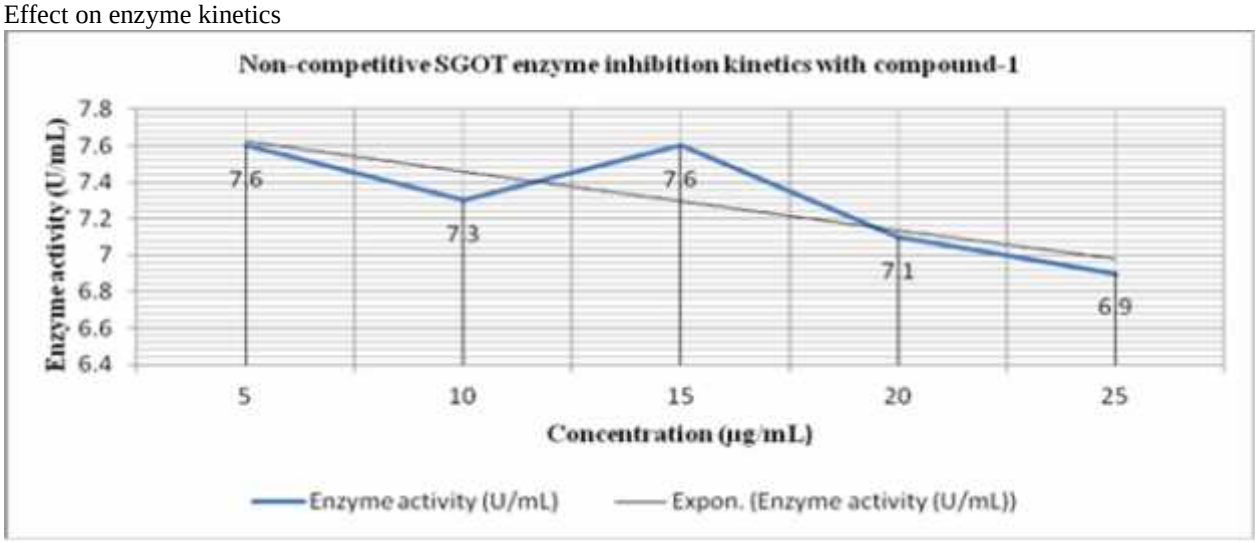


*Figure 4. Non-competitive SGOT enzyme inhibition kinetics with compound 1.*

control & standard (Atorvastatin) at pH 7.4 & $37^0$C for 80 min, oxaloacetate will form. React it with 2,4-DNPH in NaOH alkaline medium at R.T. for 10 min. Measure the Abs at 505 nm (green filter), compare test result with that of control & standard [5,6]. Allow L-alanine and α-ketoglutarate to react with SGPT with different concentration drug treated sample along with a control & standard (Atorvastatin) at pH 7.4 & $37^0$C for 50 min, pyruvate will form. React it with 2,4-DNPH in NaOH alkaline medium at R.T. for 10 min. Measure the Abs at 505 nm (green filter), compare test result with that of control & standard [5,6]. Allow phenyl phosphate to react with alkaline phosphatase at pH 10 & $37^0$C for 18 min, phenol will form. React it with aminoantipyrine with different concentration drug treated sample along with a control & standard (Atorvastatin) at R.T. for 10 min. Measure the Abs at 520 nm (green filter), compare test result with that of control & standard [5, 6].

Determination of serum lipid metabolic enzyme inhibition: Allow triglyceride to react with lipoprotein lipase & glycerol kinase, where glycerol -3-phosphate will form. React it with glycerol-3-phosphate oxidase with different concentration drug treated sample along with a control & standard (Atorvastatin) at R.T. for 15 min. Measure the Abs at 505 nm (green filter), compare test result with that of control & standard [5, 6, 7].

Statistical analysis: The values were expressed as Mean ± SD. Statistical analysis was performed by one way analysis of variance (ANOVA) followed by Student's 't' test significance are mentioned as * $P \leq 0.05$. For identification of enzyme kinetics inhibitory Vmax was determined supported by an exponential trendline. Data are analyzed in Graph Pad Prism 5.0 (trial version).

Determination of minimum inhibitory concentration (MIC): MIC was determined by addition of serial diluted drugs in different tubes containing fixed bacterial cell count (CFU) in fixed volume of medium, incubate them for 48 hrs in $37^0$C, take OD at 600 nm, identify the minimum concentration of drug that inhibit bacterial growth [8].

Antimicrobial susceptibility test: Antimicrobial susceptibility test on the isolated organism (Escherichia coli and Bacillus subtilis) was done by disc diffusion method using the Kirby-Bauer technique [9]. M-H agar plates were prepared, surface was lightly and uniformly inoculated by cotton swab. Prior to inoculation, the swab stick was dipped into bacterial suspension having turbidity 0.5 OD. The swab stick was then took out and squeezed on the wall of the test tube to discard extra suspension. Inoculated plates were incubated at $37^0$C for 24 hours. On the next day disc were placed on agar surface soaked in different concentration of drug above MIC along with solvent and a standard drug i.e. Gentamicin, plates were then incubated at $37^0$C for 48 hours, plates were read by taking measurement of zone of inhibition. Results were recorded and compared with respect to Gentamicin [10].

## RESULTS AND DISCUSSION

*In vitro* biochemical analysis shows non-competitive enzyme inhibitory kinetics profile as compound 1 is almost 4 times, 10 times & 1.3 times more effective blocker of SGOT, SGPT & ALP respectively whereas compound 2 is almost 2.8 times, 4.9 times more effective blocker of SGOT, SGPT, compound 1 can also strongly inhibit glycerol kinase (almost 90% ) at 25 µg/mL concentration. Both the compound shows high antibacterial sensitivity ≥ 200 µg concentration with maximum ≥275 µg of compound 1 against positive bacteria as compared with standard drug Gentamicin.

The results obtained from in vitro hepatic enzyme, lipid metabolic enzyme and anti-bactrial activity screening of compound 1 & compound 2 are presented in Table 1, Table 2, Table 3, Table 4 and Table 5.

Effect of compound 1 & 2 on hepatic enzyme & lipid metabolic enzyme activity

Effect on bacterial growth: Thorough examination of control serum sample for presence of aminotransferases i.e. SGOT, SGPT and alkaline phosphatase as marker of hepatic function we found that the level of SGOT 25.5 U/mL, SGPT 21.2 U/mL & ALP 8.1 KA/mL. This observation follows treatment with compound 1 & 2 taking atorvastatin as standard HMG-coA reductase inhibitor. Upon treatment with compound 1 & 2 it was observed that there are dose dependent decrease in the enzymes expression profile from where optimum concentration of compound 1 & 2 were found out as a measure of concentration of these molecules to control these enzyme expression & secretion from hepatic cells. The determined optimum inhibitory concentrations are tabulated in Table 3 which shows compound 2 is approx. 40% more potent inhibitor of aminotransferases while for alkaline phosphatase compound 1 is effective than compound 2. In conclusion we can interfere that compound 1 is almost 4 times, 10 times & 1.3 times more effective blocker of SGOT, SGPT & ALP respectively whereas compound 2 is almost 2.8 times, 4.9 times more effective blocker of SGOT, SGPT respectively in comparison with atorvastatin in equal concentration of drug and compound 1 & 2. In the same way both these compounds were analysed for their effect on lipid metabolic enzyme glycerol kinase where it was observed that compound 1 & 2 can inhibit the enzyme in higher concentration only. Compound 1 can almost 90% more strongly inhibit the enzyme at 25 µg/mL concentration whereas compound 2 can inhibit approx 20% at 15 µg/mL concentration which shows compound 1 is a strong inhibitor of enzyme glycerol kinase.

Table 4: MIC of Compound 1 & Compound 2.

| Name of drug (10 µg/ µL) | E.coli | S. aureus |
|---|---|---|
| Compound 1 | 110 µg | 160 µg |
| Compound 2 | 105 µg | 140 µg |
| Gentamicin (Standard Drug) | 125 µg | 125 µg |

Table 5: Anti-bacterial susceptibility test.

| Name of drug (10 µg/ µL) | Resistant | | Highly Sensitive | |
|---|---|---|---|---|
| | E.coli | S. aureus | E.coli | S. aureus |
| Compound 1 | ≤110 µg | ≤260 µg | ≥225 µg | ≥275 µg |
| Compound 2 | ≤205 µg | ≤240 µg | ≥ 200 µg | ≥ 255 µg |

From figure- 4, it was found that the exponential trend line follow sharp decline pattern along with increased inhibitory molecule i.e. compound 1 which shows increased inhibitory $V_{max}$. Thus we can strongly infer that the enzyme SGOT in presence of compound 1 shows non-competitive enzyme inhibition kinetics which is a type of reversible inhibition i.e. the compound 1 is not a potent toxic drug. As enzyme activity of SGOT, SGPT, ALP and glycerol kinase with both the drug shows same exponential trend line thus we also can infer that all 4 enzymes with both compound 1 & 2 follow non-competitive enzyme inhibitory kinetics.

From figure-2 it was observed that the compound 1 at concentration 25 µg/mL shows optimum enzyme activity ie. 6.9 U/mL whereas compound 2 (figure-3) at concentration 15 µg/mL shows optimum enzyme activity i.e. 7.7 U/mL on SGOT and this inhibition profile is indicated by error with standard deviation bar. As all 4 enzymes with both compound 1 & 2 follow non-competitive enzyme inhibitory kinetics, thus same statistical approach can be used for determination of optimum concentration of compound 1 & 2 for SGPT, ALP and glycerol kinase as well. Thus optimum concentration of other enzymes with respect to both the drug were determined and tabulated in table 3 which shows compound 2 is a more potent drug for hepatic and lipid profile function.

From antibacterial susceptibility study (table-5) it can be inferred that both compound 1 & 2 shows moderate antibacterial activity, where compound 2 offer more antibacterial then compound 1 as compared with established drug Gentamicin against negative bacteria whereas against positive bacteria both the drug shows less activity then standard drug. Both the compound shows high sensitivity ≥ 200 µg concentration with maximum ≥275 µg of compound 1 against positive bacteria.

## CONCLUSION

At the end we can conclude that both compound 1 & 2 has significant effect on liver function marker enzymes along with lipid metabolic enzymes where both compound 1 & 2 are not toxic as they shows increased inhibitory $V_{max}$ & exert non-competitive enzyme inhibitory kinetics. Compound 1 & 2 also have moderate to significant anti-bacterial property as well. Thus compound 1 & 2 (with more emphasis on compound 2 as more potent drug) may be suggested for clinical trial as drug of choice in pathological conditions like improper liver function & lipid metabolism and also as antibacterial agent. Finally we can say that the traditional use of the plant by the local people is justified.

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Conflict of interest: The authors have declared that there is no conflict of interest.

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