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Gastroprotective flavonoid constituents from *Oroxylum indicum* Vent.

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ABSTRACT

Chemical investigation of the stem bark of *Oroxylum indicum* resulted in the isolation and characterization of two new flavonoid glycosides (**1**, **2**), along with seven known compounds (**3–9**). Their structures were established on the basis of extensive spectroscopic (IR, MS, 2D NMR) data analysis and by the comparison with spectroscopic data reported in the literature. In addition, all the compounds were tested for their ulcer protective effects against various gastric ulceritis inducing models in rats.

Gastric or peptic ulcer constitutes major ailment that affects human gastrointestinal tract and presents major global health problem both in terms of morbidity and mortality.[1] Consumption of alcohol and analgesics have been identified as major risk factors responsible for acute gastric mucosal injury in humans and presents life-threatening hemorrhages that require surgical intervention.[2,3] Several other factors like heredity, smoking, elevated calcium level, usage of corticosteroids in high dosage etc., are also responsible for elevated risk of gastric ulceration.[4]

Presently prophylactic options for patients suffering from gastrointestinal ulceration include antacids, sucralfate, histamine-2-receptor antagonists (H_2RAs), prostaglandins, muscarinic (M_1-antagonists) and proton-pump inhibitors. However, it has been observed that long-term use of H_2-RAs and proton-pump inhibitors induces hyperplasia in enterochromal-like (ECL) cells, which may lead to further relapse of ulcer disease and induction of gastric cancer.[5] Moreover, most of these therapeutics are largely empirical and have several other adverse side-effects also.[6] Therefore, there is a need of more effective and safer antigastric ulcer agents with lesser side effects.

Plants have been used in traditional medicine systems all over the world since the dawn of human civilization. Also, plant derived metabolites have provided important basis for discovery and development of modern therapeutics.[7]

In Indian traditional medicine, the roots as well as stem bark of *Oroxylum indicum* (Family: Bignoniaceae), commonly known as '*Syonaka*', has been used for centuries for the treatment of various gastric disorders.[8] Herein, we report the isolation and structure elucidation of two new flavonoid glycosides (**1,2**), along with the seven known compounds, dihydroiso-α-lapachone (**3**), 7-O-methylchrysin (**4**), 5-hydroxy- 4′,7-di methoxy flavone (**5**), dihydro oroxylin A (**6**) oroxylin A (**7**), chrysin (**8**), and baicalein (**9**) as constituents from hexane and acetone extracts of *O. indicum* and report for the first time gastroprotective properties of these extracts and isolates in rats under various ulcer inducing conditions like aspirin induced gastric ulceritis (AIU)[9], ethanol induced ulcers (EIU)[10], cold restrain induced (CRU) gastric ulcer[11] and pylorus ligation induced gastric lesions (PLU).[12] This report further provides scientific basis for the use of *O. indicum* in traditional Indian medicinal preparations meant for antigastric ulcer activities. Except compounds **7, 8, 9** all other compounds **3–6** are being reported for the first time from this plant (Fig. 1).

The dried stem-bark (5 kg) were ground, extracted three times with hexane and acetone in a soxhlet apparatus for 72 h. The resulting extracts were evaporated to dryness under reduced pressure, affording syrupy residues. The hexane extract was subjected to column chromatography over a silica gel column (60–120 mesh, 150 × 15 cm) and eluted with a step wise gradient of chloroform: MeOH, (100, 99.5:0.5, 99:1, 98:2 by volume) to afford a total of 30 fractions of 50 ml each. Column fractions were analyzed by TLC (Silica Gel 60 F254, chloroform/MeOH, 92:8), and fractions with similar TLC patterns were combined to give two major fractions (F_1 and F_2). Flash chromatography of fraction F_1 (331.88 mg) by elution with hexane/EtOAc (80:20), yielded dehydroiso-α lapachone (**3**)[13] (0.120 g). Fraction F_2 was purified on

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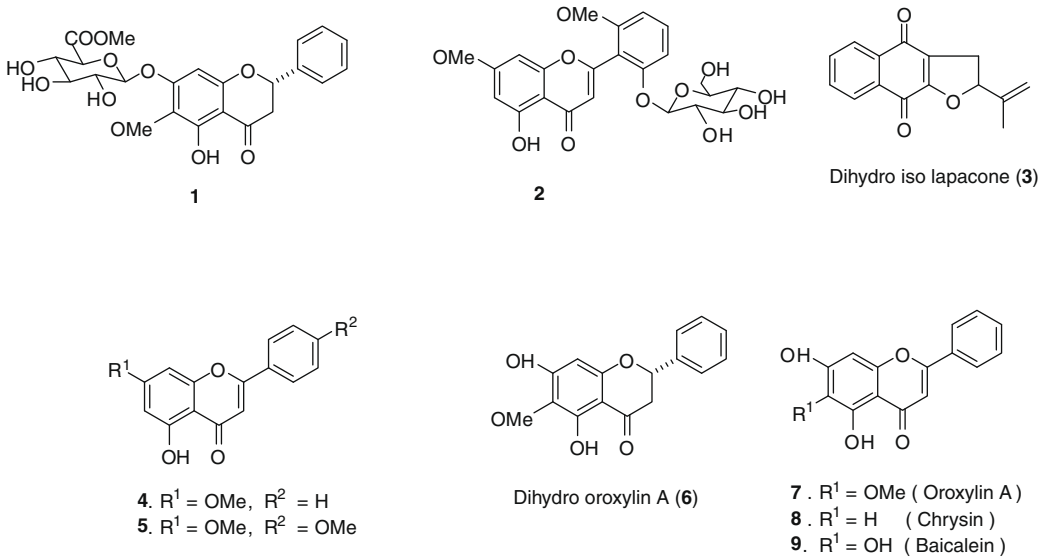

Figure 1. Compounds (**1**–**9**) isolated from *Oroxylum indicum*.

silica gel column chromatography (100–200 mesh) eluted with 0.5% MeOH in chloroform to afford 7-*O*-methyl chrysin(**4**)[14] (0.7 g), 5-hydroxy-4′,7-dimethoxy-flavonoid (**5**)[15] (0.12 g), dihydro oroxylin A (**6**)[16] (0.08 g) and eluted with 2% MeOH in chloroform to afford oroxylin A (**7**)[17] (3 g), chrysin (**8**)[17] (2 g) and baicalein (**9**)[18] (5 g). Similarly, the dark yellow colored crude acetone extract (1 g) was subjected to column chromatography over silica gel (100–200 mesh, 300 g), and eluted with chloroform/MeOH in the increasing order of polarities to afford the three fractions. Successive flash chromatography of fraction I by elution with 4% MeOH in chloroform to get the dihydrooroxylin 7-*O*-glucuronide-6′-methl ester (**1**) (0.02 g) and fraction II, elution with 6% MeOH in chloroform yielded flavonoid derivative (**2**) (0.34 g).

Compound (**1**) was isolated as pale yellow solid with $[\alpha]_D^{25}$ −46.4 (*c* 0.05 MeOH) and mp 165 °C. The IR spectrum indicated the presence of OH (3395 cm^{-1}), carbonyl (1618 cm^{-1}), ester carbonyl (1735 cm^{-1}) and aromatic functional groups. UV absorption maxima at λ_{max} 295 and 320 (sh) nm were indicative of a flavanone structure.[19] The molecular formula was determined as $C_{23}H_{24}O_{11}$ by HRESIMS, which provided a pseudomolecular ion peak at *m/z* 499.1535 (M^++Na), in conjunction with its ^{13}C NMR, displaying 23 resonances. A DEPT NMR experiment permitted differentiation of 23 carbons into twelve methine, one methylene, two methoxyls, two carbonyls and, six quaternary carbons, of which sixteen carbons were attributed to a flavonoid skeleton and six carbons to sugar moiety. Regarding the aglycone moiety, signals at δ 5.46 (1H, dd, *J* = 12.0, 3 Hz, H-2), δ 3.14 (1H, dd, *J* = 17.5, 12.0 Hz, H-3ax), and δ 2.82 (1H, dd, *J* = 17.5 3.0 Hz, H-3eq) in the ^1H NMR spectrum (in MeOH-d_4 + $CDCl_3$) together with the presence of carbonyl resonance at δ 198.78 confirmed the flavonone skeleton. Two multiplets at δ 7.57–7.48 (2H), δ 7.45–7.39 (3H), and correlations of H-2′/C-1′, C-3′, C-2; H-3′/C-4′, C-5′, H-4′/C-5′, C-3′, C-6′ in the HMBC spectrum supported that **1** having unsubstituted ring B. Further more, ^1H NMR spectrum indicated a three-proton singlet at δ 3.85, assigned to aromatic methoxyl group at C-6. On the basis of the above analyses of spectroscopic data, the flvanone aglycone was suggested as 5,7-di hydroxyl-6-methoxyflavanone, which was identical to **6**, isolated from the same plant. Acid hydrolysis[20] of **1** yielded glucuronic acid and dihydrooroxylin A (**6**), as determined by the TLC comparison with standards. The ^1H NMR spectrum also revealed one three-proton singlet at δ 3.75 assigned to ester methoxyl group, based on the HMBC correlation to C-6″(170.75) (see Fig. 2).

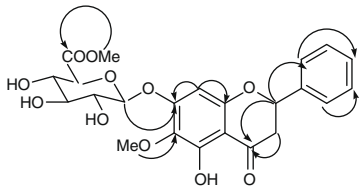

Figure 2. Key HMBC correlations of compound **1**.

The location of the glycosyl linkage was determined to be at C-7 since the anomeric proton signal at δ 5.02 showed a HMBC correlation with an oxygenated quaternary carbon at δ 156.44, assignable to C-7. The 'D' configuration of glucuronic acid was determined after acid hydrolysis of **1** by isolating the sugar using preparative HPLC and measuring the purified sugar's optical rotation. Absolute configuration of **1**was deduced from the aglycone (dihydrooroxylin A) moiety, which is known from the literature. Based on these results,[21] the structure of **1** was confirmed as methyl-3,4,5-trihydroxy-6-(5-hydroxy-6-methoxy-4-oxo-2-phenylchroman-7-yloxy)-tetrahydro-2*H*-pyran-2-carboxylate and has been trivially named as dihydrooroxylin A-7-*O*-methyl glucuronide.

Compound **2** was obtained as a white, amorphous powder with the molecular formula $C_{23}H_{24}O_{11}$, determined on the basis of NMR and HRESIMS data (*m/z* 499.1211 $[M+Na]^+$). The IR spectrum displayed absorption bands diagnostic of hydroxyl (3329 cm^{-1}), conjugated ketone (1633 cm^{-1}) and aromatic functional groups, respectively. Examination of ^1H and ^{13}C NMR data of **2** indicated that molecule consisted of a flavone and sugar moiety. The ^1H NMR spectrum of **2** (MeOH-d_4 + $CDCl_3$) showed a pair of one-proton doublets δ 6.42 (d, *J* = 2 Hz) and δ 6.34 (d, *J* = 2 Hz), were assigned to H-6 and H-8, corresponding with the HMBC cross peaks between δ_H 6.42/δ_C 161.05 (C-5), 165.39 (C-7), 92.48 (C-8) and δ_H 6.34/δ_C 98.04 (C-6), 158.76 (C-9), indicating a presence of a flavone with substitutions at C-5 and C-7. It also exhibited a singlet at δ 6.41 (1H, s), which was assigned to H-3. Further, the signals for two doublets at δ 6.80 (1H, d, *J* = 8 Hz, H-3′), δ 6.70 (1H, d, *J* = 7.8 Hz, H-5′) and a triplet at δ 7.40 (1H, *J* = 8 Hz, H-4′) in the ^1H NMR spectrum suggested the 2,6-disubstituted pattern in ring B. Two signals at δ 3.87 and δ 3.82 suggested the presence of two aromatic methoxyl groups. These assignments are clearly supported by the ^1H–1H COSY and HSQC experiments. Moreover, three

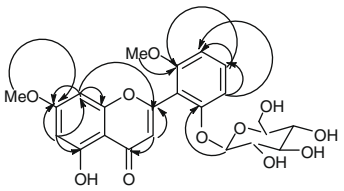

Figure 3. HMBC correlations of the compound **2**.

multiplets at δ 3.92–3.70, δ 3.55–3.40, and δ 3.10–2.90, together with a characteristic signal at δ 4.95 (1H, d, J = 7.8 Hz,) in ^{1}H NMR spectrum confirmed the glycoside linkage. Acid hydrolysis of **2** produced aglycon and D-glucose as the sole sugar, as determined by TLC, GC and comparison with the authentic sugar sample. Finally, the HMBC correlation between the anomeric proton at δ 4.95 (1H, d, J = 7.8 Hz) and C-2′ (δ 158.37) indicated the presence of a β-D-glucopyranosyl moiety, attached to C-2′ of the aglycon moiety through the anomeric carbon (Fig. 3).

In the HMBC spectrum of 2, the carbon skeleton suggested by several diagnostic correlations (H-3/C-2, C-4; H-6/C-5, C-7, C-8; H-8/C-6, C-9; H-3′/C-1′, C-4′, C-5′; H-4′/C-2′, C-3′, C-6′; H-1″/C-2′; H-2″/C-1″, C-3″; and ^{1}H–1H COSY (H-3′/H-4′; H-4′/H-5′; H-1″/H-2″; H-5″/H-6″). Based on the above data,[22] compound **2** was identified as 5- hydroxyl-7-methoxy-2-(2-methoxy-6-(3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yloxy)phenyl)-4*H*-chromen-4-one.

The Gastroprotective activities were investigated for crude extracts and the isolates against various gastric ulceration models in Wistar rats.[23,24] At two random dosage of 100 mg and 250 mg/kg body weights, hexane and acetone extracts displayed mild to moderate gastroprotective activity (Fig 4). Acetone extract displayed better activity than hexane extract. As shown in Figure 4, all the isolates displayed varying degrees of gastroprotective potentials. Among them, chrysin (**8**) displayed better activity in PLU and CRU models. Further, fractionation of active acetone extract led to the isolation of two new compounds (**1**,**2**) with the better gastroprotective potential in various ulcer inducing models (Fig. 4). Compound **1** displayed more potent activity than compound **2**. Though it is difficult to discuss the structure activity relationship criteria responsible for gastroprotective activities in this set of compounds, presence of free phenyl ring (ring B) **6, 7, 8** appears important in imparting gastroprotective activity when compared with **5**. However, absence of methoxyl group at 6th position in compound **8** may responsible for better activity in PLU and CRU animal models (Fig. 4A and B). Additionally, presence of hydroxyl group at 7th position in gastroprotection cannot be ruled out, as its absence in compounds **4** and **2** drastically decreased gastroprotective activities. However, methoxy group at 7th position appears to improve gastroprotection in EIU and AIU models (Fig. 4C and D) as is observed with the compounds **4**, **5** and **2.** Furthermore, glycosidation at 2′-position in compound **2** was observed to enhance the activity than **5**. It is important to note that glucuronization of hydroxyl group at 7th position in compound **6** significantly improved the gastroprotective properties in all the ulcer induced models as evident in the case of compound **1**.

This communication observed that compound **1** with free phenyl ring (ring B) together with glucuronide linkage is the most potent compound with gastroprotective activities among all the isolates and animal models studied, however, gastroprotective contribution of other molecules cannot be ruled out taking in to account the holistic therapeutic approach of traditional medicinal formulations. This is the first scientific report explaining gastroprotective properties in traditional Indian medicinal plant *O. indicum* based on the chemical composition and isolation of new compounds **1** and **2**.

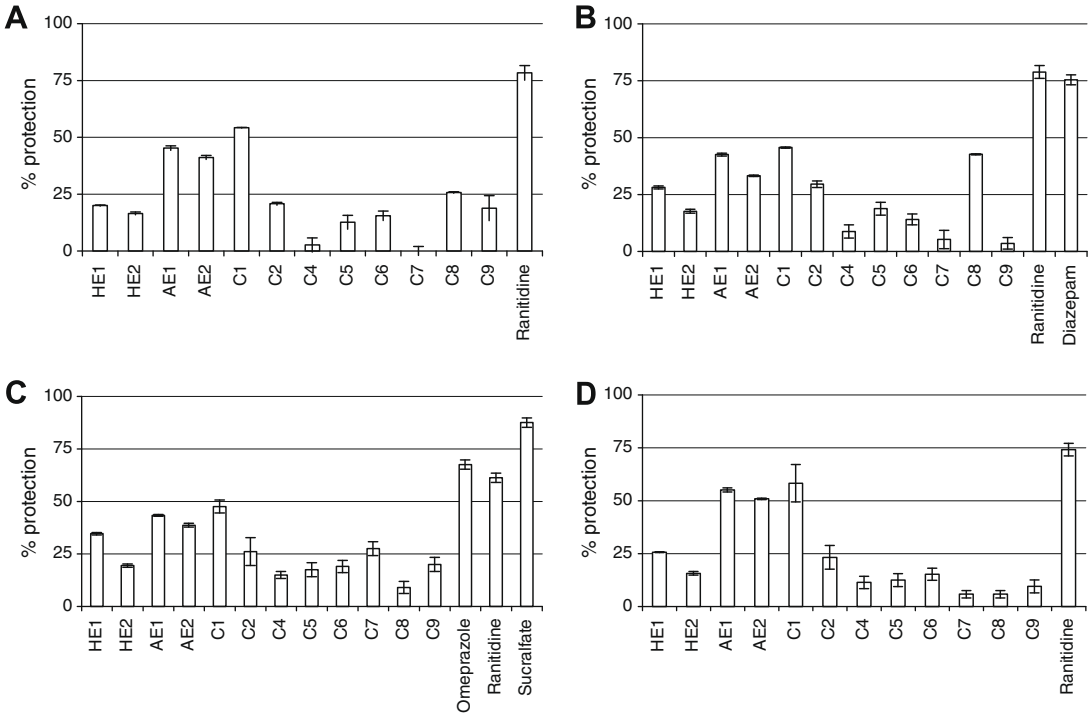

Figure 4. Ulcer protective potentials of the compounds isolated from *O. indicum*. (A) Pylorus ligation induced ulcer (PLU), (B) cold-restrain induced ulceritis (CRU), (C) ethanol induced ulceritis (EIU) and (D) aspirin induced ulceration (AIU) models in rats. Values represent% protection against individual models (mean ± SD). HE1, AE1 and HE2, AE2 represent hexane and acetone extracts at the dose of 250 and 100 mg/kg body weight, respectively. Compounds were dosed at 25-mg/kg-body weight each. Percentage of protection with standard drugs Omiprazole, Ranitidine Sucralfate and Diazepam were studied at the dose of 30 mg, 50 mg, 400 mg and 1 mg/kg body weight, respectively.

Acknowledgment

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20. *Acid hydrolysis of compound* **1**: compound **1** (10 mg) was refluxed with 7% HCl (10 mL) at 70 °C for 3 h. After cooling, the reaction mixture was diluted with H_2O and then extracted with CH_2Cl_2 (2 mL each) four times. The combined organic layers were evaporated to dryness to give residue (3 mg), which was identified as dihydrooroxylin A (**6**) on the basis of TLC and spectral data (NMR and MS).The aqueous phase was neutralized with Ag_2CO_3 powder and then filtered to remove the inorganic materials. The filtrate was concentrated to dryness and purified by preparative HPLC (Column Phenomenex Luna (C18, 150 × 4.6 mm, 5 μm); Solvent system: acetonitrile/water/0.1% acetic acid (1:1); PDA detector) to yield 2 mg of sugar, respectively. The sugar constituent of **1** was determined to be D-glucuronic acid by comparison of their HPLC retention times with that of an authentic sample (obtained from Sigma) (t_R: 4.1 min) and measurement of their optical rotations ($[\alpha]_D^{25}$ +26.4, *c* 0.05 H_2O).
21. *Spectral data for* **1**: pale yellow solid, mp 165 °C; $[\alpha]_D^{25}$ −46.4 (*c* 0.05 MeOH). ^{1}H NMR (300 MHz, $CDCl_3$ + MeOH-d_4): δ 5.46 (1H, 12.0, 3.0 Hz, H-2), 3.14, (1H, dd, *J* = 17.5, 12.0 Hz, H-3ax), 2.82 (1H, dd, *J* = 17.5 3.0 Hz, H-3eq), 3.85 (3H, s, OMe-5), 6.28 (1H, s, H-8), 7.57–7.48 (2H, m, H-2′, 6′), 7.45–7.39 (3H, m, H-3′, 4′, 5′), 5.02 (1H, d, *J* = 7.8 Hz, H-1″), 3.70–3.52 (3H, m, H-2″, 3″, 4″), 4.02 (1H, d, *J* = 4.0 Hz, H-5″), 3.78 (3H, s, OMe-6″). ^{13}C NMR (75 MHz, $CDCl_3$ + MeOH-d_4): δ 80.89 (C-2), 44.37 (C-3), 198.78 (C-4), 159.26 (C-5), 132.13 (C-6), 156.44 (C-7), 96.15 (C-8), 159.88 (C-9), 105.37 (C-10), 139.98 (C-1′), 127.48 (C-2′,6′), 129.85 (C-3′, 4′, 5′), 101.52 (C-1″), 77.00 (C-2″), 76.75 (C-3″), 74.33 (C-4″), 72.77 (C-5″), 170.75 (C-6″), 61.75 (OMe), 53.15 (OMe). ESIMS: *m*/*z* 499 $[M+Na]^+$. HRESIMS: *m*/*z* 499.1535 $[M+Na]^+$ (calcd for $C_{23}H_{24}O_{11}$, 499.1216).
22. *Spectral data for* **2**: ^{1}H NMR (300 MHz, $CDCl_3$ + MeOH-d_4): δ 6.42 (1H, s, H-3), 6.42 (1H, d, *J* = 2 Hz, H-6), 6.34 (1H, d, *J* = 2 Hz, H-8), 6.82 (1H, d, *J* = 8 Hz, H-3′), 7.40 (1H, t, *J* = 8 Hz, H-4′), 6.70 (1H, d, *J* = 8 Hz, H-5′), 4.97 (1H, d, *J* = 7.8 Hz, H-1″), 3.89–3.70 (2H, m, H-2″, H-3″), 3.10–2.90 (1H, m, H-4″), 3.55–3.40 (2H, m, H-5″, H-6″), 3.95 (3H, s, OMe), 3.90 (3H, s, OMe). ^{13}C NMR (75 MHz, $CDCl_3$ + MeOH-d_4): δ 161.87 (C-2), 113.33 (C-3), 182.64 (C-4), 161.05 (C-5), 98.04 (C-6), 165.39 (C-7), 92.48 (C-8), 158.76 (C-9), 105.54 (C-10), 112.33 (C-1′), 158.37 (C-2′), 105.73 (C-3′), 132.56 (C-4′), 108.35 (C-5′), 156.25 (C-6′), 101.46 (C-1″), 73.21 (C-2″), 76.60 (C-3″), 69.93 (C-4″), 76.80 (C-5″), 61.81 (C-6″), 56.09 (OMe), 55.76 (OMe). ESIMS: *m*/*z* 499. HRESIMS: *m*/*z* 499.1211 $[M+Na]^+$ (calcd for $C_{23}H_{24}O_{11}$, 499.1216).
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24. *Animal experiments*: male Wister rats (170–180 g Body weight) were purchased from National Institution of Nutrition, Hyderabad, India. All the experiments were performed as per the norms and permission of institutional ethical committee of University of Gulbarga, Gulbarga, India. In each group six animals were taken. In brief, experiments were carried out as follows: (i) *Pylorus ligation test*: 48-h food deprived animals were given test samples in respective dosages. After 1 h of test sample administration (1% Twen-80 suspension, 1 mL each rat), abdominal cavity was cut open under anesthesia and stomach was ligated at pylorus end. Four hours after pylorus ligation, animals were sacrificed and studied as reported.[12] (ii) *Cold restraint induced ulceritis*: experiments were performed as reported by Ogle et al.[11] After oral administration of test samples, animals were restrained to cold (4 °C) for 2 h. Thereafter, abdominal cavity was cut open. Stomach was exposed along greater curvature and severity of gastric ulcer was assessed in terms of mean ulcer index. (iii) *Ethanol induced ulceritis*: This experiment was performed as described by Jayaraj et al.[10] Forty eight-hour fasting rats were given respective dosages of the test sample and 1 h after oral test sample administration, ethanol was given intragastrically. One hour after ethanol challenge, stomach of rats was ligated at the pylorus end under anesthesia. Four hours after pylorus ligation, animals were sacrificed for the study of ulcer indexing (iv) *Aspirin induced ulceritis* was produced according to Yetkin et al.[9] In this experiment, each group of animal received respective dosages test samples for five days, followed by administration of 200 mg/kg body weight Aspirin suspended in Tween-80 (1%). At the end of the drug administration schedule, animals were fatsed for 24 h and sacrificed by cervical dislocation. Their stomach was opened along the greater curvature, washed with luke warm saline and examined under a dissecting microscope for ulcer index.