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Biological evaluation of constituents from *Grewia mollis*

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ABSATRACT

*Crude methanolic extract of G.mollis was found to have significant antimicrobial activity, at 250 µg/ml concentration (for both antibacterial and antifungal activity), anti-inflammatory activity, at 500 mg/kg concentration and significant decrease in arterial blood pressure and heart rate in both butanol extract at 3 mg/kg concentration and methanolic extract at 20mg/kg concentration. Bioassay guided fractionation led to the isolation of two known flavonoids compounds, luteloin(**1**), 7-(1-O-β-D-galacturonide)-4'-(1-O- β-glucopyranosyl)-3'4',5,7-tetrahydroxyflavone(**2**), two tripenoids, 7β-hydroxy-23-ene-deoxojessic acid(**3**), 7β-hydroxy-23- deoxojessic acid(**4**) and two steroids from the aerial part of G. mollis.*

Key words: *Grewia mollis*, Malvaceae, flavonoids, tripterenoids, steroids, antimicrobial activity.

INTRODUCTION

Grewia mollis is a shrub or small tree up to 20 ft tall, of Malvaceae family (previously belonging to Tiliacaea family) widely distributed within the Northern, middle belt of Nigeria some African countries. It is known to be a strong fireresistant.Various parts of the plant are used in food and medicine. In Nigeria, the stem bark powder or mucilage is used as a thickener in local cakes made from beans or corn flour commonly called "Kosai" and "Punkasau" in Hausa (Nigeria), respectively. The dried stem bark is ground and the powder mixed with beans or corn flour thereby enhancing the texture of the food product [1].

The flowers and young shoots are sometimes used as a soup or sauce vegetable. The infusion of the bark obtained by cold or hot maceration in water is used in beating mud floors, or mixed with the mud or the walls of huts to give a smooth surface. The mucilaginous property of the bark or leaf is used in application to cuts and sores. The Yoruba in Nigeria use it medicinally at times of child birth [1]. Some findings demonstrated that the mucilage obtained from the stem bark can serve as a good binder in paracetamol formulations [2-3]. Also the recent reports suggest that high concentration of stem bark in dietary exposure may cause some adverse effects, especially liver injury [4]. Phytochemical studies of *G. mollis* indicated the presence of tannins, saponins, flavonoids, glycosides, phenols, steroids and the absence of alkaloids in the leaves and stem bark [5] while their presence was revealed in the roots [6].

Previous phytochemical studies on *Grewia mollis* have been very rare and led to isolation of a few alkaloids. Moreover, very primitive biological studies of *Grewia mollis* have been carried up to date.

In the present phytochemical investigation biologically active methanolic extract resulted in the isolation of luteloin (**1**), 7-(1-O-β-D-galacturonide)- 4´-(1-O- β-glucopyranosyl)-3´4´,5,7-tetrahydroxyflavone (**2**), 7β-hydroxy-23-ene-deoxojessic acid (**3**), 7β-hydroxy-23- deoxojessic acid (**4**), β-sitosterol (**5**) and β-sitosterol-3-O-glucoside (**6**). This paper describes the isolation and identification of the constituents of *Grewia mollis* and their antimicrobial activities.

EXPERIMENTAL SECTION

3.1.1 General experimental procedure

Melting points were determined with a Buchi melting point apparatus. (Model B-545) and are uncorrected. Infrared spectra were obtained on a Hitachi 270-30 spectrophotometer in KBr pellets. ^{1}HNMR (500 MHz) , ^{13}CNMR (125 MHz) and 2DNMR spectra were determined on a Bruker DPX-200 spectrometer in $CDCl_3$, CD_3OD and pyridine-d_5. FAB-MS was recorded on a JEOL SX 102/DA-6000 mass spectrometer. Optical rotation was obtained on a Perkin Elmer 241 polarimeter. Elemental analytical data was recorded on Carlo Erba, Model 1106, elemental analyzer. Column chromatography was carried out using SiO_2 gel (230-400 mesh). Sephadex LH-20 was used for column chromatography (Pharmacia, 25-100µm). All the solvents were routinely distilled prior to use. Other chemicals were commercial grade without purification. TLC were carried out on precoated Si Gel F254 plates (Merck, art. 15423) and visualised by spraying with 1% ceric ammonium sulphate in 30% aqueous H_2SO_4 followed by heating the plate at 105^{o}C for 15 minutes and borinate-PEG solution (2-aminoethyldiphenylborinate, 1% in methanol :polyethylene glycol 4000, 5%in ethanol,1:1, v/v) for flavonoids.

3.1.2 Plant material

The aerial part of *Grewia mollis* juss. were collected and air-dried in April 2010 at Akba-Al-Abar, Saudi Arabia. A voucher specimen (collection no.59) was deposited in the herbarium of College of Pharmacy, King Saud University.

3.1.3 Extraction and isolation

The dried and milled Arial part of *Grewia mollis* (3000 g) was macerated overnight using MeOH at the room temperature for three times. The extract was freed of solvent on a wiped film evaporator at 50 ± 5^{0}C to get MeOH extract residue (337 g).The resultant methanol extract was suspended in a mixture of methanol-water (9:1) solution and was then followed by successive solvent partitioning to give *n*-hexane (27 g), chloroform (68.6 g), ethyl acetate (56.3 g), *n*-butanol (10.9 g) and water soluble fractions. *n*-hexane soluble fraction was chromatographed on silica gel column (*n*-hexane-$CHCl_3$-EtOAc, 12:1:1) to give four fractions (H1-H4). Fraction H3 was purified by recolumn chromatography *(n*-hexane-$CHCl_3$-EtOAc, 10:1:1) to give **1** (10 mg). Silica gel column chromatography of the $CHCl_3$ soluble fraction with $CHCl_3$-EtOAC-MeOH (10:1:1) gives six fractions (C1-C6). The major fraction C1 was chromatographed on Silica gel using $CHCl_3$-EtOAc (40:1) to yield five fractions (C11-C15). Fraction C11 was purified with EtOAc-MeOH-H_2O (8:1:0.1) to give to give **2** (10mg).The major fraction C15 was passed through Sephadex LH-20 column and eluted with MeOH-H_2O (6:4) gave **3** (15 mg) and gradually increasing the percentage of H_2O in MeOH (4:6) gave **4** (8 mg). Silica gel column chromatography of the EtOAc soluble fraction with $CHCl_3$-EtOAc-MeOH (10:1:1) gave five fractions (E1-E5). The major fraction E2 rechromatographed on silica gel column with $CHCl_3$-MeOH (8:2) to yield **5** (10 mg) and with $CHCl_3$-MeOH (5:1) yielded **6** (7 mg).

Luteolin (1)

A yellow amorphous powder, m.p. 320-321^{o}C; FAB-MS: *m/z* 287 $[M+H]^+$;, calcd for $C_{15}H_{10}O_6$; C, 62.94; H, 3.52; O, 33.54; Found: C, 62.96; H, 3.55; O, 33.56;^{1}H-NMR (DMSO, 500 MHz): 7.40 (1H, dd, *J* = 8.0, 2.0 Hz, H-6'), 7.37 (1H, d, *J* = 2.0 Hz, H-2'), 6.87 (1H, d, *J* = 8.0 Hz, H-5'), 6.64 (1H, s, H-3), 6.42 (1H, d, *J* = 2.0 Hz, H-8), 6.16 (1H, d, *J* = 2.0 Hz, H-6); ^{13}C-NMR (DMSO-*d6*, 125 MHz) d: 164.58 (C-2), 103.44 (C-3), 182.28 (C-4), 162.14 (C-5), 99.58 (C-6), 165.07 (C-7), 94.58 (C-8), 158.00 (C-9), 104.26 (C-10), 121.99 (C-1'), 113.91 (C-2'), 146.48 (C-3'), 150.64 (C-4'), 116.69 (C-5'), 119.69 (C-6').

7-(1-O-β-D-galacturonide)-4'-(1-O- β-glucopyranosyl)-3'4',5,7-tetrahydroxyflavone(2)

A yellow amorphous powder, m.p.270-272^{o}C; FAB-MS: *m/z* 625.1$[M+H]^+$, m/z 449.1$[M+H]^+$ (-galacturonic acid), *m/z* 287$[M+H]^+$ -(galacturonic acid+glucose) calcd for $C_{27}H_{28}O_{17}$; C, 51.93; H, 4.52; O, 43.55; Found: C, 51.95; H, 4.53; O, 43.57; ^{1}HNMR (DMSO, 50 MHz). δ3.083.75 (m, 17H, sugar protons), 4.50 (d, 1H, *J* = 7.21Hz, H-1´´), 5.21(d,1H, *J* = 6.82 Hz, H-1´´), 6.42 (bs,1H, H-6),6.65(bs,1H,H-8), 6.81(d, 1H, *J* = 8.42, H-5´), 7.09 (s, 1H, H-3), 7.35(Q, 1H, *J* = 8.42 and 1.8 Hz, H-6´) 7.80 (bs, 1H, H-2´); ^{13}C-NMR (DMSO, 125 MHz), δ165.36 (C-2), 104.01 (C-3), 183.19 (C-4) 160.87 (C-5), 99.41 (C-6), 163.09 (C-7), 96.31 (C-8), 157.65 C-9), 106.50 (C-10), 122.36 (C-1´), 114.10(C-2´), 145.37 (C-3´), 148.74 (C-4´), 116.81 (C-5´), 120.75 (C-6´), 101.21 (C-1´´), 73.25', (C-2´´), 77.23 (C-3´´), 70.72 (C-4´´), 76.89 (C-5´´), 62.02 (C-6´´), 103.39 (C-1´´), 75.02 (C-2´´´ and C-4´´´), 77.71 (C-3´´´), 82.07 (C-5´´´), 176.44 (C-6´´´).

7β-hydroxy-23-ene-deoxojessic acid(3)

A yellow amorphous solid; FAB-MS *m/z* 489.9 $[M+H]^+$ (calcd. For C30H49O5 H^+, 489.69) C, 73.73; H, 9.90; O, 16.37; Found: C, 73.74; H, 9.92; O, 16.38; UV (MeOH) λmax (log ε) 210 nm; IR νmax 3649, 2591, 1962, 1542, 785 cm-1; ^{1}H NMR (50 MHz, CD_3OD-*d4*):H-1: 3.55, H-3: 4.52 (dd, *J* = 6.8, 10.4 Hz) , H-5: 2.76 (dd, *J* = 4.0, 14.0 Hz), H-7: 3.56, H-8: 1.29, H-17: 1.57, H3-18:1.61, H2-19: 0.45 (d, *J* = 4.4 Hz) 0.81 (d, *J* = 4.8 Hz), H-20: 1.40, H3-21: 0.92 (d, *J* = 6.4 Hz), H-24: 5.10 (t, *J* = 12.0 Hz), H3-26: 1.01 (s), H3-27: 1.05 (s), H3-29: 1.07 (s), H3-30: 1.68 (s);

^{13}C NMR (125 MHz CD_3OD-*d4*): 73.5(C-1),37.7 (C-2), 71.4 (C-3), 46.9(C-4), 37.5 (C-5), 29.7 (C-6), 70.6 (C-7), 25.3 (C-8), 26.1 (C-9), 29.2 (C-10), 27.2 (C-11), 33.9 (C-12), 56.0 (C-13), 30.9 (C-14), 37.9 (C-15), 38.4 (C-16), 53.0(C-17), 17.8 (C-18), 22.0 (C-19), 37.2 (C-20), 19.0 (C-21), 26.0 (C-22), 34.0 (C-23), 126.3 (C-24), 131.9 (C-25), 18.2 (C-26), 19.3 (C-27), 180.6 (C-28), 9.2 (C-29), 26.1 (C-30).

7β-hydroxy-23- deoxojessic acid(4)

A yellow amorphous solid; FAB-MS *m/z* 525.359 $[M+Na]^+$ (calcd. for $C31H50O5Na^+$, 525.3556); C, 74.06; H, 10.02; O, 15.91; Found: C, 74.06; H, 10.04; O, 15.89; UV (MeOH) λmax (log ε) 210 nm; IR νmax 3655, 2327, 1550, 765 cm^{-1}; ^{1}H NMR (50 MHz, CD_3OD-*d4*): H-1: 4.0, H-3: 5.60 (dd, *J* = 4.5, 12.0 Hz) , H-5: 3.71 (dd, *J* = 4.0, 12.5 Hz), H-7: 4.12 (ddd, *J* = 4.0, 8.5, 11.0 Hz), H-8: 2.10(d, *J* = 8.5), H-17: 1.15, H3-18:1.15, H2-19: 0.55 (d, *J* = 4.5 Hz) 1.11 (d, *J* = 4.5 Hz), H-20: 1.50, H3-21: 0.98 (d, *J* = 5.0 Hz), H3-26: 1.06 (d, *J* = 7.0 Hz), H3-27: 1.05 (d, *J* = 7.0 Hz), H3-30: 1.33 (s), H-31: 4.86 (br) 4.87 (br) ^{13}C NMR (125, MHz, CD_3OD-*d4*), d: 73.0(C-1),39.4 (C-2), 70.0 (C-3), 55.8(C-4), 37.0 (C-5), 34.7 (C-6), 71.0 (C-7), 55.4 (C-8), 21.4 (C-9), 31.2 (C-10), 27.2 (C-11), 33.8 (C-12), 46.5(C-13), 49.8 (C-14), 38.0 (C-15), 29.3 (C-16), 52.5(C-17), 18.2 (C-18), 28.3 (C-19), 37.2 (C-20), 19.0(C-21), 35.9(C-22), 32.2 (C-23), 157.2(C-24), 34.5 (C-25), 22.6 (C-26), 22.5(C-27), 10.2 (C-28), 180.5 (C-29), 19.5 (C-30), 107.1(31).

β-Sitosterol (5)

A White Amorphous solid; mp 138-140^{o}C; FAB-MS *m/z* 415.431$[M+H]^+$ (calcd. for $C_{29}H_{50}O$ C, 83.99; H, 12.15; O, 3.86; Found: C, 83.98; H, 12.17; O, 3.87; ^{1}H-NMR (50 MHz, CD_3OD) δ: 3.74 (1H, m, H-3), 1.17, 1.00, 0.99, 0.98, 0.96, 0.86 (each 3H, s, CH_3), 0.95 (6H, s, 2×CH3). ^{13}C-NMR (125 MHz, CD_3OD) δ: 72.8 (C-3), 61.3 (C-10), 53.2 (C-8), 49.1 (C-4), 42.8 (C- 18), 41.7 (C-6), 39.7 (C-13), 39.3 (C-22), 38.4 (C-14), 37.8 (C-9), 37.1 (C-5), 36.1 (C-16), 35.5 (C-11), 35.3 (C- 19), 35.2 (C-29), 35.0 (C-2), 32.8 (C-21), 32.0 (C-15), 32.1 (C-28), 31.85 (C-30), 30.6 (C-12), 29.7 (C-17), 28.2 (C-20), 20.1 (C-26), 18.7 (C-27), 18.2 (C-25), 17.5 (C-1), 16.4 (C-24), 15.8 (C-7), 11.6 (C-23).

β-Sitosterol-3-*O*-glucoside (6)

A White Amorphous solid; mp 277-278^{o}C; FAB-MS *m/z* 565.21$[M+H]^+$ (calcd. for $C_{36}H_{62}O_5$ C, 72.87; H, 10.48; O, 16.64; Found: C, 72.89; H, 10.49; O, 16.66; ^{1}H-NMR (50 MHz, DMSO-*d*6) δ: 5.42 (1H, m, H-6), 4.95(1H, d, *J*=5.0, H-1'), 3.80-3.00 (sugar H), 1.05 (3H, s, 19-CH_3), 1.03-0.85 (12H, 21, 29, 27, 26-CH_3), 0.71 (3H, s, 18-CH3). ^{13}C-NMR (125 MHz, DMSO-*d*6) δ: 140.4 (C-5), 121.3(C-6), 76.8 (C-3), 56.1 (C-14), 55.3 (C-17), 49.6 (C-9), 45.1(C-24), 41.8 (C-13), 38.3 (C-12), 36.8 (C-1), 36.2 (C-10),35.4 (C-20), 33.3 (C-22), 31.4 (C-7), 31.3 (C-8), 29.2 (C-2),28.7 (C-25), 27.7 (C-16), 25.4 (C-23), 23.7(C-15), 22.6 (C-28), 20.6 (C-11), 19.7 (C-26), 19.1 (C-19), 18.9 (C-27),18.6 (C-21), 11.7 (C-29), 11.6 (C-18), 100.7 (C-1'), 76.8 (C-3'), 76.6 (C-5'), 73.4 (C-2'), 70.1 (C-4'), 61.0 (C-6').

4.Biological evaluation

4.1 Preparation of test solution:

Test solution of four different concentrations (6.25, 12.5, 25 and 50 mg/mL) were prepared by dissolving the extract in the normal saline.

4.2 Bacteria

The bacteria used in this study included three Gram-positive bacteria *Staphylococcus epidermidis* (ATCC9615), *Bacillus subtilis* (ATCC1174) and *Staphylococcus aureus* (ATCC25175) and three Gram-negative bacteria *Klebisella penumoniae* (ATCC33495), *Pseudomonas aeruginosa* (ATCC12453) and *Escherichia coli* (ATCC10536). The pathogenic bacterial strains were obtained from ATCC (American type Culture Collection, Manassas,V A) and maintained in the Microbiology Division of Pharmacology Department, Indian institute of integrative medicine (India) and maintained at usual Laboratory conditions. *S.aureus* ATCC 25175 was maintained by subculturing on Trypticase soy agar (Difco Laboratories, Detroit, MI) at 37^{o}C.

4.3 Animals

Wistar rats were maintained in a standard environmental condition and fed with standard laboratory rat diet and water ad libitum. The rats were acclimatized to our laboratory conditions for a week before the experiments.

4.4 MIC determination of *G. mollis* (Methanolic extract)

The MIC was determined as per the guidelines of Clinical and Laboratory Standards Institute (formally, the National Committee of Clinical Laboratory Standards) [21]. All bacteria used in this study were grown to stationary phase for 24 h at 37^{o}C. Bacterial suspension were prepared by suspending 24-h- grown culture in brucella broth (Difco Laboratories) (for anaerobic bacteria) and sterile normal saline (for aerobic bacteria).The turbidity of bacterial suspensions was adjusted to a McFarland standard of 0.5, which is equivalent to 1.5x 10^8 CFU/ml. The twofold serial dilutions of *G. mollis* extracts were prepared in Muller Hinton broth (Difco Laboratories) for aerobic

bacteria. BHI broth for 5% CO_2 cultures, and Wilkins-Chalgren broth for anaerobic bacteria in amounts of 100µl per well in 96-well U-bottom microtiter plates (Trason, Mumbai, India). The above mentioned bacterial suspension was further diluted in respective growth medium, and a 100µl volume of this diluted inoculums was added to each well of the plate, resulting in a final inoculums of 5x 10^5 CFU/ml in the well: final concentrations of *G. mollis* extract ranged from 15.6 to 4,000µg/ml. The plates were incubated at 37^{o}C for 24 h. The plates were read visually, and the minimum concentration of the compound showing no turbidity was recorded as the MIC.

4.5 Time-kill studies against *S.aureus*

S.aureus ATCC 25175 was grown in BHI broth at 37^{o}C for 24 h. The turbidity of the suspension was adjusted to 0.5 McFarland standard in sterile normal saline. A total of 200µl of this suspension was used in inoculate 20ml of BHI broth containing increasing concentration of methanolic extract (*G.mollis*) ranging from 125 to 1,000 µg/ml. Dimethyl sulfoxide controls were also included in the study. Suspensions were incubated at 37^{o}C, and the number of CFU was determined on BHI agar using a serial dilution method at various time points [22].

4.6 Antimicrobial activity against adherent *S.aureus* in water-insoluble glucan

The formation of water-insoluble glucan by *S.aureus* was performed by a previously described method [23]. Briefly, aliquots of 100µl of culture of *S.aureus* ATCC 25175 (1x10^7 to 1x 10^8 cells/ml) were inoculated into 10 ml of fresh BHI broth containing 2% sucrose (w/v) in the test tubes and incubated at 37^{o}C for 24 h at an inclination of 30^o. The fluid containing planktonic cells was gently removed. The water –insoluble glucan containing cells of *S.aureus* ATCC 25175 were gently washed with 10ml of sterile water and resuspended in 10ml of citrate buffer (10 mM, pH 6.0) containing 1,000 µg/ml methanolic extract (*G.mollis*), followed by incubation at 37^{o}C for 5 min.The mixture was gently washed again with sterile water containing 0.1% Tween 80 (w/v). After incubation of cells at 37^{o}C for 6, 12, 18 and 24 h, the acid produced by the culture was measured by using a pH meter. The fluid containing free cells of *S.aureus* ATCC 25175 was gently removed. The water-insoluble glucan was resuspended in 10 ml of sterile water and homogenized using five 30-s ultrasonic bursts, and the turbidity was measured at 610 nm.

4.7 Postantibiotic effect (PAE)

The postantibiotic effect (PAE) of methanolic extract (*G.mollis*) was determined by the method described by Crag and Gudmundsson [24]. Bacterial suspension of *S.aureus* ATCC 25175 was prepared by suspending 24-h growth in sterile normal saline. Methanolic extract of *G.mollis* was added at the MIC and 2 x MIC into test tubes containing 10^6 CFU of each isolate per ml in BHI broth. After a brief exposure (5min) to the methanolic extract (*G.mollis*), samples were diluted methanolic extract (*G.mollis*) to 1: 1,000 to effectively remove methanolic extract (*G.mollis*). Samples were taken every hour, and the number of CFU was determined until turbidity was noted. The PEA = T-C, where T represents the time required for the count in the test culture to increase 1 $\log_{10}$ CFU/ml above the count observed immediately after drug removal and C represents the time required for the count of the untreated control tube to increase by 1 $\log_{10}$ CFU/ml.

4.8 Selection of resistant mutant in *vitro*

The first-step mutants of *S.aureus* ATCC 25175 was selected using a previously described method [25]. A bacterial suspension containing 109 CFU (100 µl) was plated on BHI agar containing methanolic extract (*G.mollis*) at concentration equal to 2 x, 4x, and 8x MIC. Mutation frequency was calculated by counting the total number of colonies appearing after 48 h of incubation at 37^{o}C in 5% CO_2 on the methanolic extract (*G.mollis*) -containing plate and by dividing the number by the total number of CFU plated. All mutation prevention concentration determinations were made in triplicate and results were identical.

4.9 Anti-inflammatory effect

The anti-inflammatory activity was evaluated using rat paw edema as a model for acute inflammation induced in male Wistar rats by formaldehyde [26-27]. Rats were divided into 2 groups. The first group served as a negative control received I.P. injection of vehicle, 0.25% sodium carboxy methyl cellulose. Rats in second group were divided into subgroups and treated with the methanolic and fractions (chloroform, ethyl acetate and butanol). The organic extracts were suspended in 0.25% sodium carboxymethyl cellulose using sonicator and injection via I.P. route one hour before formaldehyde. Edema was induced by injecting 0.2 ml formaldehyde solution into the rat hind paws. The volume of the rat paw before, 1 and 2 hours injection of formaldehyde were measured. The volume of edema is expressed in ml (mean±SEM). Protection Against inflammation is expressed as % inhibition of edema 1 and 2 hours after formaldehyde injection in comparison with the control group. Difference between the control and the treated groups was analyzed using Student's *t*-test for the methnolic extract and one way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test for the fractions. A *p* value of 0.05 or less was taken as a criterion for statistically significant differences.

4.10 antihypertensive activity

The male Wistar rats were kept in room with 12h dark and 12h light cycle. Water and food were provided *ad-libitum*. The rats were anesthetize using 25% (w/v) aqueous urethane at a dose of (1.25 g/kg b. wt. i.p.). the carotid artery was cannulated and connected to an ITT blood pressure transducer for measurement of the arterial blood pressure. The heart rate was calculated from the blood pressure pulse by increasing the rate of recording from 0.05 to 1 cm sec^{-1} in the Narco Physiograph Coupler. All tested extracts in a dose of 20 mg/kg b. wt except butanol extract 3mg/kg b. wt. were injected intraperitoneally. Changes in blood pressure were quantified in mm Hg using the calibration system built-in the physiograph. Changes in the heart rate were calculated as percentage change compared with the pre-drug level [28].

4.11 Diuretic effect:

A rat weight 200-250 g is placed in a metabolic cage (TECHNI PLAST – ITALY, 170022) for 24 hours where the urine is collected from both control (which is given saline) and different extracts of *G. mollis*. All volumes of urine are collected and measured according to the method of Nedi, T., et al [28].

4.12. Statistical analysis

Results were expressed as % decrease and Mean + SEM where applicable. Statistical significance was tested using Student`s *t*-test. The difference was taken to be statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

2.1 Chemistry :

Methanol extract of the aerial part of *Grewia mollis* afforded six compounds after repeated column chromatographic separation and purification of each fraction. They were identified as two flavonoids, two triterpenoids, and two steroids by spectroscopic analysis.

Compound **1** (Figure 1) was obtained as a yellow amorphous powder after crystallization from MeOH. An $[M+H]^+$ peak at 287 in FAB-MS along with the analysis of ^{13}C-NMR, DEPT showed its molecular formula $C_{15}H_{10}O_6$. The ^{1}H-NMR spectrum of **1** showed six methyl singlet signals. The singlet peak at δ4.85 (1H, s) was identified as H-19 proton signal attached on an olefinic double bond. In the ^{13}C-NMR spectrum, 14 carbon signals were observed, which included two olefinic carbons at δ 142.7 and δ 129.7 and an oxygenated carbon at δ 79.0. As the result, compound **1** was identified as Luteolin, which was reported in the literature [7-11].

Copmound **2** (Figure 1) was obtained as a yellow amorphous powder soluble in water. Positive ion FAB-MS gave a molecular ion peak at *m/z* at 625.1, ($[M+H]^+$ -galacturonic acid) at 449.1 and ($[M+H]^+$ -galaturonic acid-glucose) at *m/z* 287 indicates the presence of galactournic acid and glucose moieties. Based on this data the **2** had the molecular formula $C_{27}H_{28}O_{17}$ (mw 624). Based on the analysis of the ^{1}H and ^{13}C NMR, HMQC and HMBC spectrum and a comparison of the data with the literature [12]. Compound **2** was determined to be 7-(1-O-β-D-galacturonide)- 4´-(1-O- β-glucopyranosyl)-3´4´,5,7-tetrahydroxyflavone.

Compound **3** (Figure 1) was isolated as a yellowish amorphous solid. Positive ion FAB-MS gave a molecular ion peak at *m/z* 489.9 $[M+H]^+$, consistent with the molecular composition of C30H48O5. The IR spectrum of **3** showed a broad band at 3649 cm^{-1}, and a sharp band at 1542 cm^{-1} indicating the presence of hydroxyl and carbonyl groups. The ^{1}H and ^{13}C NMR spectra indicated that this compound belonged to the class of triterpenoids. The ^{1}H spectrum of **3** indicated that the compound was pure with three oxygenated protons and six methyl groups. A highly shielded peak at δH 0.45 (d, J = 4.4 Hz, H-19) suggested the presence of a cyclopropyl ring [13]. The ^{13}C spectrum of compound **3** contained 30 signals: six methyls, eight methylenes, five methines, three oxygenated carbons, and eight quaternary carbon peaks. The ^{1}H and ^{13}C NMR signals in methanol-*d4* showed typical signals for the cyclopropane methylene protons with peaks at δC/δH 22.0 (C-19)/0.45 (d, J = 4.4 Hz, Hβ-19) and 0.81 (d, J = 4.8 Hz, Hα-19); two quaternary carbons at δC 29.1 (C-9) and δC 29.2 (C-10). Three oxygenated methine peaks were observed at δC/δH 73.5 (C-1)/3.55 (br, Hβ-1); δ C/δH 71.4 (C-3)/4.52 (dd, J = 10.4 Hz, 6.8 Hz, Hα-3), δC/δH 70.6 (C-7)/3.56 (br, Hβ-7). From HSQC and HMBC correlations, the proton-carbon pairs were connected to each other and 1*J*CH correlations were determined to obtain a cycloartane-type triterpene. HMBC correlations for the fusion of the A/B rings was confirmed by the correlation of H-29 (δH 1.07, s) to C-3 (δC 71.4), C-5 (δC 37.5), C-28 (δC 180.6); H-5 (δH 2.76, dd, J = 4.0 Hz, 14.0 Hz) to C-4 (δC 46.9), C-3 (δC 73.5), C-6 (δC 29.6), C-7 (δC 70.6) and C-19 (δC 22.0). Similarly, the B/C ring fusion was confirmed by correlation of H-19 to C-8 (δC 25.3), C-9 (δC 26.1), and C-11(δC 27.2), and H-8 (1.29, s) to C-7, C-11, C-14 (δC 30.9), and C-19 (δC 22.0). After confirming the fusion of the C/D ring at position C-13 and C-14, the attachment of the alkyl side chain was determined. A strong HMBC correlation from H-17 (δH 1.57, m) to C-14 (δC 30.9), C-21 (δC 19.0), C-22 (δC 26.0), and C-27 (δC 17.8) indicated the point of attachment of the alkyl chain at C-17 thereby confirming the cycloartane skeleton. Based on

the NMR spectral evidences and a comparison of the data with the literature [14]. compound **3** was determined as 7β-hydroxy-23-ene-deoxojessic acid.

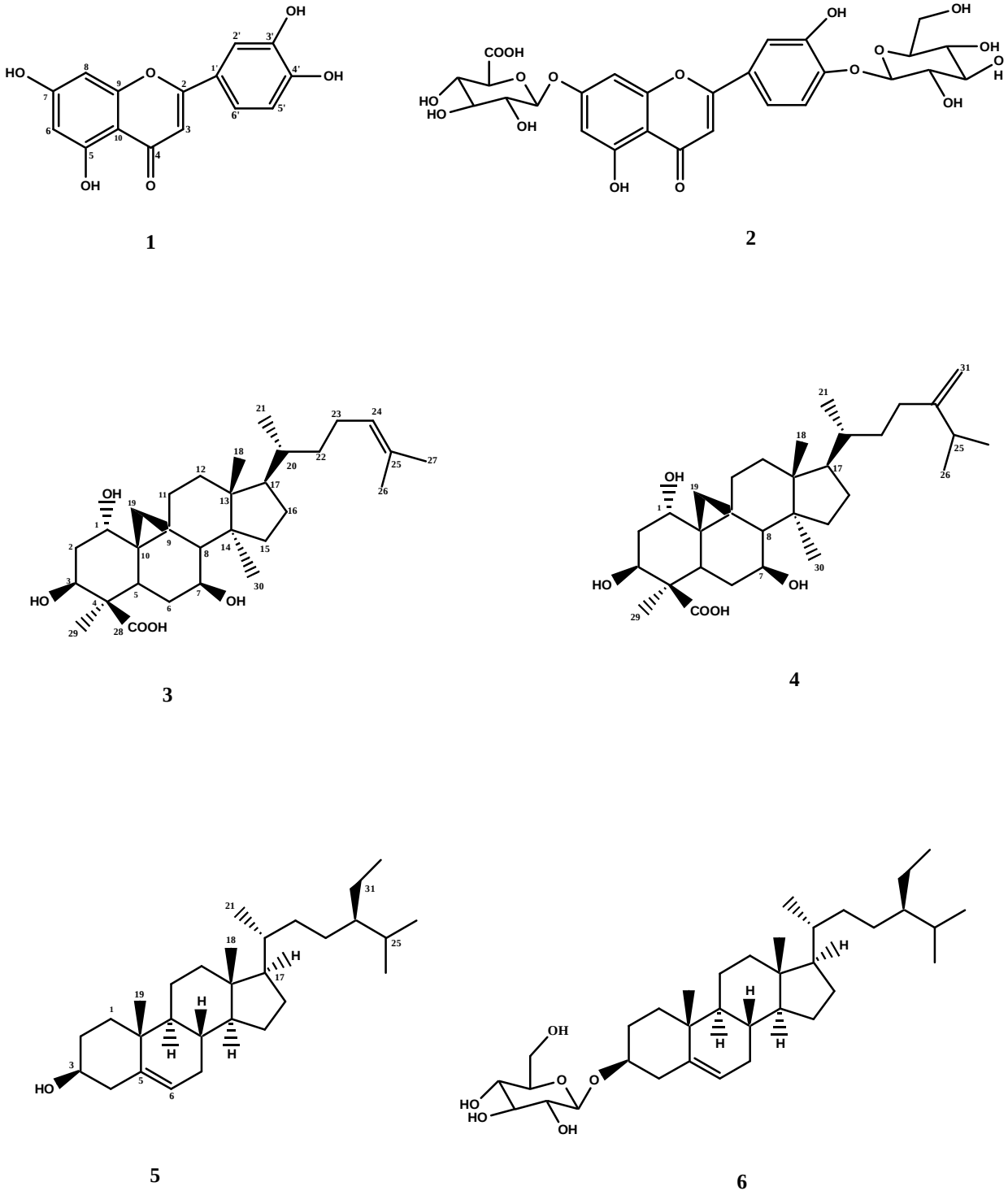

Figure 1: Chemical structures isolated from aerial part of *Grewia mollis*.

Compound **4** (Figure 1) was also isolated as a yellowish amorphous solid. Positive ion FAB-MS gave a molecular ion peak at *m/z* 525.3591 $[M+Na]^+$, confirming the molecular composition of C31H50O5. The IR spectrum of **4** showed a broad band at 3655 cm^{-1} and a sharp band at 1550 cm^{-1} indicating presence of hydroxyl and carbonyl groups. The ^1H and ^{13}C NMR spectra indicated that this compound also belonged to the class of triterpenoids. The ^1H NMR spectrum of **4** in methanol-*d4* indicated that the compound had three oxygenated protons and six methyl groups. A highly shielded doublet at δH 0.59 (d, *J* = 4.5 Hz, H-19) suggested the presence of a cyclopropyl ring

[13]. The ^{13}C spectrum of compound **4** contained 31 signals: six methyls, nine methylenes, five methines, four oxygenated carbons, and seven quaternary carbon peaks. The ^{1}H and ^{13}C NMR signals in methanol-*d4*showed typical signals for the cyclopropane methylene protons with peaks at δC /δH 28.3 (C-19)/0.59 (d, *J* = 4.5 Hz, Hβ-19) and 1.14 (d, *J* = 4.8 Hz, Hα-19); two quaternary carbons at δC 21.4 (C-9) and δC 31.2 (C-10). Three oxygenated methine peaks were observed at δC /δH 73.0 (C-1)/4.02 (br, Hβ-1); δC /δH 71.0 (C-3)/4.14 (br, Hα-3), δC /δH 70.0 (C-7)/5.63 (dd, *J* = 12.4 Hz, 4.5 Hz, Hβ-7). The HSQC and HMBC correlations confirmed that compound **4** had a similar cycloartane-type skeleton as that of **3.** HMBC correlations for the fusion of the A/B rings was confirmed by the correlation of H-29 (δH 1.80, s) to C-3 (δC 71.0), C-5 (δC 37.3), C-28 (δC 180.5); H-5 to C-4 (δC 55.8), C-3, C-6 (δC 34.7), C-7 (δC 70.0), and C-19 (δC 28.3). After connecting the fragments together from HMBC correlations, as discussed above, compound **4** has also the flat structure. The only difference between compound **3** and compound **4** was the position of the double bond in the alkyl side chain. The position of the double bond in **4** was confirmed from the HMBC correlations of H-25 (δH 2.28, m) to the two methyl doublets, C-26 (δC 22.6) and C-27 (δC 22.5).

To determine the stereochemistry of compound **4**, 1D and 2D ROESY experiments were carried out and the carbon chemical shifts were compared to those in literature [14]. The *β*-orientation of H-3 (δH 5.63, dd) was confirmed from its coupling constants (*J* = 12.4 Hz, 4.5 Hz). A strong correlation of H-3 to H-5 (δH 3.74, dd, *J* = 14.0 Hz, 4.0 Hz); H-5 to H-7 (δH 4.14, br) and H-7 to H-8 (δH 2.12, br) showed *β*-orientation of these four protons. Further, the *trans* fusion of the C/D rings was established from the ROESY correlations of H-8 to H-18 (δH 1.18, s) and H-7 to H-30 (δH 1.33, s).From the ROESY data and its comparison to literature values [14], the structure of compound **4** was confirmed to be 7*β*-hydroxy-23-deoxojessic acid.

Compounds **5** and **6** (Figure 1) were identified as β-sitosterol (**5**) and β-sitosterol-3-O-glucoside (**6**) by comparing physicochemical and spectral data with published literatures [15-18].

2.2 Biological Evaluation:

The *invitro* antibacterial activity of the aerial part of *Grewia mollis* was investigated against various strains of bacteria such as *Staphylococcus epidermidis, Bacillus subtilis, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Escherichia coli* by Kirby – Bauer disc diffusion method. The methanolic extract showed significant activity against *Staphylococcus epidermidis, Bacillus subtilis and Staphylococcus aureus* when compared with the standard antibiotics Cloxacillin (5 mcg/disc), Amoxicillin (10 mcg/disc) and Ciprofloxacin (5 mcg/disc) respectively. Methanolic extract of *G.mollis* aerial part showed inhibitory activity against oral cavity pathogen. It exhibited an inhibitory effect on oral cavity pathogen tested (MIC of 62.5 to 500 µg/mL) with minimal bacterial concentration that was twofold greater than the inhibitory concentration. Methanolic extract exhibited concentration-dependent killing of *Staphylococcus aureus* ATCC25175 up to 4 x MIC and also prevented the formation of water-insoluble glucan. The anti-inflammatory activity of the aerial part of *G.mollis* was investigated, the methanolic extract exerted the highest anti-inflammatory effect after two hours.

Table 1: Evaluation of antibacterial activity of methanolic aerial part extract of *Grewia mollis*

S. No	Microorganism	Standard antibiotic used and concentration	Zone of inhibition of standard in mm	Zone of inhibition (mm)				
				250 mcg	500 mcg	750 mcg	1000 mcg	1250 mcg
1	*Staphylococcus epidermidis*	Cloxacillin 5 mcg	21 mm	9	10	12	13	15
2	*Bacillus subtilis*	Amoxicillin 10 mcg	19 mm	9	11	13	14	16
3	*Staphylococcus aureus*	Ciprofloxacin 5 mcg	20 mm	14	20	20	21	22
4	*Klebisella penumoniae*	Levofloaxin 5 mcg	35 mm	2	4	4	6	8
5	*Pseudomonas aeruginosa*	Amikacin 10 mcg	26 mm	5	6	8	8	10
6	*Escherichia coli*	Sparfloaxin 5 mcg	22 mm	-	-	4	6	7

Antibacterial Evaluation

Kirby – Bauer disc diffusion method [19] was used for the antibacterial study. Cloxacillin (5 mcg/disc), Amoxicillin (10 mcg/disc), Ciprofloxacin (5mcg/disc), Levofloxacin (5 mcg/disc), Amikacin (10 mcg/disc) and Sparfloxacin (5 mcg/disc) were used as the standard antibiotics for the organisms *Staphylococcus epidermidis, Bacillus subtilis, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Escherichia coli* respectively. The filter paper impregnated with methanolic extract (at the concentrations of 250 mcg/ml, 500 mcg/ml, 750 mcg/ml, 1000 mcg/ml and 1250 mcg/ml.) and standard antibiotic disc were placed aseptically on the Muller-Hinton agar

medium (Hi-Media, Mumbai) which was already swabbed with the test organism and incubated at 37°C for 16 to 18 hours. The zone of inhibition in mm was measured and the results were given in Table 1.

The observation of the minimum inhibitory concentration study for methanolic extract against *Staphylococcus epidermidis, Bacillus subtilis, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Escherichia coli*. From the data it is evident that methanolic extract is active against both gram positive and gram negative bacteria, but more active against gram positive bacteria such as *Staphylococcus epidermidis*, *Bacillus subtilis* and *Streptococcus aureus*. With standard antibiotic such as Cloxacillin (5mcg), Amoxicillin (10mcg) and Ciprofloxacin (5mcg) respectively.

To evaluate the antimicrobial activity against oral microorganism, MIC was determined and the result of methanolic extract is shown in Table 2. Methanolic extract exhibited an MIC range of 64 to 250 µg/ml against the oral cavity pathogen, *Staphylococcus aureus.*

Antibacterial activity

Table 2: MIC determination of the active samples

Plant code	MIC (µg/ml)	
	S.aureus ATCC	***MRSA 15187***
G.Mollis (Methanolic extract)	**64**	128

Ciprofloxacin was used as a standard antibacterial agent in this study
MRSA*: Methicillin-resistant Staphylococcus aureus*

The MIC of extract (250 µg/ml) showed a 3-log reduction in growth in 10 h, compared to untreated control, while 2x MIC and 4x MIC could reduce the CFU count *S. aureus* ATCC25175 below the detection limit (>50 CFU/mL) in 8 h and 4 h respectively. The kill kinetics study showed that methanolic extract exhibit a time and concentration-dependent killing effect against *S. aureus* ATCC25175.

The adherent cells of *S. aureus* ATCC25175 treated with extract at 1,000 µg/ml grew slowly and water-insoluble glucan synthesis was inhibited in the presence of sucrose. In contrast, adherent cells without extract treated treatment grew well and there was synthesis of water-insoluble glucan in the presence of sucrose. Exposure of extract resulted in the slower acidification of the broth in the presence of sucrose, and a pH of 6.7 was recorded after a 42 h incubation.

However preliminary phytochemical analysis of methanolic extract revealed the presence of carbohydrate, glycosides, phytosterols, flavonoids triterpenoids and phenolic compounds. The location of antibacterial agents from components of *G. mollis* is in progress to identify potent antibacterial agents. The antibacterial activity in methanolic extract of *G. mollis* by Kirby −Bauer disc diffusion method showed good antibacterial activity against gram positive organism.

Table 3: Effect of the successive fractions of *G. mollis* on formaldehyde-induced rat paw edema after one, two hours of injection.

Treatment group (500mg/kg, i.p)	% inhibition of inflammation After One hour	% inhibition of inflammation After Two hours
Methanolic ext.	38.9 ± 1.3	38.7±2.3
Chloroform	14.4 ± 1.9	13.9±0.9
Ethyl acetate	27.0 ± 2.1	0.7 ±0.1
Butanol	39.6 ± 1.1	21.7 ± 0.3

Anti-inflammatory effect

The anti-inflammatory activity was indicated by the ability of the test materials to decrease the volume of edema induced by formaldehyde after one and two hours of its injection comparing with the control. Percentage of edema inhibition produced by extracts was calculated in Table 3. Methanolic extract 500 mg/kg I.P. showed pronounced anti-inflammatory effect by 38.9±1.3 and 38.7±2.3 after one and two hours of injection respectively. This effect may be due to its steroidal and/or triterpenoidal composition which has proved as anti-inflammatory activity [20] on the other hand, butanol extract 500 mg/kg is approximately as potent as methanolic extract 39.6 ±1.1 after one hour injection, however, is less potent as anti-inflammatory as methanolic extract by 21.7±0.3 after two hours of injection. Chloroform extract has shown to inhibit the induced inflammatory response to formaldehyde to a lesser extent by 14.4±1.9 and 13.9±0.9 after one and two hours respectively, followed by ethyl acetate fraction by 27.0±2.1

and 0.7±0.1 after one and two hours respectively. The present findings give enough information that fractions of *G. mollis* provide inhibitory affects in inflammatory process in which supports the use of this plant for the treatment of arthritis, dermatitis and wounds in the Saudi medicine. However, toxicity studies are required to prove the safety of this plant.

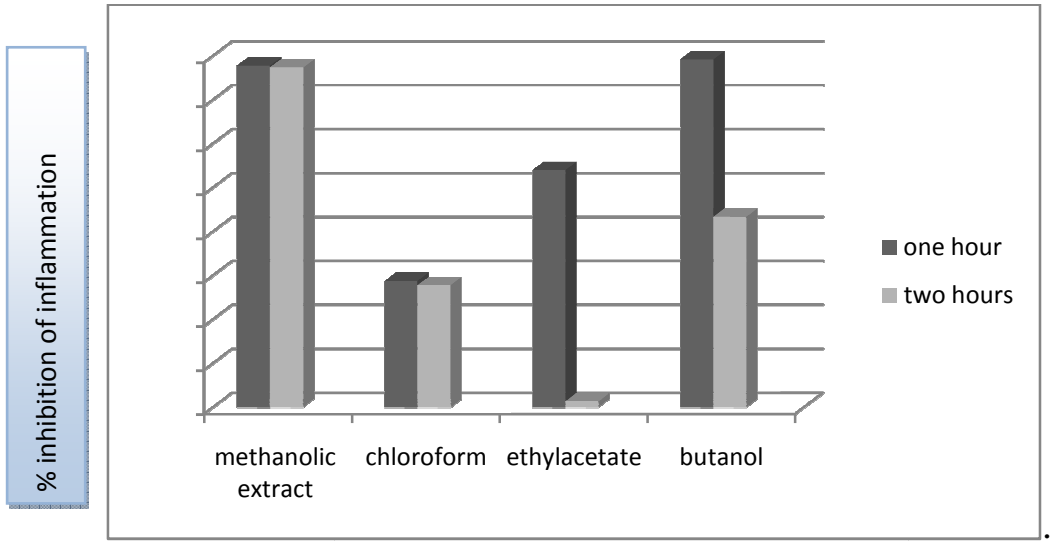

Figure 2: effect of the successive fractions of *G. mollis* on formaldehyde-induced rat paw edema after one, two hours of injection.

Antihypertensive activity

Intraperitoneal administration of methanolic extract (20 mg/kg b. wt.), chloroform extract (20 mg/kg b. wt.), ethyl acetate extract (20 mg/kg b. wt.) and butanol extract (3 mg/kg b. wt.) into the urethane anesthetized rats induced dose dependent decrease in the arterial blood pressure and heart rate. Table 4 presented the effect of methanolic and different extracts of *G. mollis.* Most of the cardiovascular depressant activity is found more pronounced in butanol extract at the small dose of 3.0 mg/kg which produced a perceptible fall in arterial pressure by 22.5 mmHg when compare with a control group. The effect of methanolic, chloroform and ethylacetate extracts at the dose of 20 mg/kg b. wt. in the arterial blood pressure indicating that the methanolic extract possess vasodepressant activity by 27 mmHg followed by chloroform extract by 18 mmHg, however, ethylacetate extract have minor effect on decreasing blood pressure at the dose of 20 mg/kg b. wt. by 13 mm Hg when compare with a control group. Also, the percentage decrease in heart rate was more pronounced in butanol at a small dose of 3.0 mg/kg by 32.1. The cardio-depressant activity of the methanolic extract at a dose of 20 mg/kg b. wt. showed significant decrease in heart rate by 35.7 mmHg. On the other hand, the chloroform extract was decreased heart rate by 27.6 at a dose of 20 mg/kg, while ethylacetate shown little change in heart rate. Most of the cardio-depressant activity seemed to be contained in the butanol and methanolic extracts at a dose dependent manner while ethylacetate extract has not shown any significant decrease in arterial pressure and heart rate. It would be evident from above that the butanol and methanolic extracts of *G. mollis* possess a marked potency for lowering the blood pressure and heart rate in rats at a dose-dependent manner, indicating that they possess a vasodilation action.

Table 4: Effects of methanolic and different extracts of *G.mollis* on arterial pressure and heart rate.

Extract	Decrease in Arterial pressure mmHg	% Decrease in heart rate
Methanolic ext.(20 mg/kg)	27±1.2	35.7±1.1
Chloroform (20mg/kg)	18± 2.0	27.6±1.5
Ethylacetate (20 mg/kg)	13±1.4	5.0±0.4
Butanol (3 mg/kg)	22.5±1.7	32.1±1.1

Table 5: Results of diuretic effect of *G.mollis* extracts

Extracts (500mg/kg, i.p)	Percentage change	Urine color
Methanolic ext.	↓9.3	Light amber
Chloroform	↓6.2	Light amber
Ethyl acetate	↓37.3	Reddish amber
Butanol	↓31.2	Yellow

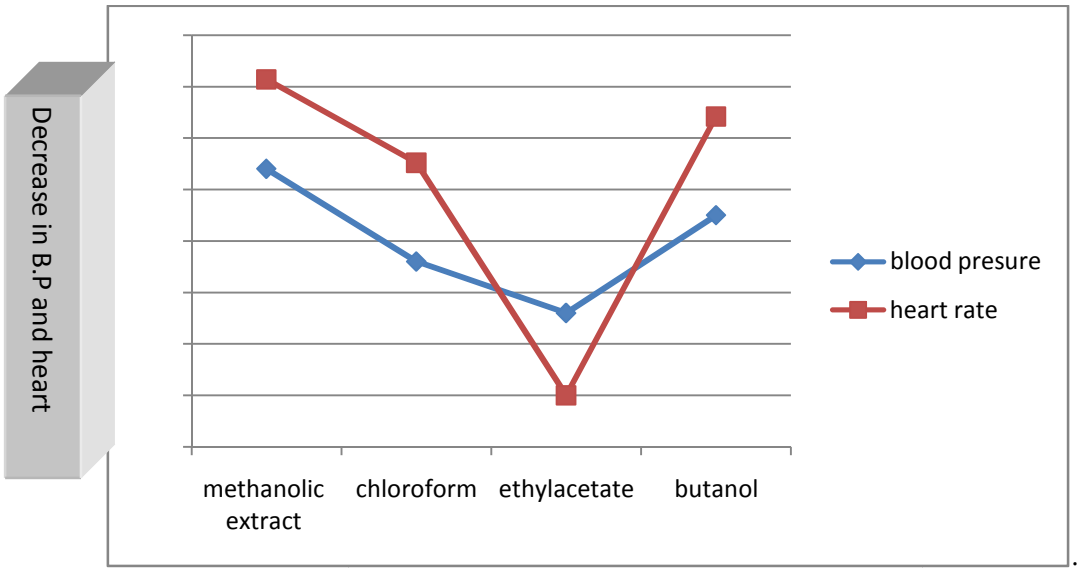

Figure 3: effect of the successive fractions of *G. mollis* on blood pressure and heart rate.

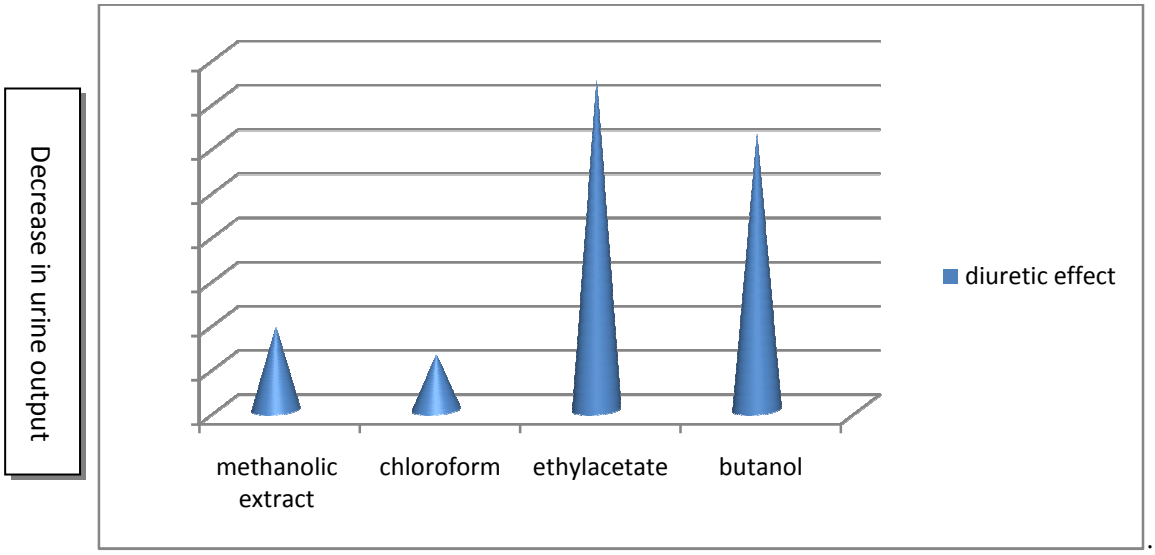

Figure 4: effect of the successive fractions of *G. mollis* on urine output.

Diuretic activity

The diuretic activity of different extracts of *G. mollis* in a dose of 500 mg/kg i.p. was studied in comparison with a control group using normal saline in rats. Ethyl acetate and butanol extracts showed a significant decreased on the urine output by 37.5% and 31.2% respectively. While chloroform and methanolic extracts produced slightly decreased in urine output by 6.2% and 9.3% at a dose of 500 mg/kg respectively when comparison to control group, Table 5. The results of this study indicated that *G.mollis* extracts may contain compound (s) that mediate effect by decreasing in blood flow in the kidneys and decreasing in the glomerular filtration rate resulting in decrease urine output, i.e., *G. mollis* extracts act as oliguresis (oliguria) that decrease the volume of the urine. Further experimentation is needed in order to understand the precise mechanism of action of the diuretic effect on different extracts.

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