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EXPLORING THE MECHANISMS OF GASTROPROTECTIVE ACTION OF ALSTONIA SCHOLARIS IN RATS

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Abstract:

Background and Aim: *Alstonia scholaris (A. scholaris) is used traditionally in folk medicines for inflammation, wound, dyspepsia, gastritis and ulcer. Previously, it has been reported to possess gastro-protective activity in ethanol-induced ulcer model. However, it has not been established that how does A. scholaris protect the stomach from exogenous inflammatory agents. Keeping in view above statement, the study was aimed to investigate various pathways possibly involved in protecting the gastric mucosa from agent like ethanol.*

Materials and Methods: *Five different pathways; α-2 adrenergic receptor, ATP-dependent K^+ channel, Histamine, Prostaglandin and Nitric oxide were examined through their specific inhibitors to explore their involvement in gastro-protective action of aqueous extract of A. scholaris leaves. In every pathway, animals were pretreated with their specific agonist or antagonist for 30 mins following administration of aqueous extract of A. scholaris leaves for an hour. After that ethanol was administered for an hour and then animals were sacrificed. Biochemical and physiological parameters such as volume of gastric acid, its pH, calculation of ulcer index by examining the score of ulcer, calculation of total acidity, protein contents and mucous adherence to gastric wall were determined. The data were analyzed by using one way ANNOVA followed by Dunnett's test.*

Key Results: *A. scholaris caused a significant ($p < 0.001$) gastro-protective effect in ethanol induced ulcer model. Glibenclamide (5mg/kg, p.o.), inhibitor of K_{ATP}-channel and Histamine (3mg/kg, s.c.), an agonist of histamine pathway suppressed the gastro-protective action of A. scholaris. However, its gastro-protective activity was observed in α-2 adrenergic receptor, Prostaglandin and Nitric oxide pathway in the presence of their respective antagonists.*

Conclusions and Implications: *The data suggests that gastro-protective activity of aqueous extract of A. scholaris leaves may involve blockade of histamine receptors most likely H-2 receptor. In addition, it also showed its ability to stimulate K_{ATP} channels in ATP-dependent K^+ channel mediated pathway. Therefore, it is concluded that A. scholaris has shown a tendency to modify proton pump in a way to be protective of gastric mucosa.*

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INTRODUCTION:

Alstonia scholaris is evergreen tropical plant native to Subcontinent, Nepal, Pakistan, China, Srilanka, Southeast Asia, Sub-Himalayan belt and Africa. It grows widely in fugacious, dried, evergreen forests of west Bengal, India, Pakistan and in even plains and landscapes. It is an elegant verdure tree and has turned out to be most mainstream decorative tree in gardens, landscapes, parks, in the warm and temperate regions of Texas, California and Florida in USA because of its large canopy like appearance. It belongs to the Apocynaceae family. This family comprises of more than 250 or more genera and 2000 types of tropical trees, vines and bushes and exhibits high biological activities and medicinal properties. Well known of this family are *Alstonia venenata*, *Alstonia scholaris* and *Rauwolfia serpentina* because of their medicinal properties. *Alstonia scholaris* is commonly called devils tree, Black board tree, milk wood, cheesewood; Sanskrit (Saptaparna) local urdu name Shaitan wood, Chatiana; Trade name (Pulai).
Nearly 400 compounds have been identified. Alkaloids, flavonoids, steroids, saponins, tannins, phenols, coumarins were reported as chief chemical constituents (Khyade & Vaikos, 2009). Important alkaloids such as echitamine, tubotaiwine, akuammcine, echitamidine, picrinine, strictamine, ditamine, echitenine are found in *Alstonia scholaris. A.S.* leaves contains various alkaloids such as scholarcine, 19-epischolarcine, nareline ethyl-ether, lagunamine (19-hydroxytubotaiwine), angustilobine B acid, isoalschomine and netulin (Arulmozhi *et al.*, 2007).

The indole alkaloids alstonamine and rhazmanine have been separated from leaves (Khyade & Vaikos, 2009). Non-alkaloids constituents such as ursolic acid, β-sitosterol, cycloeucalenol, α-amyrin acetate, squalene, α-tocopherol, Alstonic acid A and Alstonic acid B. (Hui *et al.*, 2009). Flowers contains alkaloids, carbohydrates, amino acids, saponins, fixed oils and fats (Thankamani *et al.*, 2011). Non-alkaloids constituents of flowers are n-hexacosane, ursolic acid, palmitic acids and β-amyrin. Flowers of *A.S.* yields mostly an essential oil by steam distillation process (Meena *et al.*, 2011). The alkaloids found in bark and latex of *A.S.* are echitamine, ditamine and echitenine (Dey, 2011; Meena *et al.*, 2011). The leaves of *A.S.* also contains flavonoids like kaempferol, quercetin, isorhamnetin-3-O-β-D-galactopyranoside, isorhamnetin (Hui *et al.*, 2009). Different phytoconstituents of *A.S.* incorporates bgr-amyrin, agr-amyrin, venenative, lupeol acetate, rhazine, linalool, alpha-terpineol, 2-phenylethyl acetate, cis/trans linalool oxides, yohimbine and steroids also reported (Dey, 2011). *A.S.* ethanolic leaves extract showed remarkable antiulcer activity by pyloric ligation method (Arulmozhi *et al.*, 2012).

MATERIALS AND METHODS:

Chemicals were imported from Sigma-Aldrich chemical Co. USA and some chemicals were purchased from local market. Chemicals utilized in experiments are absolute ethanol, Formalin (Merck, KGaA-64721 Darmstadt, Germany), Di-ethyl ether (Labscan Asia Co.Ltd, Thailand), Albumin Bovine Serum (BioShop, Canada), Alcian Blue 8GX (UNI-Chemicals Reagent, China), Folin and Ciocalteu's Phenol reagent (Central Drug House Ltd, New Delhi, India), Tris-Hydrochloride (Sigma-Aldrich Chemical Co.USA), Sodium Carbonate, Sodium Hydroxide and Magnesium Chloride (BDH Chemicals Ltd. Poole, England), Copper(II) Sulphate Pentahydrate (Merck, Germany), Sucrose (RDH LaborchemiKalien GmbH & co. Seeize), Potassium-Sodium Tartrate terahydrate (Sigma, Germany), Yohimbine (Sigma-Aldrich Chemical Co.USA), Misoprostol (Getz Pharma, pvt Ltd.), L-Name (Sigma-Aldrich Chemical Co. Korea), L-Arginine (Sigma-Aldrich Chemical Co.USA), Glibenclamide (Pfizer pvt. Ltd.), Histamine, Ranitidine, Piroxicam (Sigma-Aldrich co.Uk), Chloroform (VW chemicals, India), Phenolphthalein (Fisher Scientific), Sodium carbonate, Sodium hydroxide and magnesium chloride (BDH Chemicals Ltd. Poole, England), Sucrose (RDH GmbH & Co. Seeize), Normal Saline (Otsuka, Pakistan Ltd). The instruments used in experimental study are analytical weighing balance (AB54-S by Mettle Toledo, Switzerland), pH meter (pH 720 VELP Scientifica), Rotary evaporator (Heidolph Lab 4002 Sigma Aldrich, Germany), Homogenizer (Wise Stir, HS 30E, Daihan Sceintific Co. Ltd. Korea), Oven (model U30, Memert West U.S.A), Chiller (MDF-U32V, Sanyo electric Co. Ltd), Refrigerator (Dawlance, Pak), Sonicator (Decent store, India), Ice crusher (KT 108), Top load balance (Smith process instrumentation, South Africa), Centrifuge machine (16-PK Sigma lab, Germany), UV-Spectrophotometer (UV 2500, Shimadzu corporation, Kyoto , Japan),Vortex mixer (Biosciences, Korea).

Data was statistically analyzed by the one way analysis of variance (ANOVA) followed by Dunnett's test by using GraphPad Prism version 5.0 and probability level lowers than 0.05 ($p < 0.05$) was considered significant statistically. Results were multifaceted. Hence, multiple comparisons were possible. We compare normal versus control, control versus all other groups and extract group versus antagonist group.

Experimental Protocols

For this set of experiments, 500 mg/kg dose of *A.S.* used since it didn't cause poisonous quality and was intense in diminishing the instigated harm in all experimental models of gastric ulcer. Specific antagonist were used for example Yohimbine (2 mg/kg, i.p.) for alpha-2 (α_2) adrenergic receptor, Piroxicam (200 mg/kg, p.o.) for prostaglandins, For NO synthesis L-Name (10 mg/kg, i.p.), for KATP channels, Glibenclamide (5 mg/kg, p.o.) and Ranitidine for Histamine H_2 receptors or a specific agonists such as L-arginine (600 mg/kg, p.o.) as a control for L-Name and Misoprostol (0.016 mg/kg, p.o.) as a control for Piroxicam. All chemicals were dispersed in saline water (Júnior *et al.*, 2014). In each experimental pathway, rats were administered with their specific agonist or antagonist for 30 min before the oral dose of *A.S.*. The volume of 7 ml/kg, p.o. of 96% absolute ethanol was orally administered one hour after the oral dose of *A.scholaris*. After one hour, rats were relinquished and their stomach were isolated, cautiously cut along the more prominent bend, washed with ordinary saline and subjected to determine various parameters.

RESULTS:

Stomach of rats, after excision and removal of gastric juice and contents were washed with normal saline and observed for ulceration macroscopically. Figures 1 to 10 represent the macroscopic view of streaks, red colouration, red spots, deep ulcer and perforations in experimental pathways. From these, numbers of ulcers were counted and severity score determined accordingly, which was used to calculate ulcer index (UI) and percentage protection.

Figure 11-15 represents ulcer index, Figure 16-20 represents percentage of ulcer protection, Figure 21-25 represents PH value. Figure 26-30 represents gastric volume , Figure 31-35 represents total acidity, Figure 36-41 represents protein contents , Figure 42-47 represents mucus adherence to gastric wall of different pathways used in this experiments.

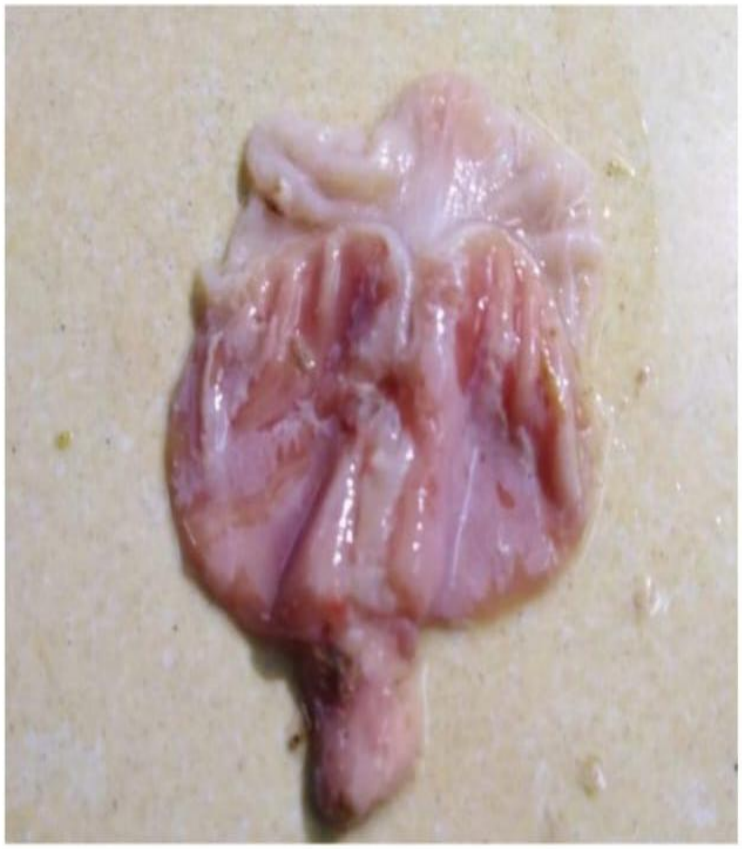

Figure 1. Macroscopic View of Rat Stomach of Normal Group

Each ulcer has score used to calculate the severity score as; Normal (0), Red colouration (0.5), Spot ulcer (1.0), Hemorrhage streak (1.5), Deep ulcer (2.0) and Perforation (3).

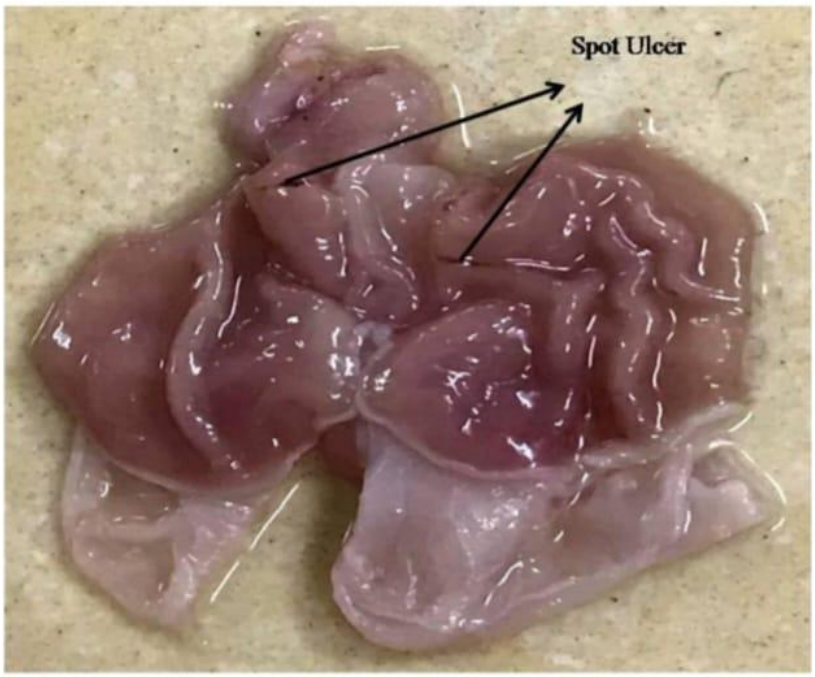

Figure 2. Macroscopic View of Rat Stomach of *Alstonia scholaris* + Ethanol Group

Each ulcer has severity score, used to calculate the ulcer index as; Normal (0), Red colouration (0.5), Spot ulcer (1.0), Hemorrhage streak (1.5), Deep ulcer (2.0) and Perforation (3).

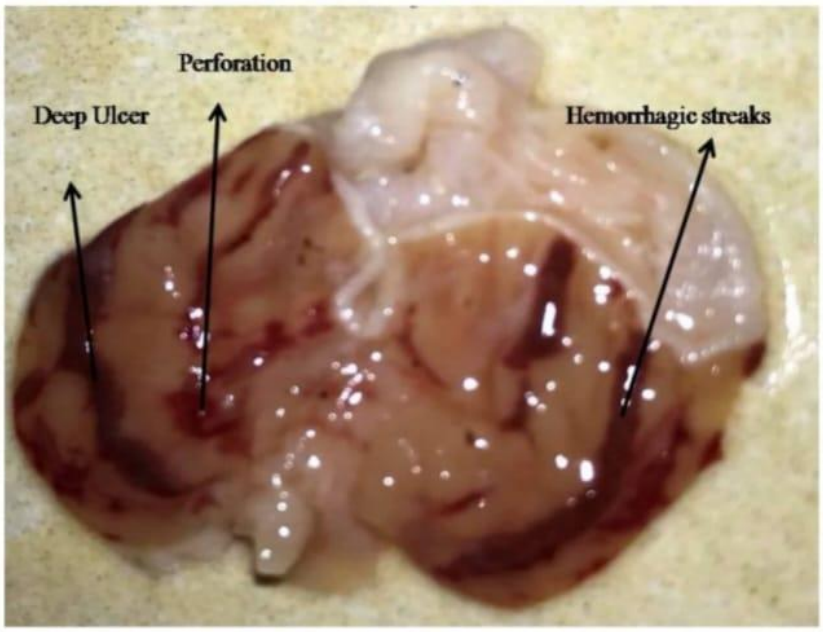

Figure 3. Macroscopic View of Rat Stomach of Histamine + A.S. + Ethanol Group in Histamine Pathway

Each ulcer has severity score, used to calculate the ulcer index as; Normal (0), Red colouration (0.5), Spot ulcer (1.0), Hemorrhage streak (1.5), Deep ulcer (2.0) and Perforation (3).

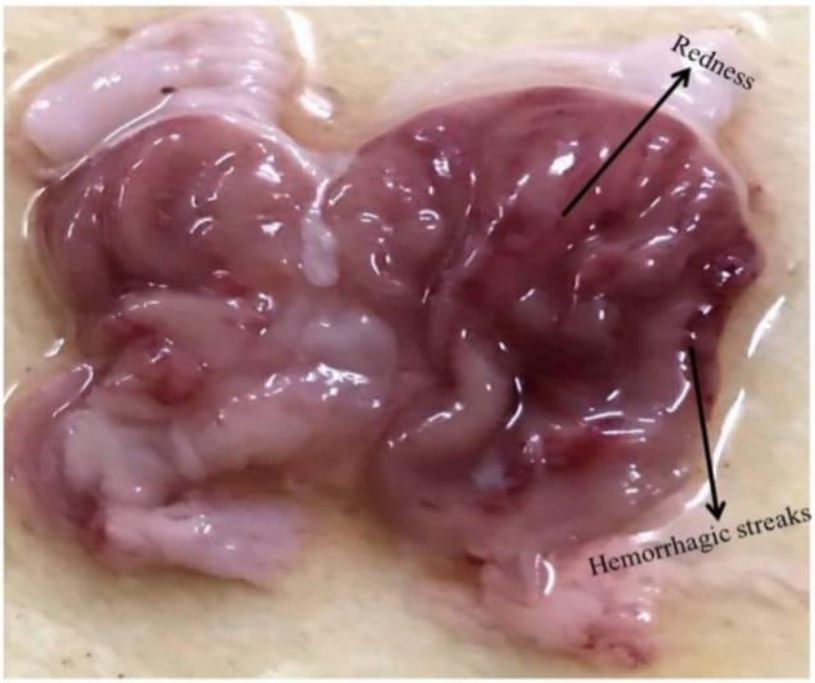

Figure 4. Macroscopic View of Rat Stomach of Glibenclamide + Ethanol Group in ATP-dependent K^+ Channel Pathway

Each ulcer has severity score used to calculate the ulcer index as; Normal (0), Red colouration (0.5), Spot ulcer (1.0), Hemorrhage streak (1.5), Deep ulcer (2.0) and Perforation (3).

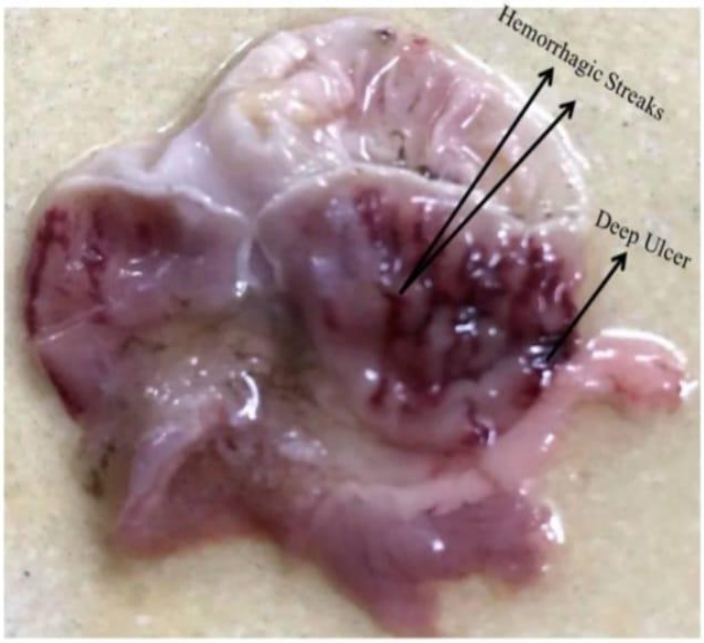

Figure 5. Macroscopic View of Rat Stomach of Glibenclamide + A.S. + Ethanol Group inATP-dependent K^+ Channel Pathway

Each ulcer point has severity score used to calculate the ulcer index as; Normal (0), Red colouration (0.5), Spot ulcer (1.0), Hemorrhage streak (1.5), Deep ulcer (2.0) and Perforation (3).

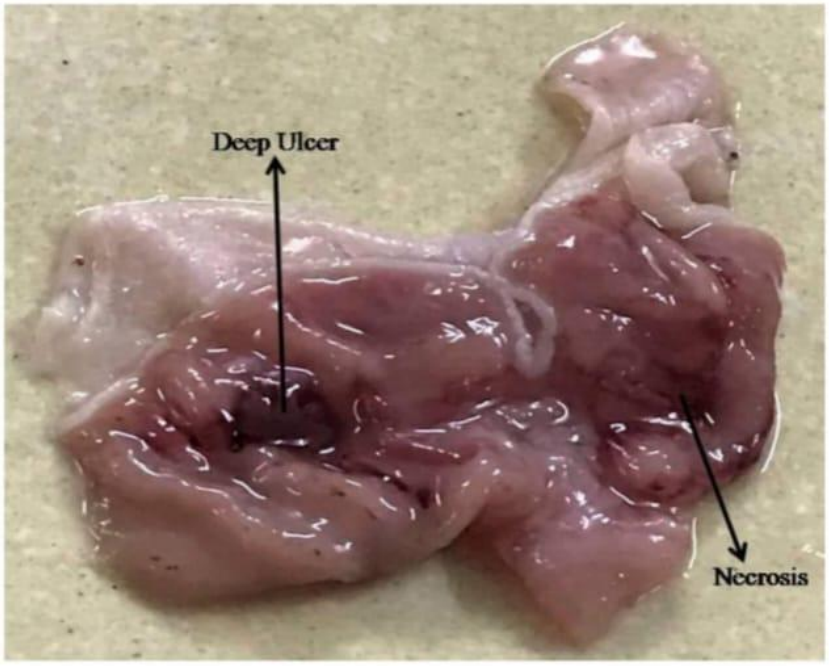

Figure 6. Macroscopic View of Rat Stomach of Piroxicam + Ethanol Group in Prostaglandin Pathway

Each ulcer has severity score used to calculate the ulcer index as; Normal (0), Red colouration (0.5), Spot ulcer (1.0), Hemorrhage streak (1.5), Deep ulcer (2.0) and Perforation (3).

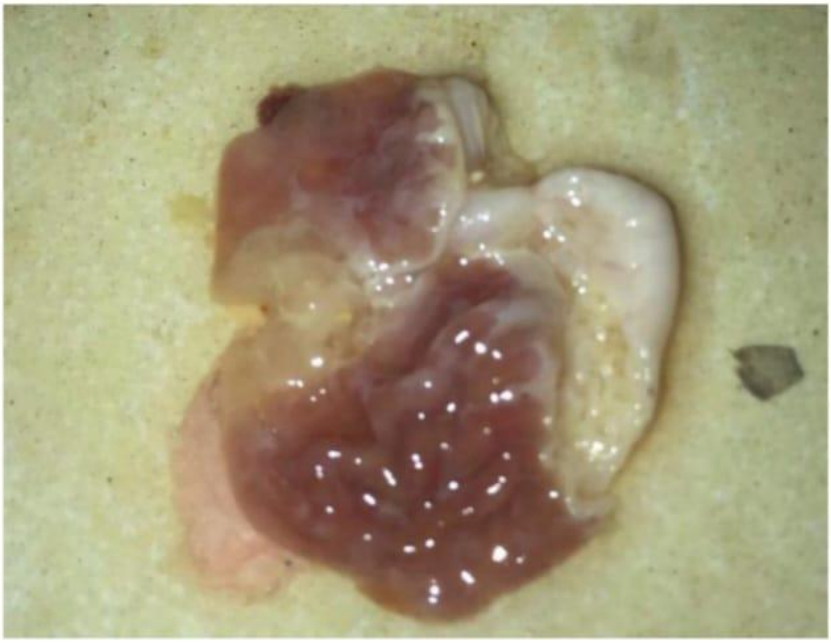

Figure 7. Macroscopic View of Rat Stomach of Misoprostol + Ethanol Group in Prostaglandin Pathway

Each ulcer has severity score used to calculate the ulcer index as; Normal (0), Red colouration (0.5), Spot ulcer (1.0), Hemorrhage streak (1.5), Deep ulcer (2.0) and Perforation (3).

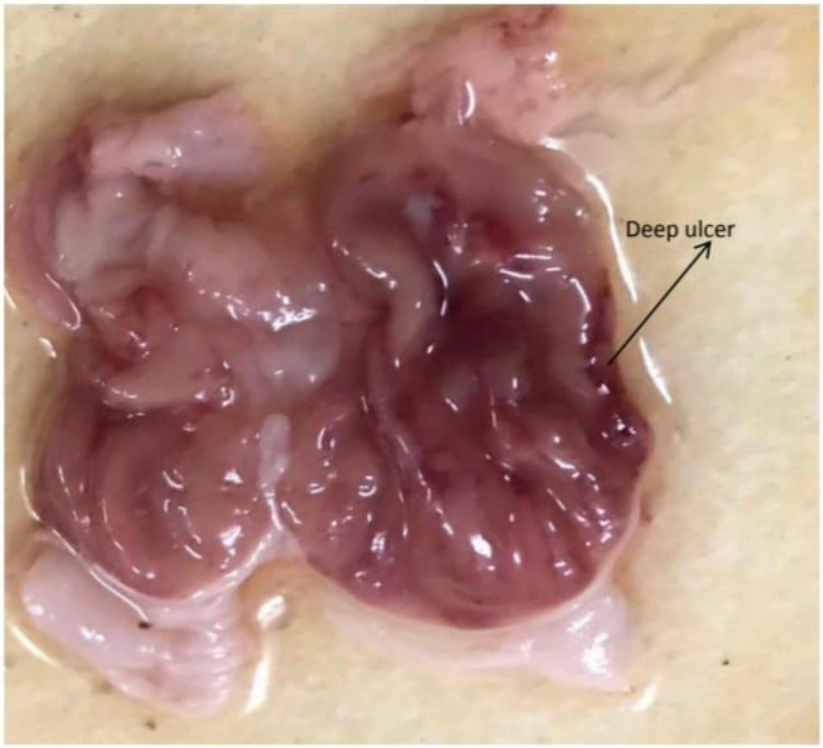

Figure 8. Macroscopic View of Rat Stomach of Yohimbine + Ethanol Group in Alpha-2 Pathway

Each ulcer has score used to calculate the ulcer index as; Normal (0), Red colouration (0.5), Spot ulcer (1.0), Hemorrhage streak (1.5), Deep ulcer (2.0) and Perforation (3).

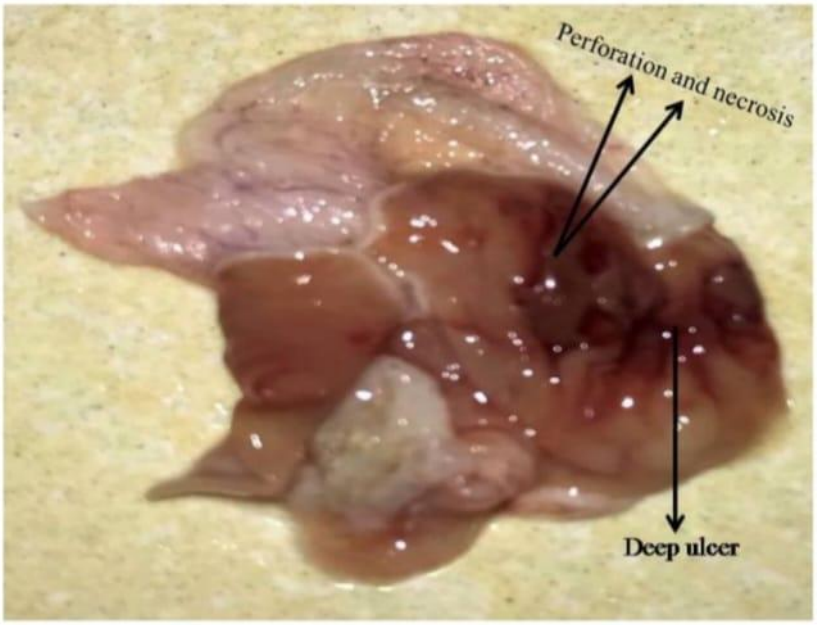

Figure 9. Macroscopic View of Rat Stomach of L-NAME + Ethanol Group in NO Pathway

Each ulcer has score used to calculate the ulcer index as; Normal (0), Red colouration (0.5), Spot ulcer (1.0), Hemorrhage streak (1.5), Deep ulcer (2.0) and Perforation (3).

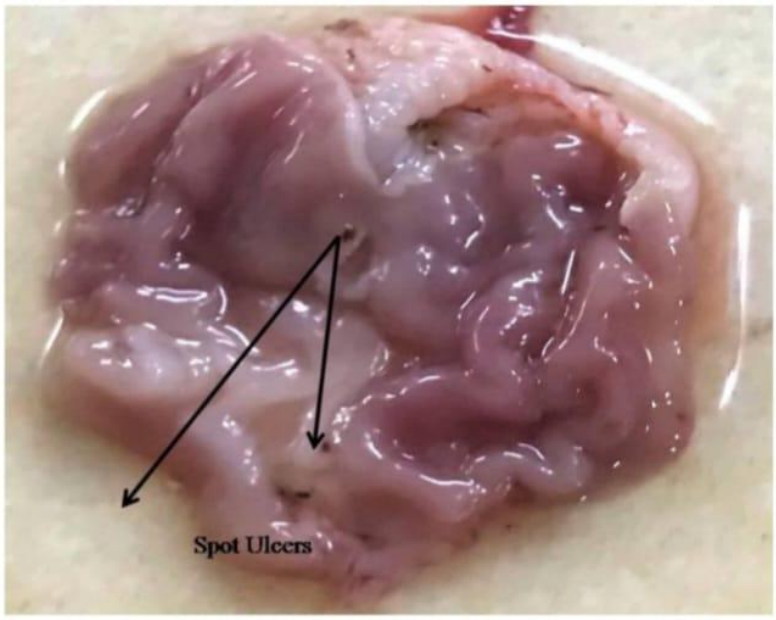

Figure 10. Macroscopic View of Rat Stomach of L-Arginine + A.S. + Ethanol Group in Nitric oxide (NO) Pathway

Each ulcer has score used to calculate the ulcer index as; Normal (0), Red colouration (0.5), Spot ulcer (1.0), Hemorrhage streak (1.5), Deep ulcer (2.0) and Perforation (3).

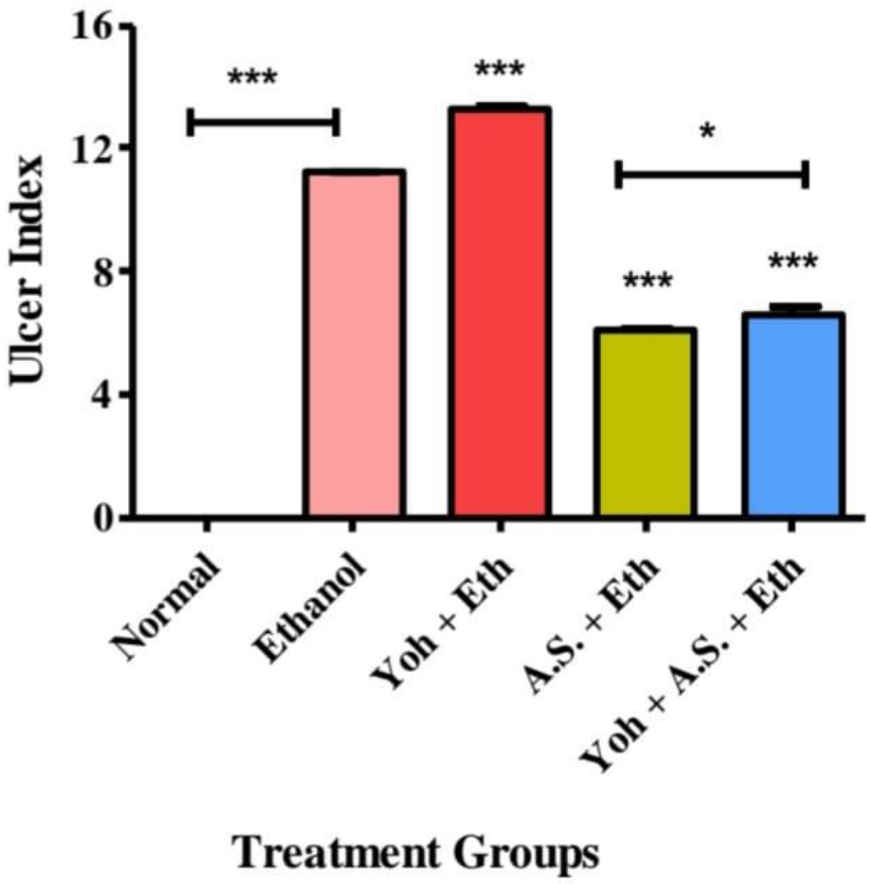

Figure 11. Ulcer Index of Alpha-2 (α-2) Receptor Pathway

Each bar shows mean of 5 rats, whereas upright line above each bar represents S.E.M. Line above the column shows the comparison of Normal versus Ethanol group and Extract (A.S.+ Eth) versus Extract + Antagonist (Yoh + A.S. + Eth) group. *** represents the significance level $p < 0.001$, ** and * are non-significant with respect to Ethanol group by on-way ANOVA followed by Dunnett's test. (Yoh: Yohimbine, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

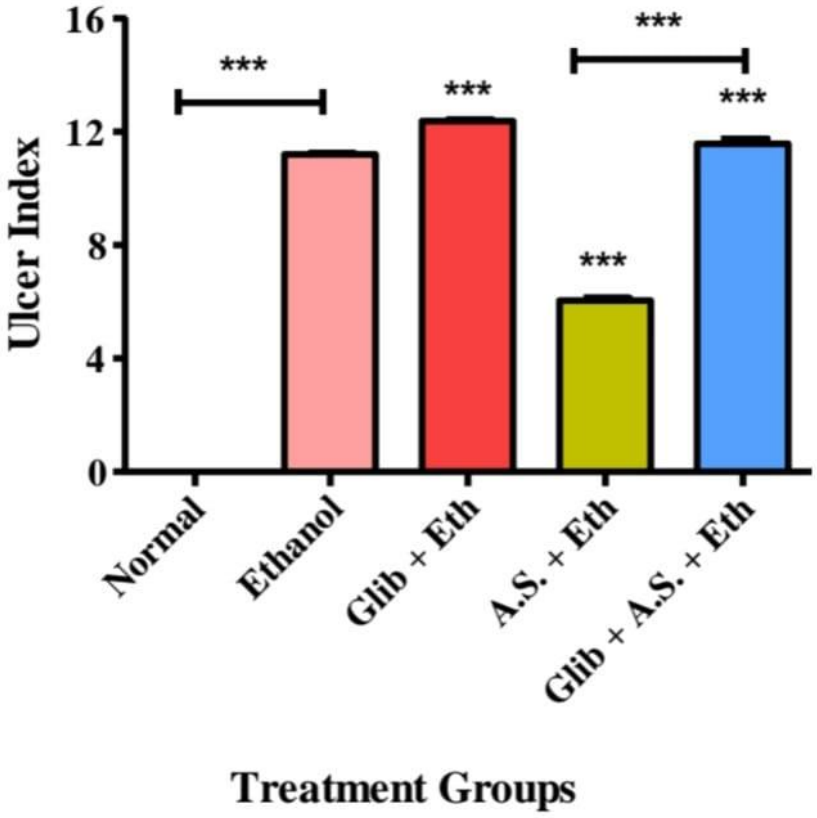

Figure 12. Ulcer Index of ATP-dependent K^+ Channel Pathway

Each bar shows mean of 5 rats, whereas upright line above each bar represents S.E.M. Line above the column shows the comparison of Normal versus Ethanol group and Extract (A.S.+ Eth) versus Extract + Antagonist (Glib + A.S. + Eth) group. *** represents the significance level $p < 0.001$, ** and * are non-significant with respect to Ethanol group by on-way ANOVA followed by Dunnett's test. (Glib: Glibenclamide, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

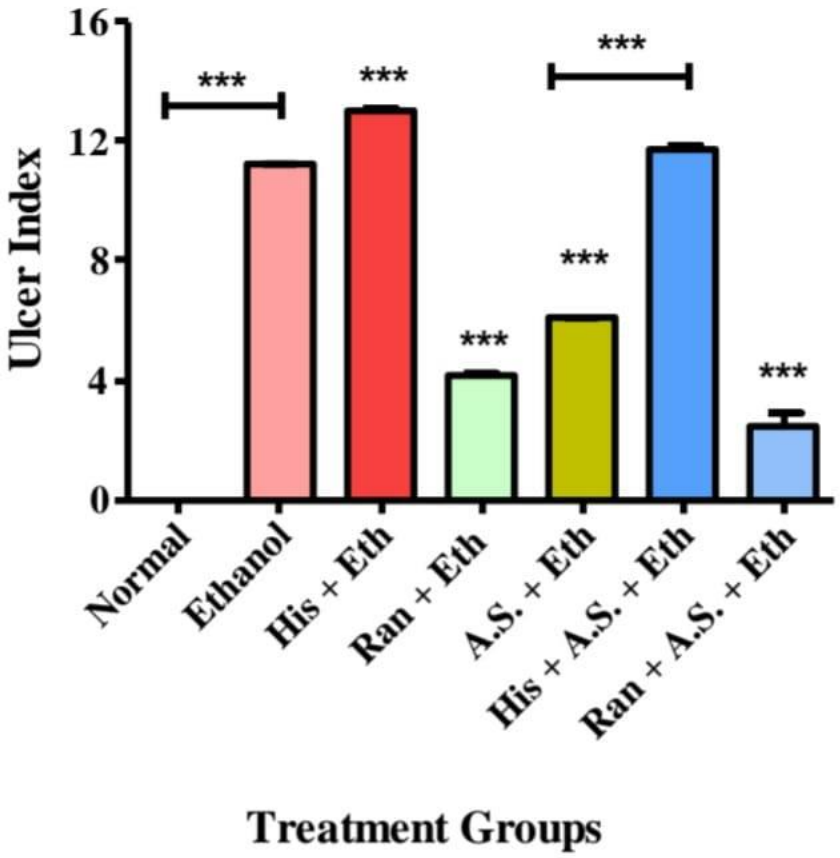

Figure 13. Ulcer Index of Histamine Pathway

Each bar shows mean of 5 rats, whereas upright line above each bar represents S.E.M. Line above the column shows the comparison of Normal versus Ethanol group and Extract (A.S.+ Eth) versus Extract + Agonist (His + A.S. + Eth) group. *** represents the significance level $p < 0.001$, ** and * are non- significant with respect to Ethanol group by on-way ANOVA followed by Dunnett's test. (His: Histamine, Ran: Ranitidine, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

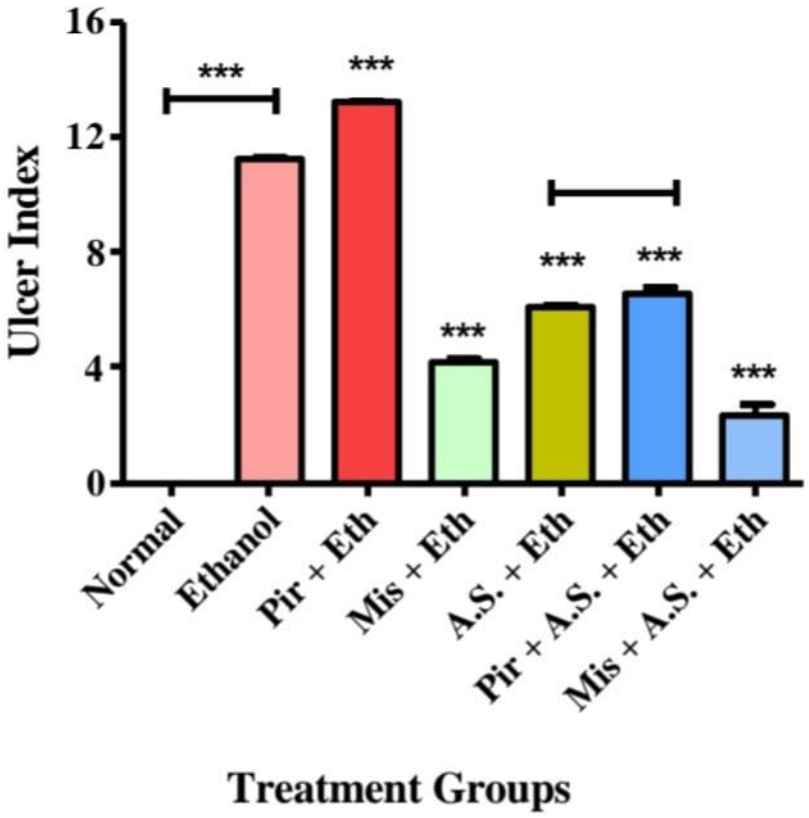

Figure 14. Ulcer Index of Prostaglandin Pathway

Each bar shows mean of 5 rats, whereas upright line above each bar represents S.E.M. Line above the column shows the comparison of Normal versus Ethanol group and Extract (A.S.+ Eth) versus Extract + Antagonist (Pir + A.S. + Eth) group. *** represents the significance level $p < 0.001$, ** and * are non-significant with respect to Ethanol group by on way ANOVA followed by Dunnett's test. (Pir: Piroxicam, Mis: Misoprostol, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

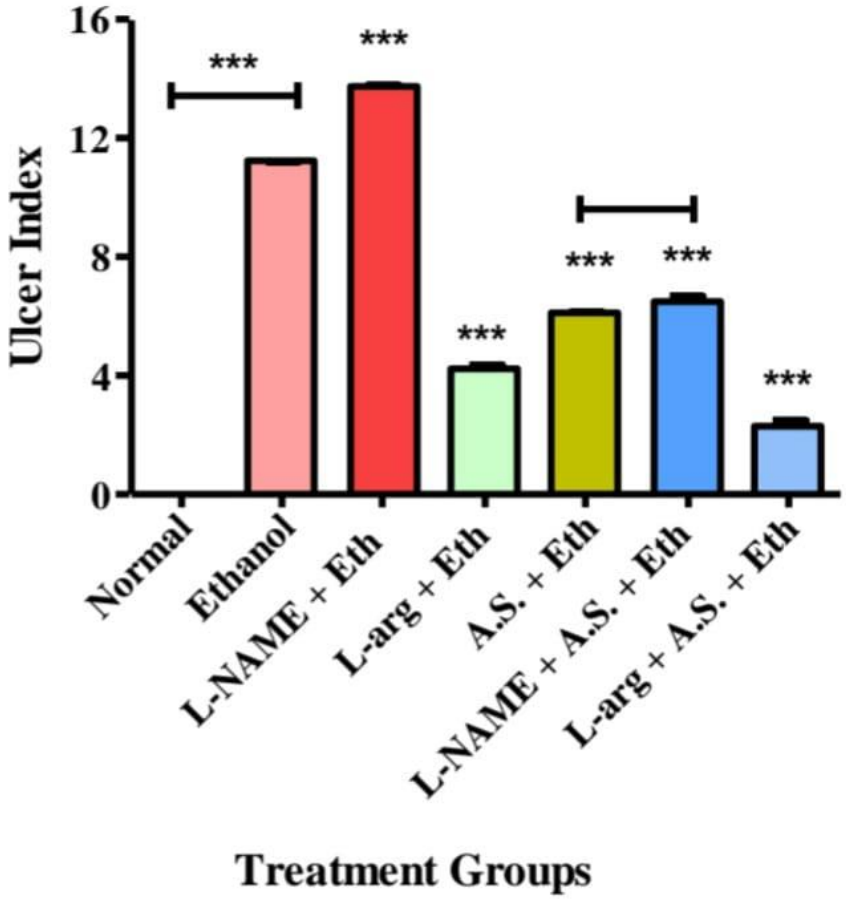

Figure 15. Ulcer Index of Nitric oxide (NO) Pathway

Each bar shows mean of 5 rats, whereas upright line above each bar represents S.E.M. Line above the column shows the comparison of Normal versus Ethanol group and Extract (A.S.+ Eth) versus Extract + Antagonist (L-NAME + A.S. + Eth) group. *** represents the significance level $p < 0.001$, ** and * are non-significant with respect to Ethanol group by on-way ANOVA followed by Dunnett's test. (L-NAME; L-Nitro-Arginine Methyl Ester, L-Arg: L-Arginine, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

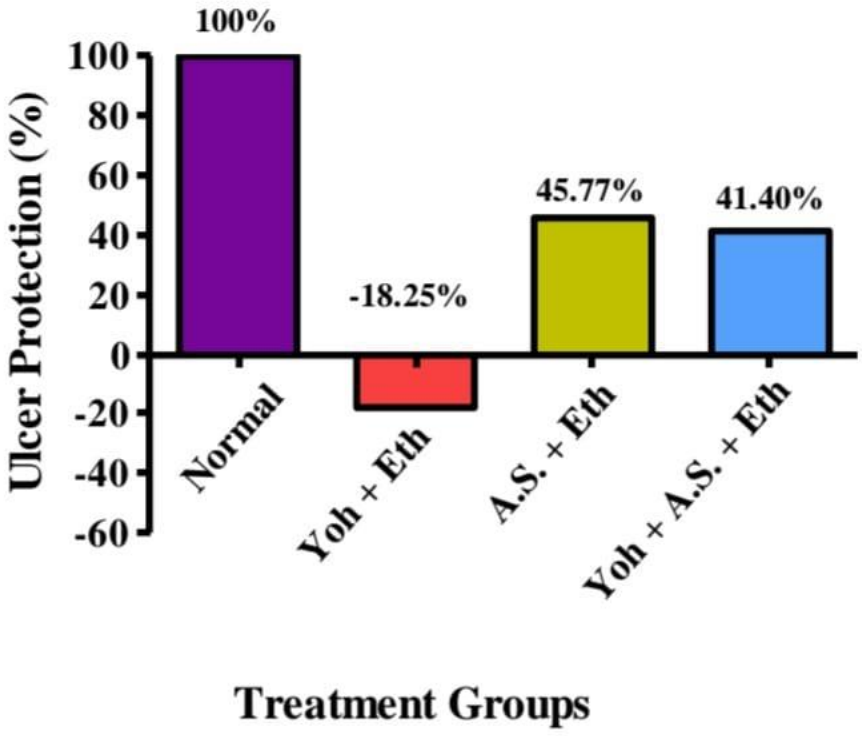

Figure 16. Ulcer Protection (%) of Alpha-2 (α-2) Receptor Pathway

Each bar shows the percentage of ulcer protection calculated for treatment groups when compared with the Ethanol group. (Yoh: Yohimbine, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

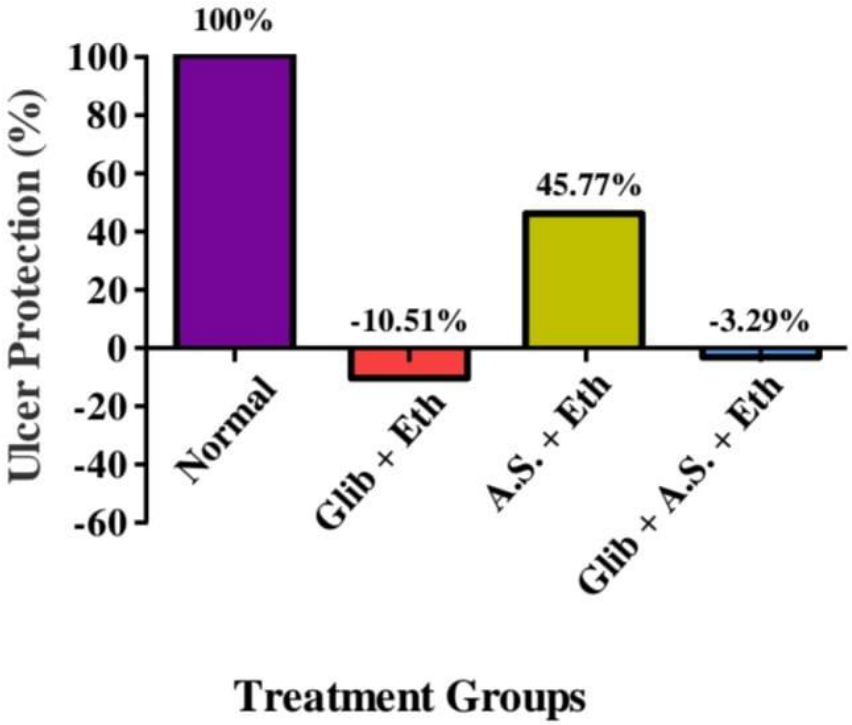

Figure 17. Ulcer Protection (%) of ATP-dependent K^+ Channel Pathway

Each bar shows the percentage of ulcer protection calculated for treatment groups when compared with the Ethanol group. (Glib: Glibenclamide, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

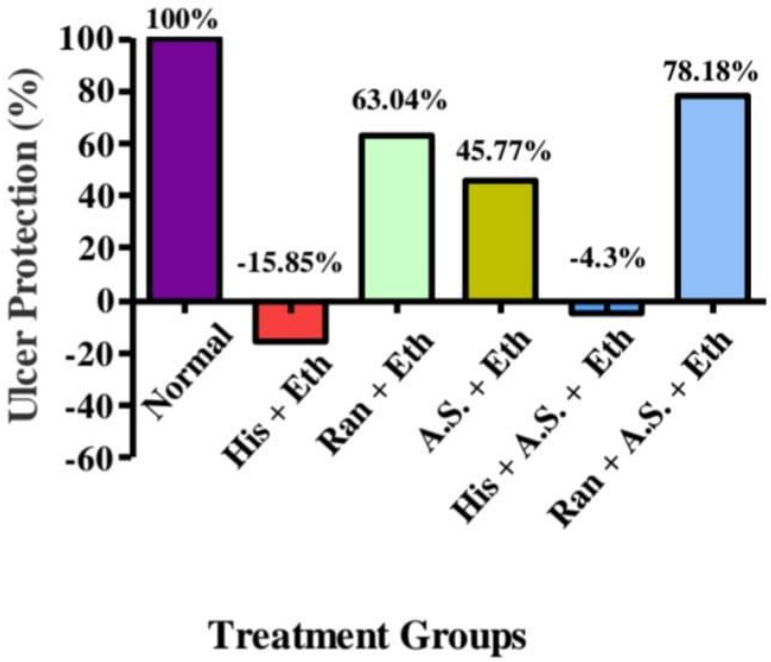

Figure 18. Ulcer Protection (%) of Histamine Pathway

Each bar shows the percentage of ulcer protection calculated for treatment groups when compared with the Ethanol group. (His: Histamine, Ran: Ranitidine, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

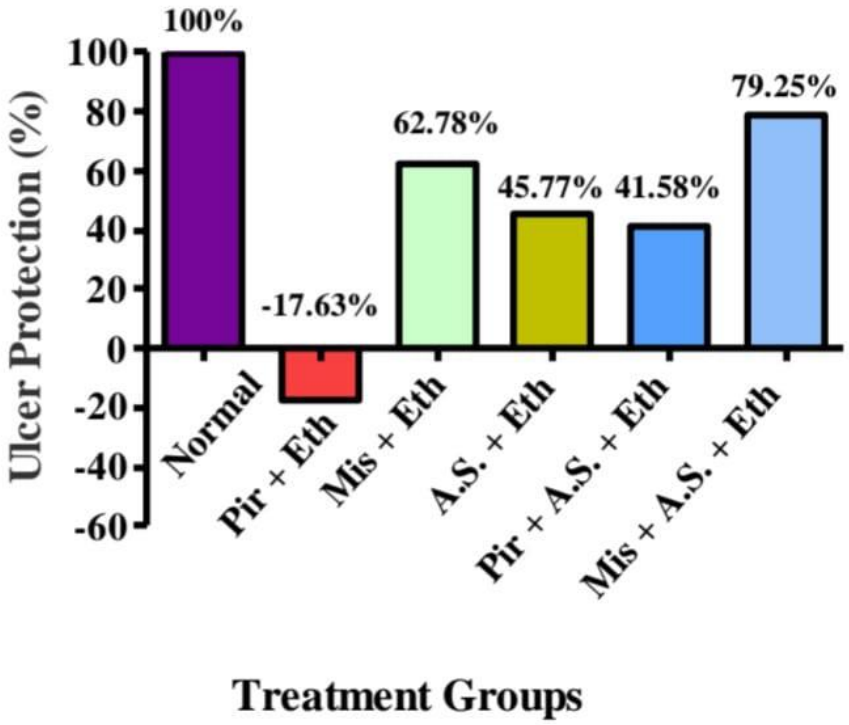

Figure 19. Ulcer Protection (%) of Prostaglandin Pathway

Each bar shows the percentage of ulcer protection calculated for treatment groups when compared with the Ethanol group. (Pir: Piroxicam, Mis: Misoprostol, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

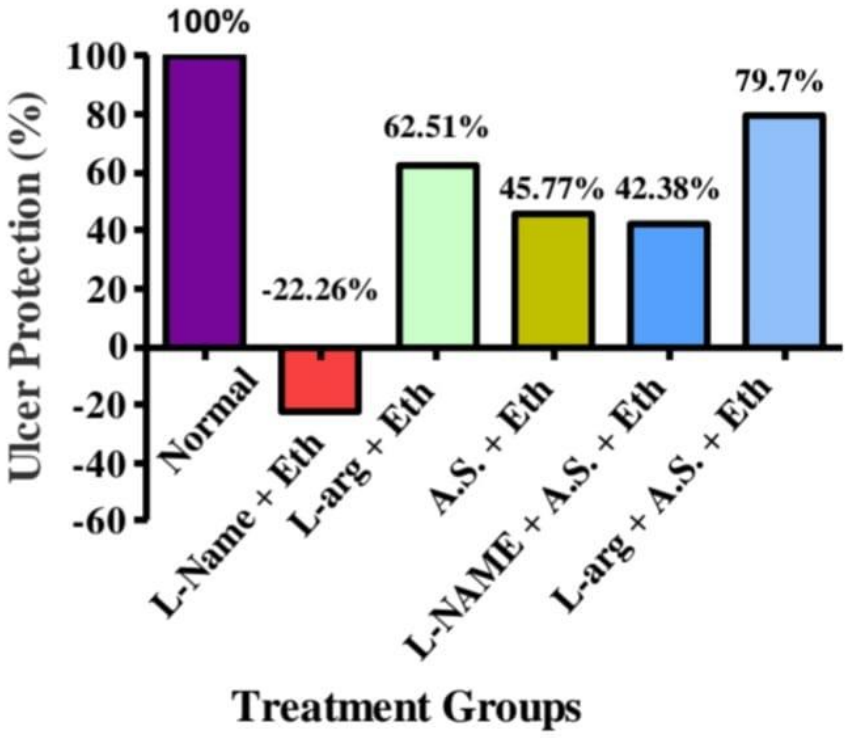

Figure 20. Ulcer Protection (%) of Nitric oxide (NO) Pathway

Each bar shows the percentage of ulcer protection calculated for treatment groups when compared with the Ethanol group. (L-NAME: L-Nitro-Arginine Methyl Ester, L-Arg: L-Arginine, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

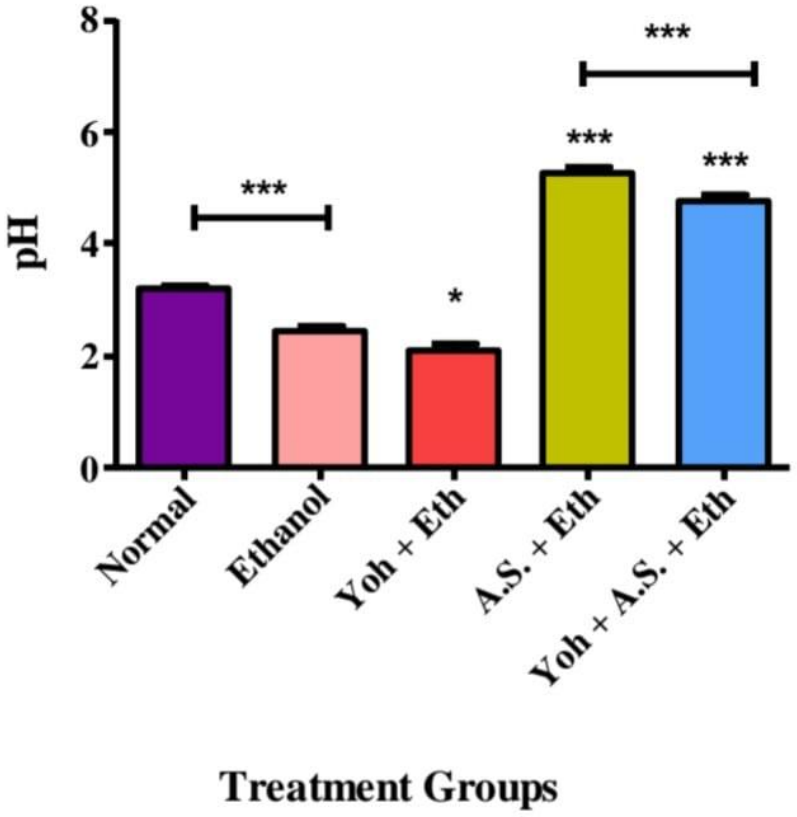

Figure 21. pH of Alpha-2 (α-2) Receptor Pathway

Each bar shows mean of 5 rats, whereas upright line above each bar represents S.E.M. Line above the column shows the comparison of Normal versus Ethanol group and Extract (A.S.+ Eth) versus Extract + Antagonist (Yoh + A.S. + Eth) group. *** represents the significance level $p < 0.001$, ** and * are non-significant with respect to Ethanol group by on-way ANOVA followed by Dunnett's test. (Yoh: Yohimbine, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

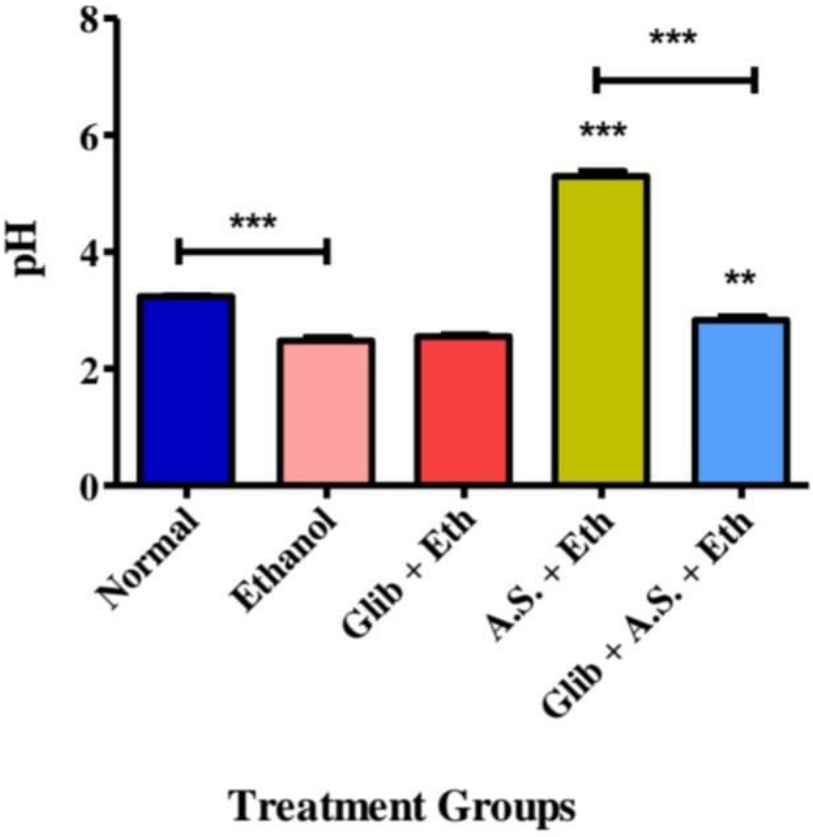

Figure 22. pH of ATP-dependent K^+ Channel Pathway

Each bar shows mean of 5 rats, whereas upright line above each bar represents S.E.M. Line above the column shows the comparison of Normal versus Ethanol group and Extract (A.S.+ Eth) versus Extract + Antagonist (Glib + A.S. + Eth) group. *** represents the significance level $p < 0.001$, ** and * are non significant with respect to Ethanol group by on way ANOVA followed by Dunnett's test. (Glib: Glibenclamide, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

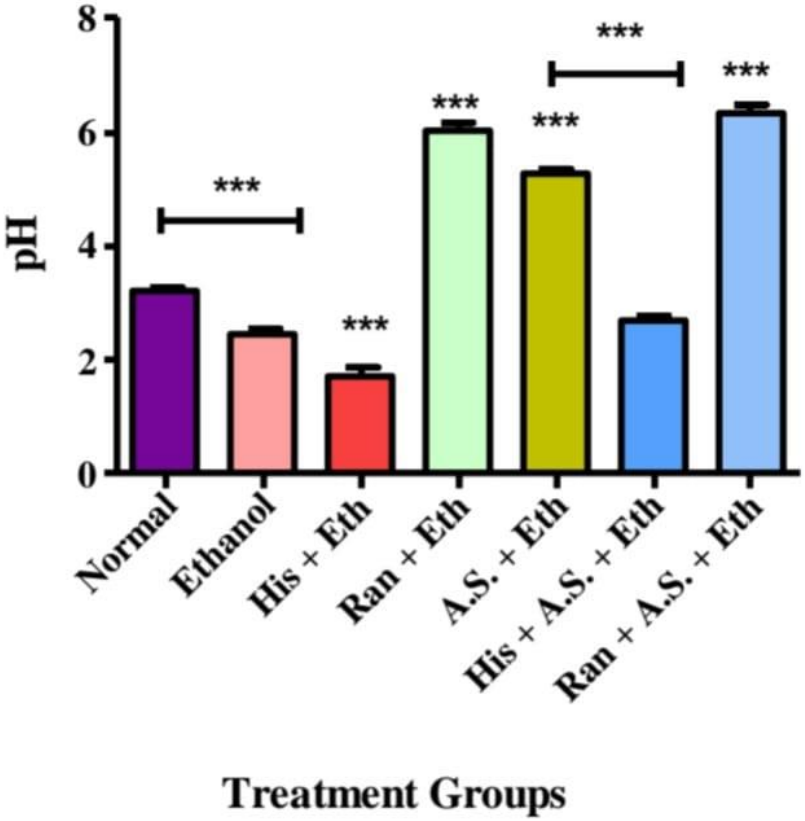

Figure 23. pH of Histamine Pathway

Each bar shows mean of 5 rats, whereas upright line above each bar represents S.E.M. Line above the column shows the comparison of Normal versus Ethanol group and Extract (A.S.+ Eth) versus Extract + Agonist (His + A.S. + Eth) group. *** represents the significance level $p < 0.001$, ** and * are non-significant with respect to Ethanol group by on-way ANOVA followed by Dunnett's test. (His: Histamine, Ran: Ranitidine, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

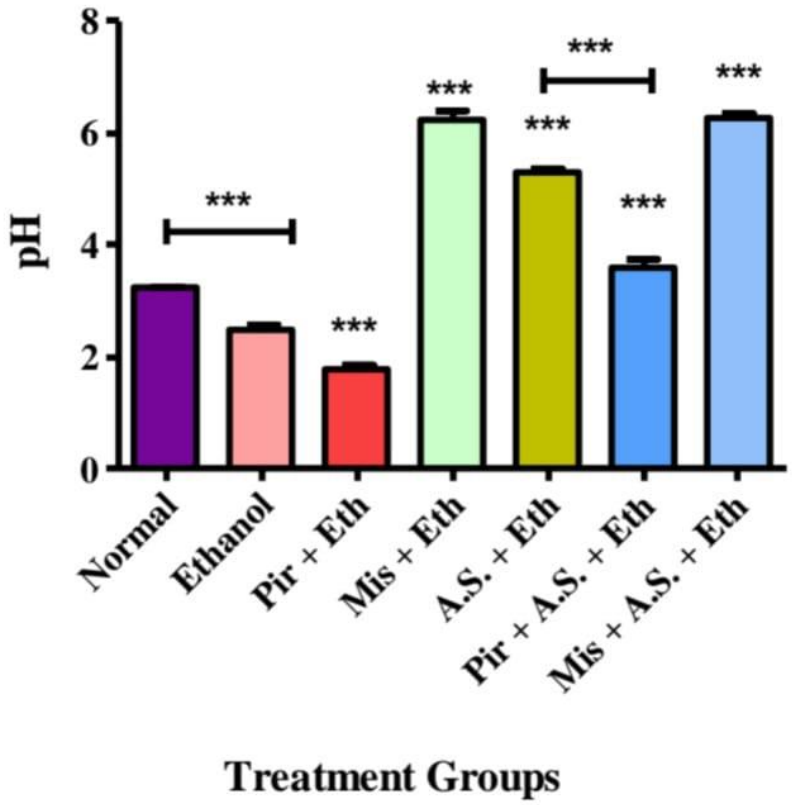

Figure 24. pH of Prostaglandin Pathway

Each bar shows mean of 5 rats, whereas upright line above each bar represents S.E.M. Line above the column shows the comparison of Normal versus Ethanol group and Extract (A.S.+ Eth) versus Extract + Antagonist (Pir + A.S. + Eth) group. *** represents the significance level $p < 0.001$, ** and * are non-significant with respect to Ethanol group by on-way ANOVA followed by Dunnett's test. (Pir: Piroxicam, Mis: Misoprostol, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

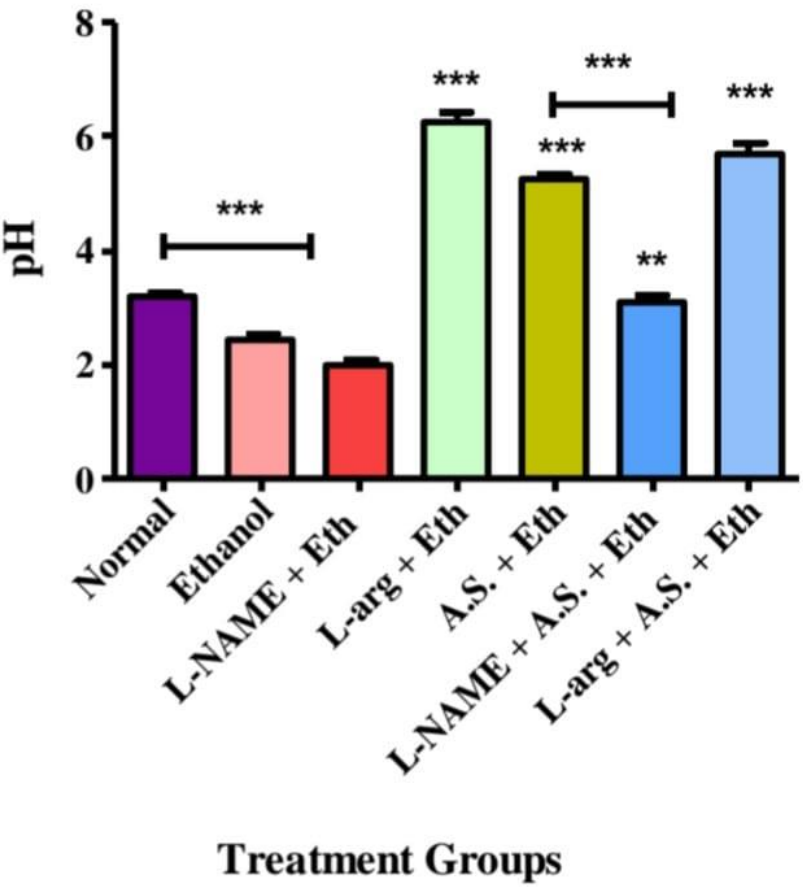

Figure 25. pH of Nitric oxide (NO) Pathway

Each bar shows mean of 5 rats, whereas upright line above each bar represents S.E.M. Line above the column shows the comparison of Normal versus Ethanol group and Extract (A.S.+ Eth) versus Extract + Antagonist (L NAME + A.S. + Eth) group. *** represents the significance level $p < 0.001$, ** and * are non-significant with respect to Ethanol group by on-way ANOVA followed by Dunnett's test. (L-NAME; L-Nitro-Arginine Methyl Ester, L-Arg: L-Arginine, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

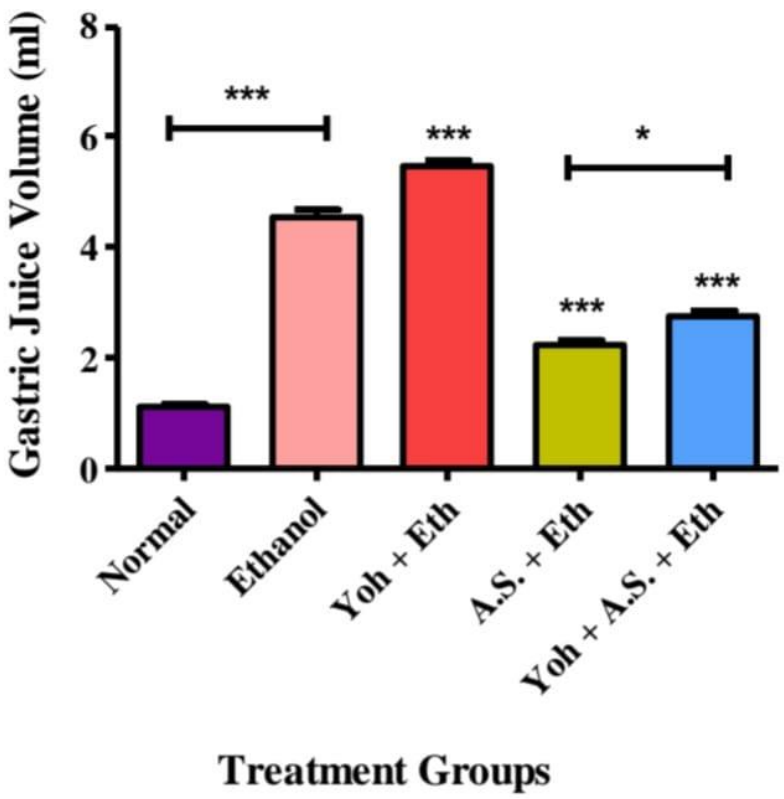

Figure 26. Gastric Juice Volume (ml) of Alpha-2 (α-2) Receptor Pathway

Each bar shows mean of 5 rats, whereas upright line above each bar represents S.E.M. Line above the column shows the comparison of Normal versus Ethanol group and Extract (A.S.+ Eth) versus Extract + Antagonist (Yoh + A.S. + Eth) group. *** represents the significance level $p < 0.001$, ** and * are non-significant with respect to Ethanol group by on-way ANOVA followed by Dunnett's test. (Yoh: Yohimbine, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

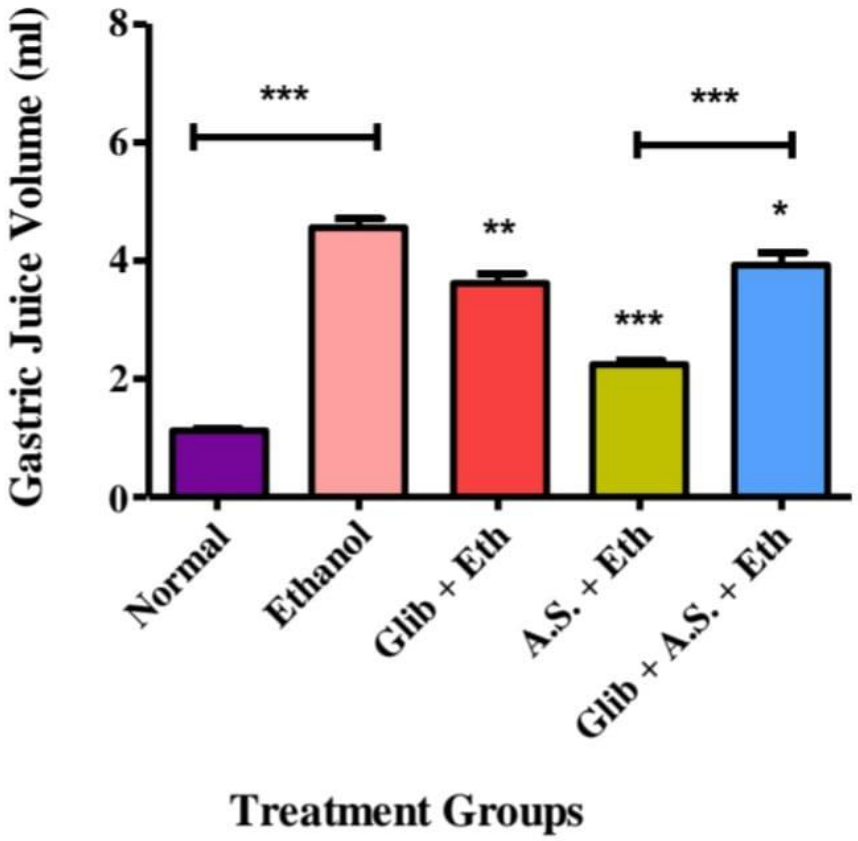

Figure 27. Gastric Juice Volume (ml) of ATP-dependent K^+ Channel Pathway

Each bar shows mean of 5 rats, whereas upright line above each bar represents S.E.M. Line above the column shows the comparison of Normal versus Ethanol group and Extract (A.S.+ Eth) versus Extract + Antagonist (Glib + A.S. + Eth) group. *** represents the significance level $p < 0.001$, ** and * are non-significant with respect to Ethanol group by on-way ANOVA followed by Dunnett's test. (Glib: Glibenclamide, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

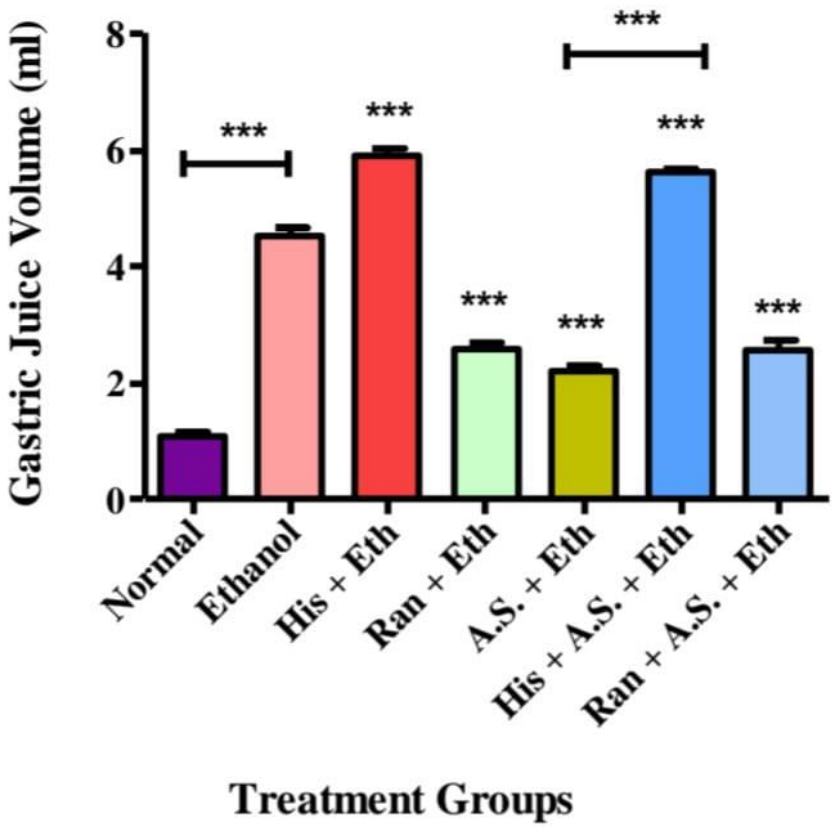

Figure 28. Gastric Juice Volume (ml) of Histamine Pathway

Each bar shows mean of 5 rats, whereas upright line above each bar represents S.E.M. Line above the column shows the comparison of Normal versus Ethanol group and Extract (A.S.+ Eth) versus Extract + Agonist (His + A.S. + Eth) group. *** represents the significance level $p < 0.001$, ** and * are non-significant with respect to Ethanol group by on-way ANOVA followed by Dunnett's test. (His: Histamine, Ran: Ranitidine, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

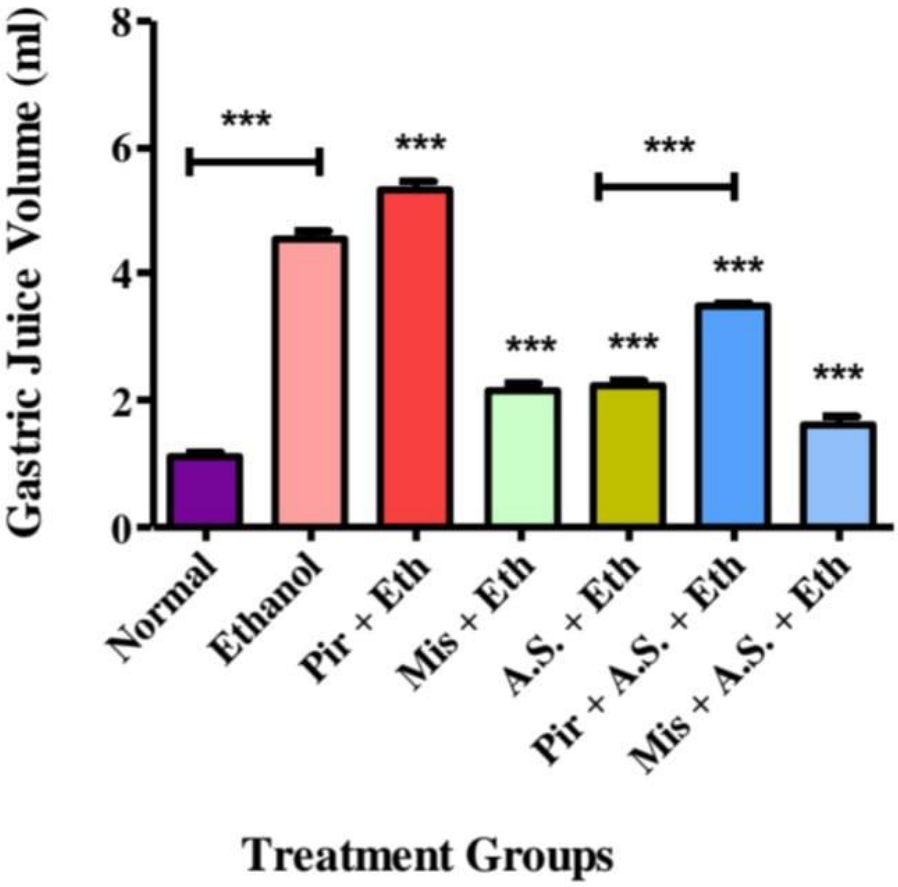

Figure 29. Gastric Juice Volume (ml) of Prostaglandin Pathway

Each bar shows mean of 5 rats, whereas upright line above each bar represents S.E.M. Line above the column shows the comparison of Normal versus Ethanol group and Extract (A.S.+ Eth) versus Extract + Antagonist (Pir + A.S. + Eth) group. *** represents the significance level $p < 0.001$, ** and * are non-significant with respect to Ethanol group by on-way ANOVA followed by Dunnett's test. (Pir: Piroxicam, Mis: Misoprostol, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

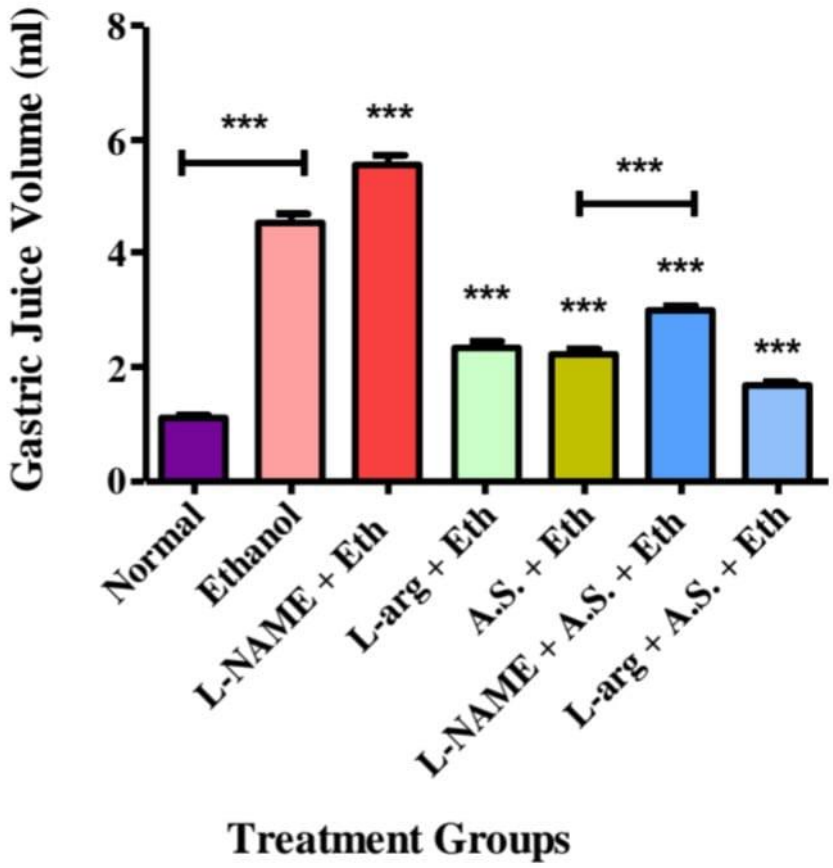

Figure 30. Gastric Juice Volume (ml) of Nitric oxide (NO) Pathway

Each bar shows mean of 5 rats, whereas upright line above each bar represents S.E.M. Line above the column shows the comparison of Normal versus Ethanol group and Extract (A.S.+ Eth) versus Extract + Antagonist (L-NAME + A.S. + Eth) group. *** represents the significance level $p < 0.001$, ** and * are non-significant with respect to Ethanol group by on-way ANOVA followed by Dunnett's test. (L-NAME; L Nitro Arginine Methyl Ester, L-Arg: L-Arginine, Eth:Ethanol, A.S.: *A. scholaris* 500mg/kg).

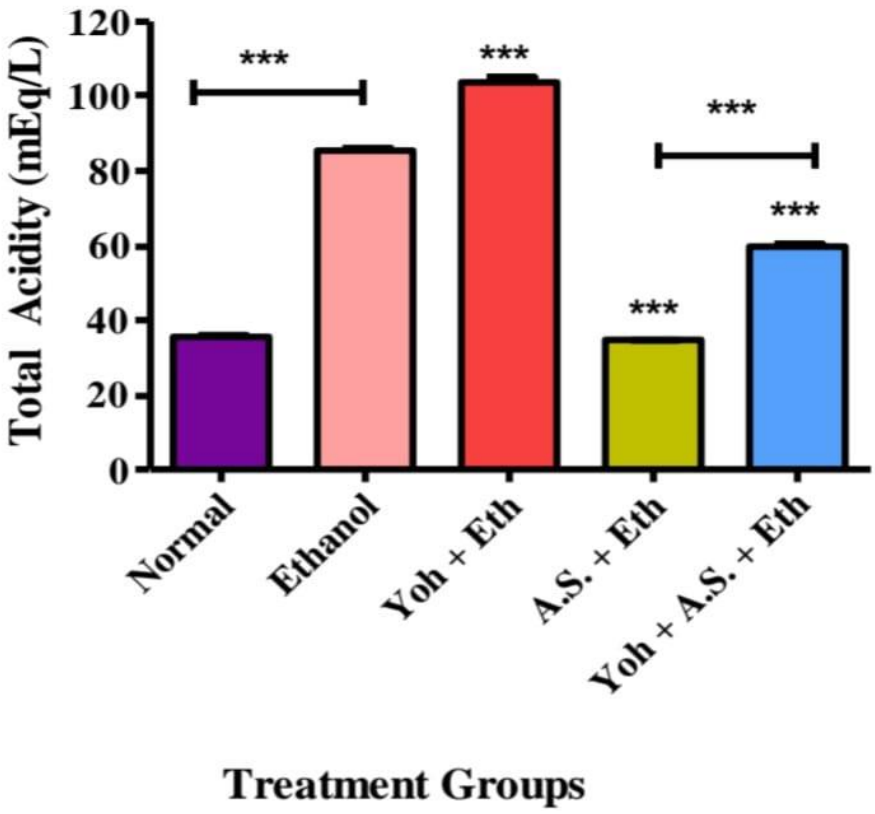

Figure 31. Total Acidity of Alpha-2 (α-2) Receptor Pathway

Each bar shows mean of 5 rats, whereas upright line above each bar represents S.E.M. Line above the column shows the comparison of Normal versus Ethanol group and Extract (A.S.+ Eth) versus Extract + Antagonist (Yoh + A.S. + Eth) group. *** represents the significance level $p < 0.001$, ** and * are non-significant with respect to Ethanol group by on-way ANOVA followed by Dunnett's test. (Yoh: Yohimbine, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

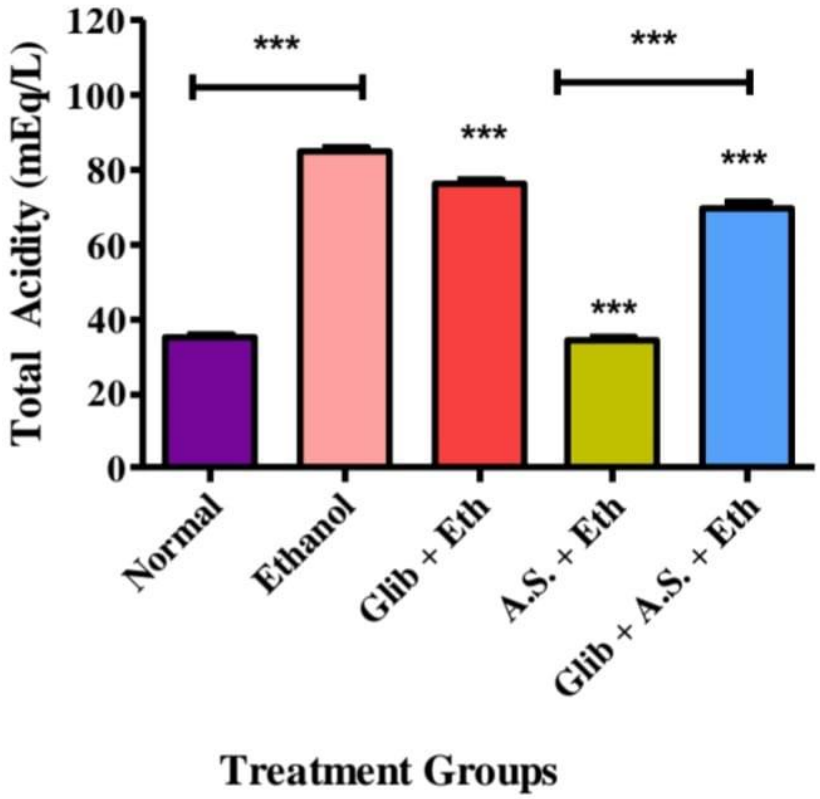

Figure 32. Total Acidity of ATP-dependent K^+ Channel Pathway

Each bar shows mean of 5 rats, whereas upright line above each bar represents S.E.M. Line above the column shows the comparison of Normal versus Ethanol group and Extract (A.S.+ Eth) versus Extract + Antagonist (Glib + A.S. + Eth) group. *** represents the significance level $p < 0.001$, ** and * are non-significant with respect to Ethanol group by on-way ANOVA followed by Dunnett's test. (Glib: Glibenclamide, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

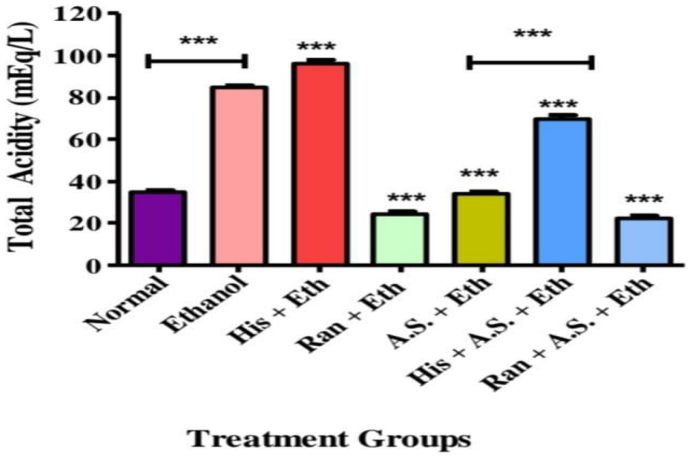

Figure 33. Total Acidity of Histamine Pathway

Each bar shows mean of 5 rats, whereas upright line above each bar represents S.E.M. Line above the column shows the comparison of Normal versus Ethanol group and Extract (A.S.+ Eth) versus Ex tract + Agonist (His + A.S. + Eth) group. *** represents the significance level $p < 0.001$, ** and * are non-significant with respect to Ethanol group by on-way ANOVA followed by Dunnett's test. (His: Histamine, Ran: Ranitidine, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

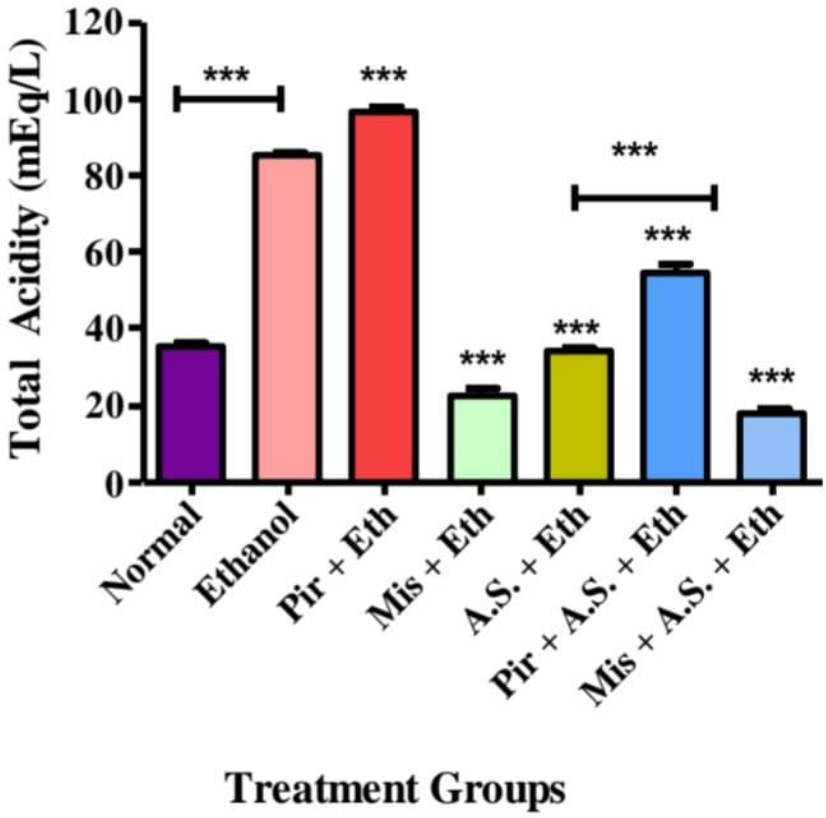

Figure 34. Total Acidity of Prostaglandin Pathway

Each bar shows mean of 5 rats, whereas upright line above each bar represents S.E.M. Line above the column shows the comparison of Normal versus Ethanol group and Extract (A.S.+ Eth) versus Extract + Antagonist (Pir + A.S. + Eth) group. *** represents the significance level $p < 0.001$, ** and * are non-significant with respect to Ethanol group by on-way ANOVA followed by Dunnett's test. (Pir: Piroxicam, Mis: Misoprostol, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

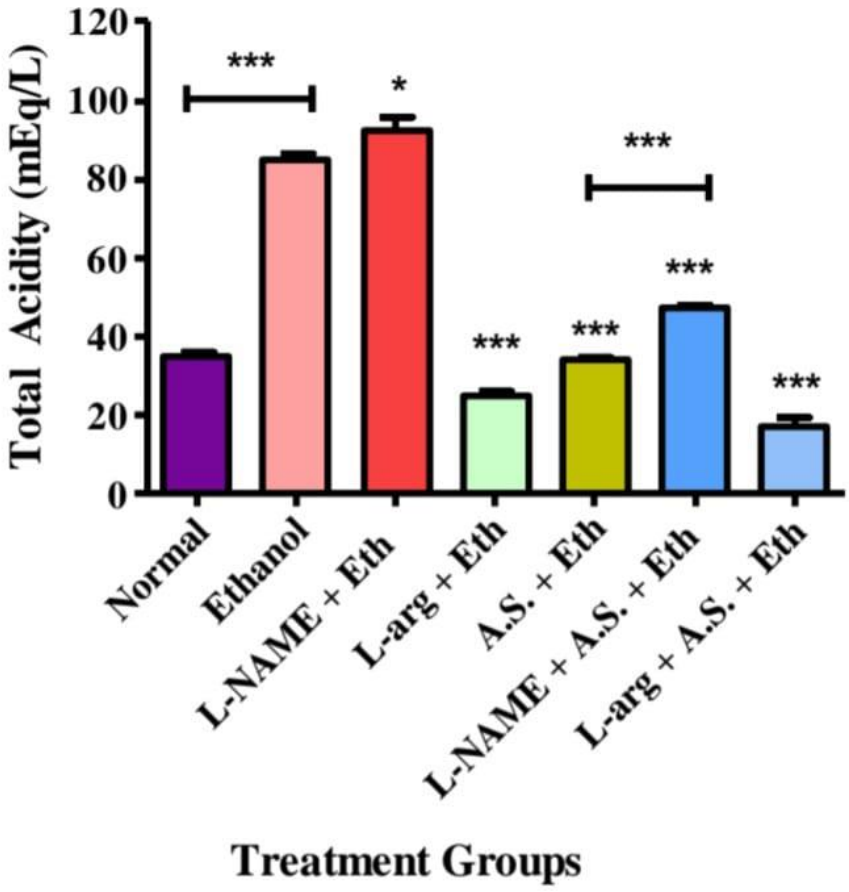

Figure 35. Total Acidity of Nitric oxide (NO) Pathway

Each bar shows mean of 5 rats, whereas upright line above each bar represents S.E.M. Line above the column shows the comparison of Normal versus Ethanol group and Extract (A.S.+ Eth) versus Extract + Antagonist (L-NAME + A.S. + Eth) group. *** represents the significance level $p < 0.001$, ** and * are non-significant with respect to Ethanol group by on-way ANOVA followed by Dunnett's test. (L-NAME; L-Nitro-Arginine Methyl Ester, L-Arg: L-Arginine, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

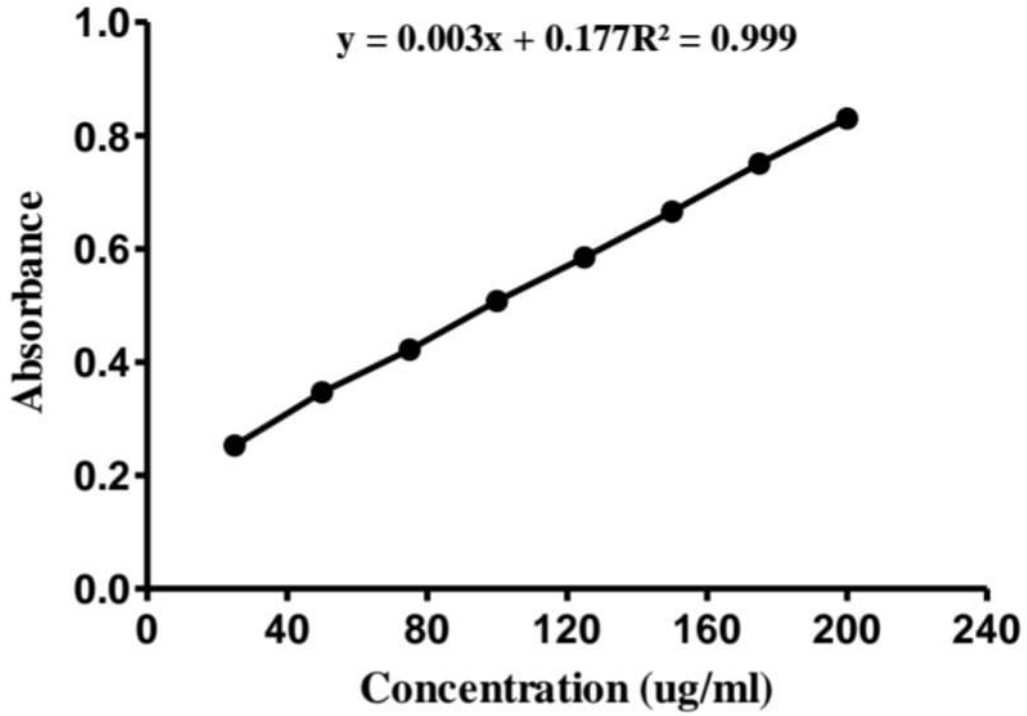

Figure 36. Standard Curve of Total Protein Content of Gastric tissue.

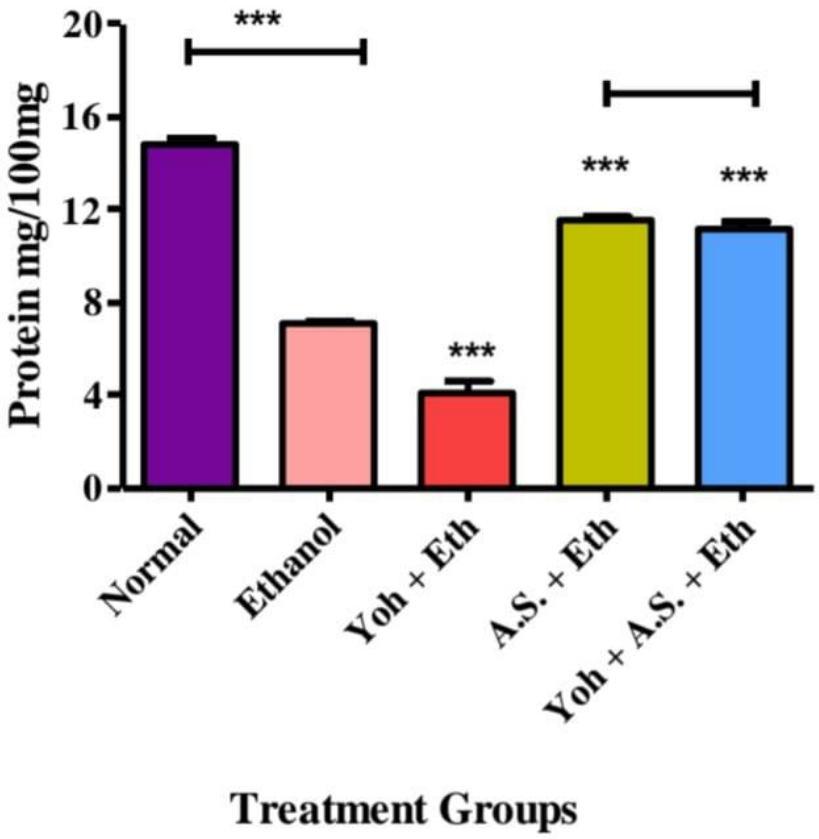

Figure 37. Protein Contents (mg/100mg) of Alpha-2 (α-2) Receptor Pathway

Each bar shows mean of 5 rats, whereas upright line above each bar represents S.E.M. Line above the column shows the comparison of Normal versus Ethanol group and Extract (A.S.+ Eth) versus Extract + Antagonist (Yoh + A.S. + Eth) group. *** represents the significance level $p < 0.001$, ** and * are non-significant with respect to Ethanol group by on-way ANOVA followed by Dunnett's test. (Yoh: Yohimbine, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

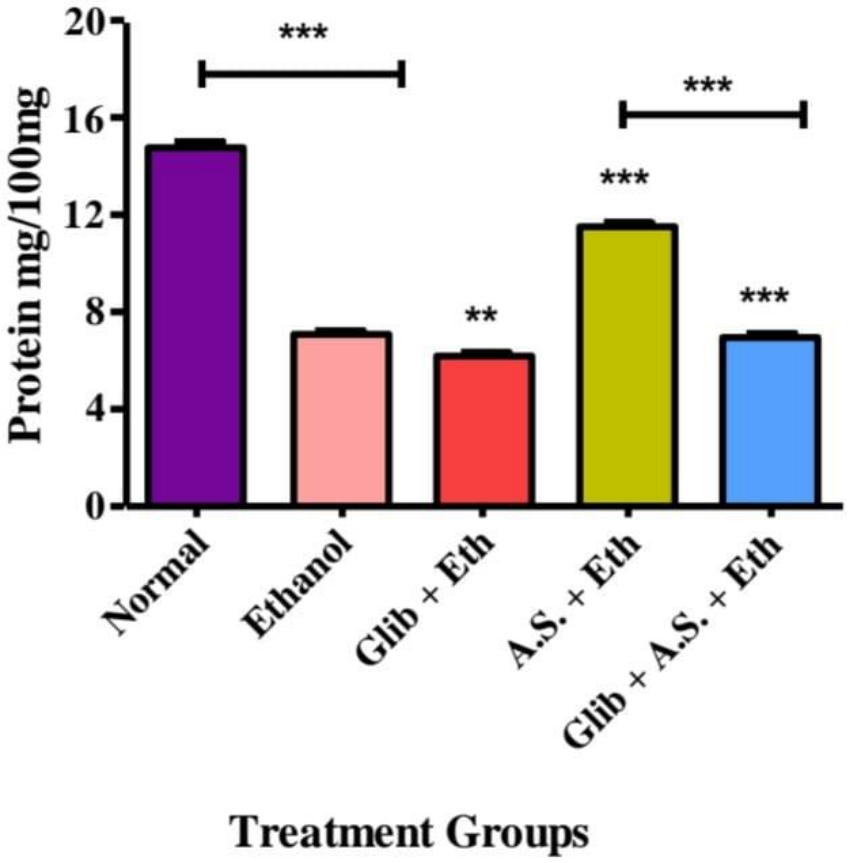

Figure 38. Protein Contents (mg/100mg) of ATP-dependent K^+ Channel Pathway

Each bar shows mean of 5 rats, whereas upright line above each bar represents S.E.M. Line above the column shows the comparison of Normal versus Ethanol group and Extract (A.S.+ Eth) versus Extract + Antagonist (Glib + A.S. + Eth) group. *** represents the significance level $p < 0.001$, ** and * are non-significant with respect to Ethanol group by on-way ANOVA followed by Dunnett's test. (Glib: Glibenclamide, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

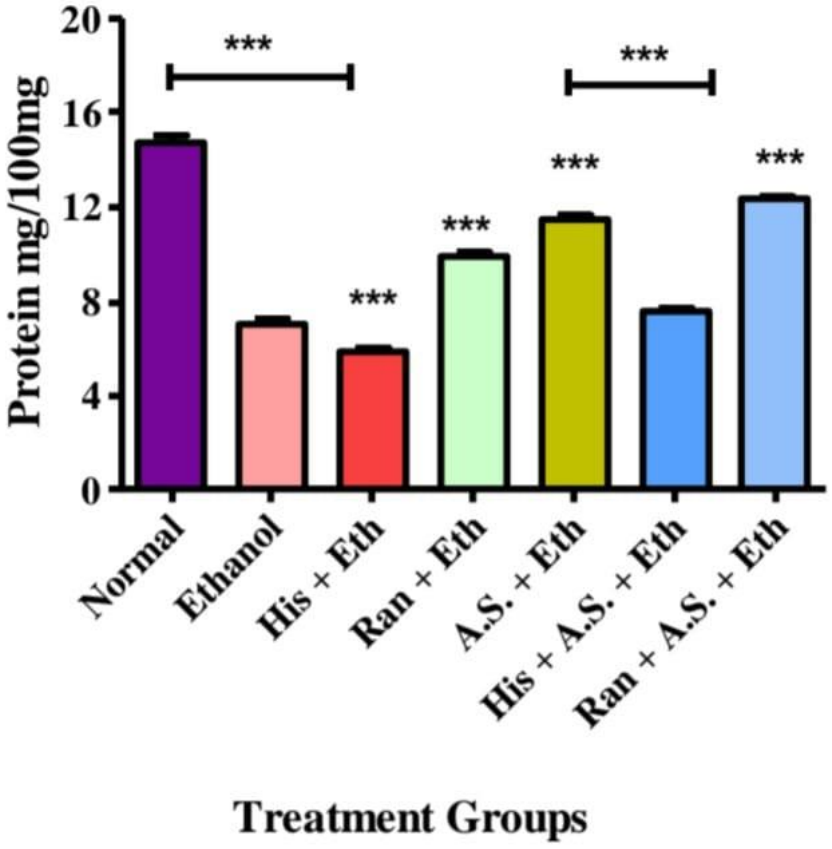

Figure 39. Protein Contents (mg/100mg) of Histamine Pathway

Each bar shows mean of 5 rats, whereas upright line above each bar represents S.E.M. Line above the column shows the comparison of Normal versus Ethanol group and Extract (A.S.+ Eth) versus Extract + Agonist (His + A.S. + Eth) group. *** represents the significance level $p < 0.001$, ** and * are non-significant with respect to Ethanol group by on-way ANOVA followed by Dunnett's test. (His: Histamine, Ran: Ranitidine, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

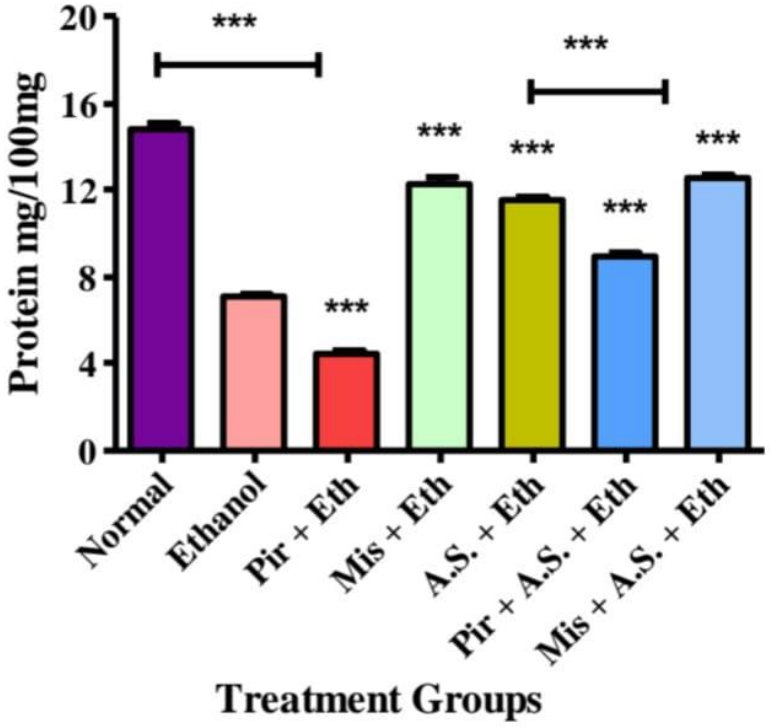

Figure 40. Protein Contents (mg/100mg) of Prostaglandin Pathway

Each bar shows mean of 5 rats, whereas upright line above each bar represents S.E.M. Line above the column shows the comparison of Normal versus Ethanol group and Extract (A.S.+ Eth) versus Extract + Antagonist (Pir + A.S. + Eth) group. *** represents the significance level $p < 0.001$, ** and * are non-significant with respect to Ethanol group by on-way ANOVA followed by Dunnett's test. (Pir: Piroxicam, Mis: Misoprostol, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

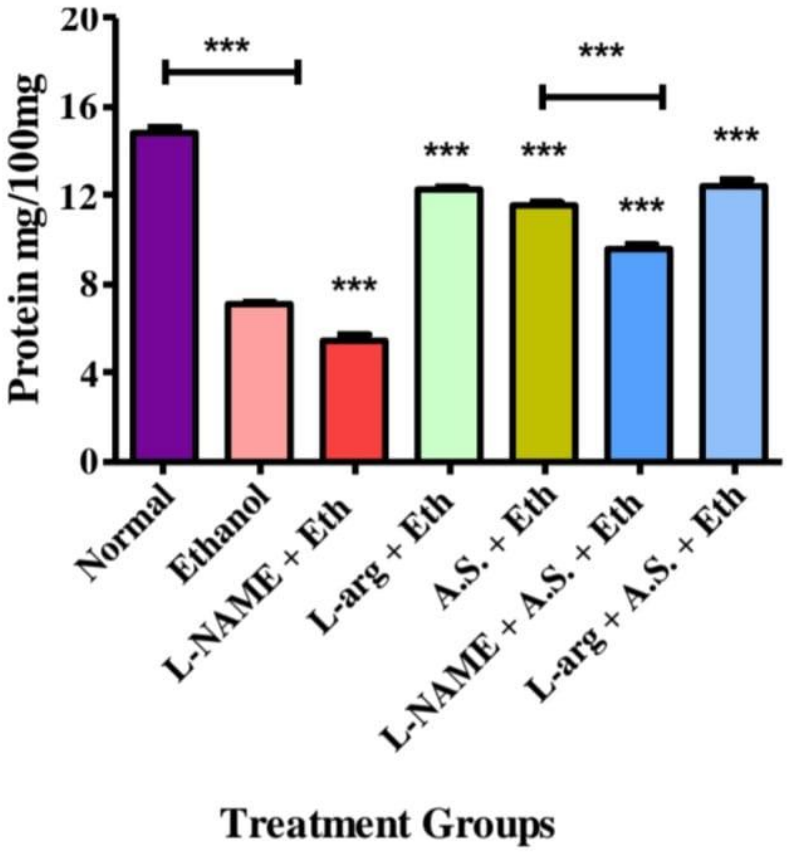

Figure 41. Protein Contents (mg/100mg) of Nitric oxide (NO) Pathway

Each bar shows mean of 5 rats, whereas upright line above each bar represents S.E.M. Line above the column shows the comparison of Normal versus Ethanol group and Extract (A.S.+ Eth) versus Extract + Antagonist (L-NAME + A.S. + Eth) group. *** represents the significance level $p < 0.001$, ** and * are non-significant with respect to Ethanol group by on-way ANOVA followed by Dunnett's test. (L-NAME; L-Nitro-Arginine Methyl Ester, L-Arg: L-Arginine, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

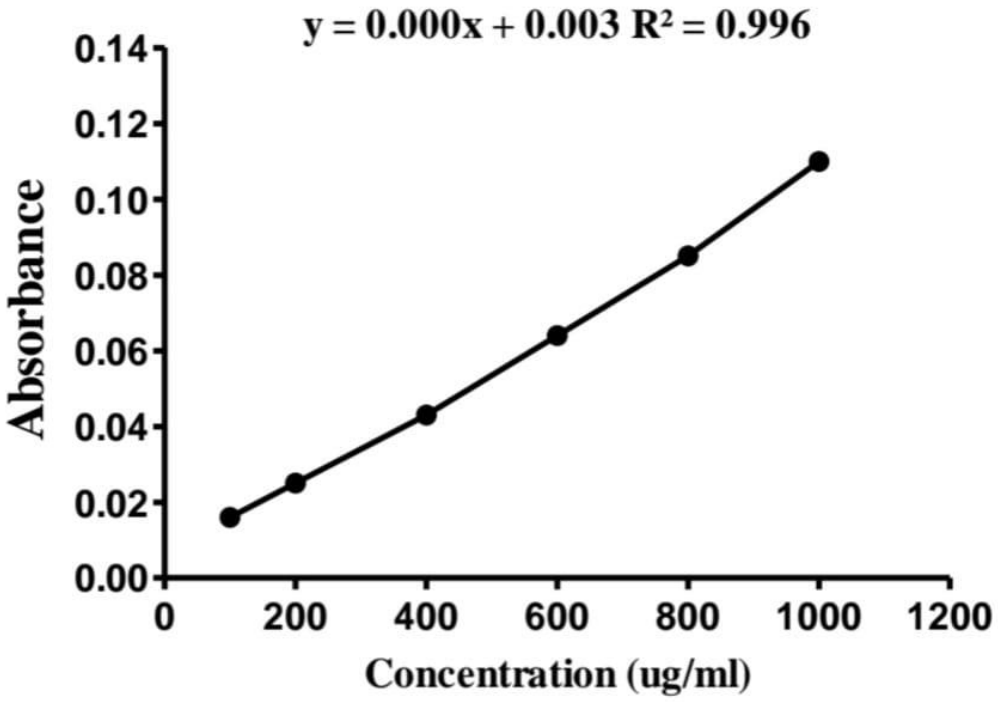

Figure 42. Standard Curve of Mucus Adherence to Gastric Wall.

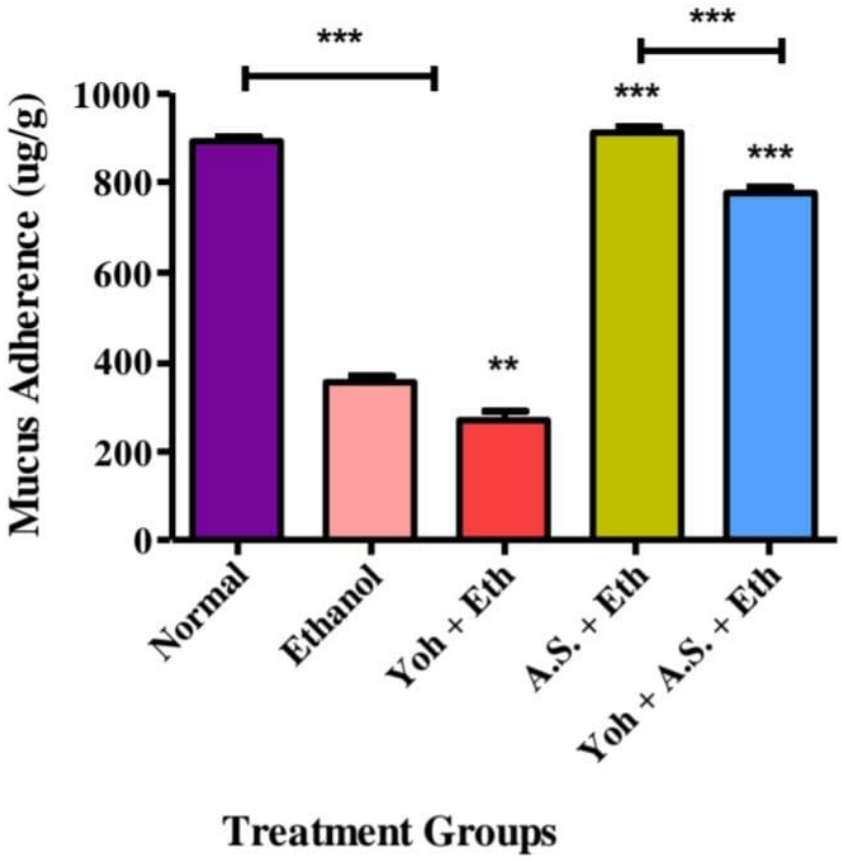

Figure 43. Mucus Adherence (ug/g) to Gastric Wall of Alpha-2 (α-2) Receptor Pathway

Each bar shows mean of 5 rats, whereas upright line above each bar represents S.E.M. Line above the column shows the comparison of Normal versus Ethanol group and Extract (A.S.+ Eth) versus Extract + Antagonist (Yoh + A.S. + Eth) group. *** represents the significance level $p < 0.001$, ** and * are non-significant with respect to Ethanol group by on-way ANOVA followed by Dunnett's test. (Yoh: Yohimbine, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

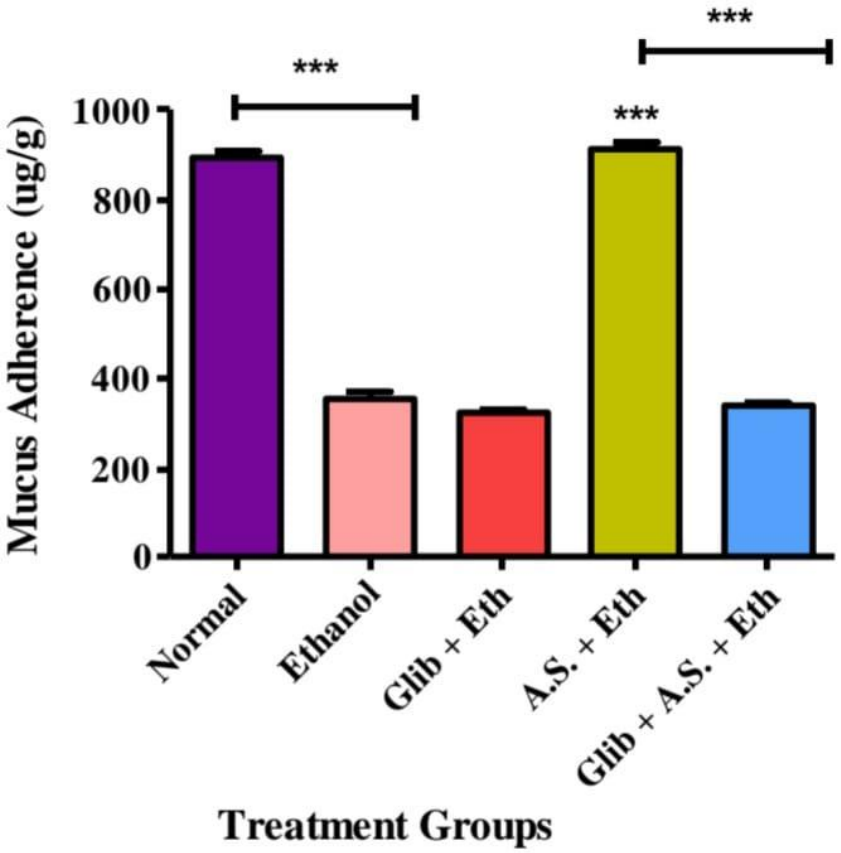

Figure 44. Mucus Adherence (ug/g) to Gastric Wall of ATP-dependent K^+ Channel Pathway

Each bar shows mean of 5 rats, whereas upright line above each bar represents S.E.M. Line above the column shows the comparison of Normal versus Ethanol group and Extract (A.S.+ Eth) versus Extract + Antagonist (Glib + A.S. + Eth) group. *** represents the significance level $p < 0.001$, ** and * are non-significant with respect to Ethanol group by on-way ANOVA followed by Dunnett's test. (Glib: Glibenclamide, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

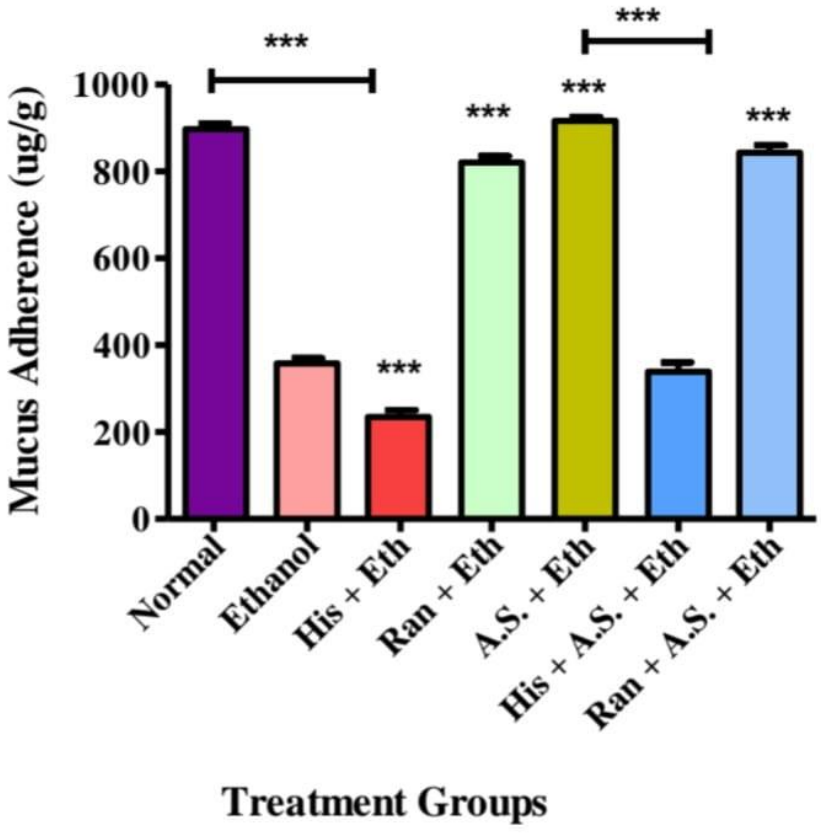

Figure 45. Mucus Adherence (ug/g) to Gastric Wall of Histamine Pathway

Each bar shows mean of 5 rats, whereas upright line above each bar represents S.E.M. Line above the column shows the comparison of Normal versus Ethanol group and Extract (A.S.+ Eth) versus Extract + Agonist (His + A.S. + Eth) group. *** represents the significance level $p < 0.001$, ** and * are non-significant with respect to Ethanol group by on-way ANOVA followed by Dunnett's test. (His: Histamine, Ran: Ranitidine, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

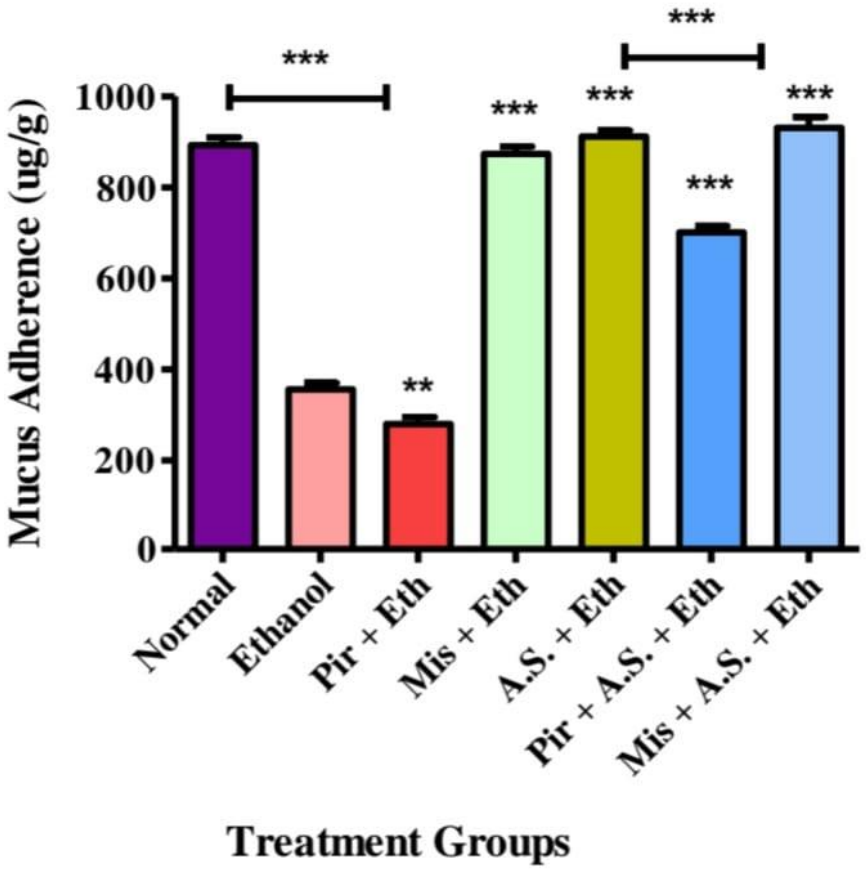

Figure 46. Mucus Adherence (ug/g) to Gastric Wall of Prostaglandin Pathway

Each bar shows mean of 5 rats, whereas upright line above each bar represents S.E.M. Line above the column shows the comparison of Normal versus Ethanol group and Extract (A.S.+ Eth) versus Extract + Antagonist (Pir + A.S. + Eth) group. *** represents the significance level $p < 0.001$, ** and * are non-significant with respect to Ethanol group by on-way ANOVA followed by Dunnett's test. (Pir: Piroxicam, Mis: Misoprostol, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

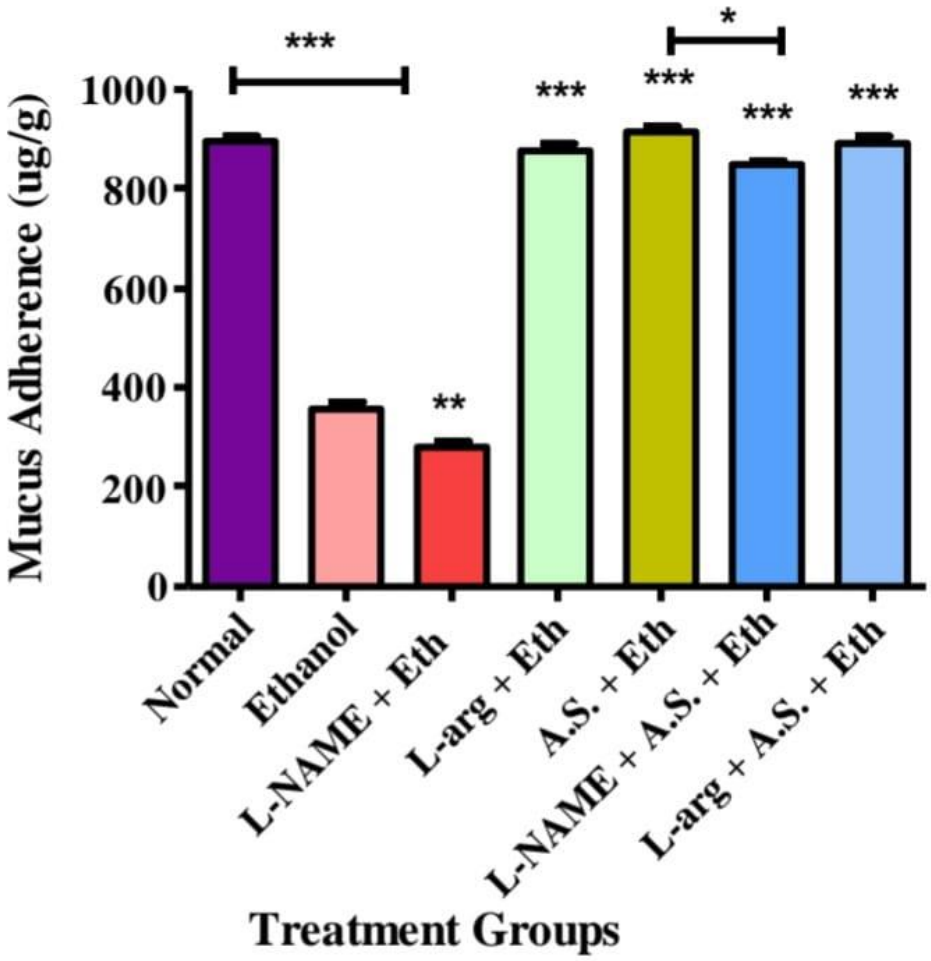

Figure 47. Mucus Adherence (ug/g) to Gastric Wall of Nitric oxide (NO) Pathway

Each bar shows mean of 5 rats, whereas upright line above each bar represents S.E.M. Line above the column shows the comparison of Normal versus Ethanol group and Extract (A.S.+ Eth) versus Extract + Antagonist (L-NAME + A.S. + Eth) group. *** represents the significance level $p < 0.001$, ** and * are non-significant with respect to Ethanol group by on-way ANOVA followed by Dunnett's test. (L-NAME; L-Nitro-Arginine Methyl Ester, L-Arg: L-Arginine, Eth: Ethanol, A.S.: *A. scholaris* 500mg/kg).

4. DISCUSSION:

Natural sources of drugs from animal and plant origin have been utilized in medicines from thousands of years. These sources of drugs have many medicinal potential still to be explored by the scientists. They are always keen to be used in medicines due to fewer side effects as compared to the synthetically available drugs (Patwardhan & Vaidya, 2010). Various endogenous as well as exogenous factors lead to the gastric ulceration like stress, alcohol, smoking and NSAIDs. Gastro-protective activity of Alstonia scolaris have been reported by our colleagues in our laboratory but the pathway involved in gastro-protective activity was unknown (unpublished data).

Five pathways; Nitric Oxide (NO) (Freitas et al., 2004), ATP-dependent K^+ channel (Silva et al., 2011), Histamine (Kim et al., 2005), Prostaglandin (Júnior et al., 2014) and Alpha-2 (α-2) receptor pathway (e Silva et al., 2012) were studied using gastric ulcer related parameters; ulcer index, pH, total acidity, gastric juice volume, protein contents of gastric tissue and mucous contents were analyzed. All these mechanisms are involved in the gastro-protective action as well as in gastric ulceration. The principle behind the inclusion of the pathway was the agonist/antagonist activity over the pathway and examination of the outcomes with the control group and extract treated group. In the presence of antagonist the activity over particular target could not be preceded as the target was pre-occupied, based on the observation of gastroprotective action of the extract in that particular group, we decided either the pathway was engaged with the action or not (Júnior et al., 2014). To induce the ulcer in rats we used ethanol which was treated by A. scholaris extract as observed by macroscopic examination. Ethanol-induced gastric ulceration has widely been studied as an exogenous factor of ulcer causing agent as well as to study the gastro-protective effect of many natural and synthetic drugs. Ethanol induces ulceration by direct damage to the gastric tissue and by producing free radicals/reactive oxygen species resulting in ethanol characteristics; deep ulcer with hemorrhagic streaks and perforations. It produces acute inflammation directly damaging to the tissue resulting in infiltration of the blood cells (Zamora Rodríguez et al., 2007). A. scholaris has been shown to reduce the ulcer due to ethanol induction as it has anti-oxidant potential by blocking the synthesis of free radicals which produces ulceration and damage to the gastric mucosa (Rawal et al., 2008).

It is evident from the past literatures that prostaglandins and nitric oxide collaborate with sensory nerves for maintaining gastric mucosal integrity and cytoprotection (Brzozowski et al., 1999). Prostaglandins impart its crucial role in modulating gastric mucosal integrity through stimulation of K_{ATP}-channels in the presence of gastric acid secretions (Curtis et al., 1995). These channels contribute their role in blood pressure control as they are linked with the relaxation of vascular smooth muscle (Alqasoumi et al., 2008). Potassium channels are the most diversified and broadened family of ion channels located in all cellular membrane. K_{ATP}channels are ligand gated proteins. They have been accounted for to be contributed in different physiologic functions of the stomach like gastric acid secretion, gastric mucosal blood stream guideline and contractility of stomach (Garcia, 1997). It is thought that K_{ATP}-channels provide gastric mucosal protection by increasing blood flow in gastric mucosa (Doi et al., 1998). Endogenously K_{ATP}-channels are activated by calcitonin gene-related peptide (Nelson et al., 1990), prostacyclin (Brayden, 2002), nitric oxide (Murphy & Brayden, 1995) and adenosine (Dart & Standen, 1993). K_{ATP}-channels are activated by adenylate cyclase system resulting increase in intracellular cAMP level which in turn activates protein kinase A. Phosphorylation by protein kinase A results in opening of KATP-channels (Quayle et al., 1994). Activation of K_{ATP}channels causes membrane hyperpolarization leading to closing of voltage-gated Ca^{2+} channels, which decreases intracellular calcium Ca^{2+} and causes vasodilation (Brayden, 2002). Due to vasodilation blood flow in gastric mucosa is increased, which is responsible for gastro-protective effect. Calcitonin gene related peptide (CGRP) is important endogenous vasodilator in stomach which is responsible for increase in gastric mucosal blood flow and protects the gastric mucosa from acid injury (Li et al., 1992).

In our study experiment, Glibenclamide which is a potent blocker of these K_{ATP}-channels used to explore the pathway of A. scholaris for its gastroprotective activity. Glibenclamide belongs to sulfonylurea class which hinders K_{ATP}-channels and causes membrane depolarization, due to which Ca^{2+} channels opens, Intracellular calcium Ca^{2+} level increases leading to vasoconstriction and decreased mucosal blood flow, which potentiate the ulceration in ethanol- induced ulcer model. Our study manifested that when A. scholaris given simultaneously with a sulfonylurea (glibenclamide) that hinders K_{ATP} channels (Alqasoumi et al., 2008), the gastroprotective impact is turned around, showing that the defensive

movement of A. scholaris was interceded by stimulation of K_{ATP} channels. Therefore our data suggests that there is involvement of K_{ATP} channels in gastro-protective activity of A. scholaris. Similar gastroprotective effect was studied in sulfated-polysaccharide fraction of Hypnea musciformis (Damasceno et al., 2013), Cenostigma macrophyllum (Viana et al., 2013), Quebrachitol, a bioactive component islolated from Magonia glabrata (De Olinda et al., 2008), friedelin, triterpenoid isolated from Azima tetracantha (Antonisamy et al., 2015), Carbenoxolone, a semi-synthetic triterpenoid islolated from Glycyrrhiza glabra (Chávez-Piña et al., 2011), Violacein Isolated from Chromobacterium violaceum (Antonisamy et al., 2014), thymol (Ribeiro et al., 2016), menthol (Rozza et al., 2013), Sildenafil (Medeiros et al., 2008), Nicorandil (Ismail et al., 2007) and eugenol (Morsy & Fouad, 2008).

Histamine is an important part of local immune response and plays its role in inflammatory processes. Histamine released from the mast cells and has inflammatory role in various pathophysiological conditions. A particular response is generated when histamine binds to its specific receptor like inflammation, production of gastric acids or mucus. Histamine produces ulceration through its receptors (H_1, H_2 & H_3), all these receptors have role in the gastroprotection and various drugs like Ranitidine have been used pharmacologically for ulceration affecting H_2 receptors (Dembiński et al., 2005). The secretion of HCl by parietal cells is stimulated by histamine through the activation of H_2 receptors (Perez-Zoghbi et al., 2008). Histamine is the predominant chemo-stimulator of hydrogen ion secretions in the stomach. H_2 receptors causes activation of adenylate cyclase system which leads to increase concentration of calcium, resulting increased cAMP and activation of protein kinase A, due to which proton pump gets activated and leads to increase in gastric acid production and causes ulcer (Dembiński et al., 2005; Al-Mofleh et al., 2006). Gastric acid secretions follow cAMP/Protein kinase A/proton pump pathway (Takeuchi et al., 1999).

In our experimental study, when Histamine administered in ethanol-induced ulcer model, it worsened the lesion areas. When Histamine co-administered with A. scholaris gastro-protective effect was suppressed. However, gastro-protective effect of A. scholaris was maximized in the presence of antagonist (Ranitidine). It is likely that protection by the A. scholaris against ethanol- induced gastric ulceration is achieved by the suppression of acid secretion. This effect is similar to the effect of antiulcer drugs which act by providing cytoprotective defense against gastric acid secretions. A. scholaris remarkably terminated the histamine induced proton efflux in the stomach. The gastro-protective mechanism of A. scholaris is considered by competitive binding to H_2 receptors which is required for histamine to provoke H^+ efflux in the stomach. The antihistamine activity of A. scholaris is due to presence of several phytochemicals in its leaves. Flavonoids are present in high quantity in A. scholaris leaves, thought to nullify the effect of histamine which is dominant mediator in ulceration (Macander, 1986; Sharma et al., 1996).

Therefore, our data suggests that there is a role of histamine pathway in gastro-protective mechanism of A. scholaris. Alstonia scholaris exerts its gastro-protective activity by blocking histamine H_2 receptors. Similar gastro-protective mechanism was also studied in extract of Buchholzia coriacea (Enechi & Nwodo, 2014), Curcuma longa (Kim et al., 2005), Voacanga africana (Tan & Nyasse, 2000), Bryophyllum pinnatum (Pal & Chaudhuri, 1991) Cassia singueana (Ode et al., 2011). Several drugs Cimetidine (Levine et al., 1979), Famotidine (Yamao et al., 2006), Nizanitidine (Sontag, 1990) and Ranitidine (Brogden et al., 1982) employed to treat gastric ulcers work by blocking of H_2 receptors.

To assess the impact of endogenous No on the gastro-protective impact of A. scholaris, rats were pretreated with antagonist of nitric oxide synthase (NOS), L-NAME, an analogue of Larginine, which is hydrolyzed to yield L-nitro arginine and thwarts NOS movement. Nitric oxide (NO) has a huge defensive effect on the GI tract. Preclinical examinations have affirmed that NO contributes in keeping up peptic mucosal quality, hindering adherence of leucocyte to the endothelial layer, relocation of neutrophil and furthermore supportive in fixing harm brought about by NSAIDs. In addition, NO has vasodilatory impact and upgrades gastric blood stream (Lanas, 2008). In our experimental study treatment with L-NAME (NOS inhibitor) didn't hinder the defensive impact of A. scholaris against injury instigated by absolute ethanol, and treatment with L-arginine (NOS substrate) additionally did not meddle with A. scholaris defensive movement. Actually, NO is a putative dispatcher and gas, which contributes in the signal transduction joined by assortment of physiological processes like neurotransmission, smooth muscle relaxation and vasodilation, regulation of pro and antiapoptotic systems and keeping up pulse and blood stream (Szlachcic et al., 2013). It likewise involved in controlling gastrointestinal motility, enlistment of neutrophils, gastric blood stream and emission of mucus (Kubes

et al., 1991). The enzymatic biosynthesis of nitric oxide and its bioavailability is limited by the articulation and action of NOS (Szlachcic et al., 2013). So our data proposed nonassociation of nitric oxide pathway in gastro-protective activity of A. scholaris.

Prostaglandins (PGs) have a role in keeping mucosal integrity intact, COX-1 catalyzes biosynthesis of prostaglandins in the gastrointestinal tract whereas, in pathophysiological conditions such as inflammation COX-2 mainly provides PGs (Peskar, 2001). It has been established that prostaglandin E2 (PGE2) has a gastro-protective impact against ethanol-induced sores because of ascend in intracellular guanosine-30, 50-cyclicmonophosphate (cGMP), that is intervened by an over the top intracellular free calcium concentration and generation of nitric oxide (Sakai et al., 1995). In our experimental study, misoprostol, which is PGE1 analogue, guarded the gastric mucosa against the harm brought about by ethanol, which confirms the value of PGs in keeping mucosal integrity intact. Our data shows that pre-treatment with A. scholaris protects the gastric mucosa against gastric ulcers induced by piroxicam. Piroxicam, non-selective inhibitors of Cox-1 and Cox-2, when given concurrently with A. scholaris in ethanol-induced ulcer models, the effect was not stifled indicating non-inclusion of prostaglandins pathways in gastro-protective effect of A. scholaris. This data clearly implies that there is no role of prostaglandins in gastro-protective activity of A. scholaris.

Alpha-2 (α-2) noradrenergic receptors are present on smooth muscles of blood vessels. α-2 agonists initiates smooth muscle contraction and cause vasoconstrictions, thereby stimulating the response of sympathetic adrenergic nerve activation to the blood vessels. (Gyires et al., 2000) explained that activation of α-2 adrenergic receptors is liable for gastro-protective effects. α-2 receptor stimulation mediates the suppression of GIT functions involving gastro duodenal secretions. α-2 noradrenergic receptors control gastric acid secretion and render assurance against destructive vesicant specialists like ethanol and NSAIDs. The modulation of α-2 receptors in peripheral parasympathetic ganglia intramural reduce the arrival of vagal release acetylcholine, which stifles the gastric emissions and motility, and upgrades blood stream (Yelken et al., 1999). In our study based experiments administration of Yohimbine (α-2 antagonist) an alkaloid that boosts neurotransmitter release by impeding presynaptic α-2 receptor didn't counter the defensive impact of A. scholaris against ethanol-induced gastric ulcers, which demonstrates that α-2 noradrenergic receptors are not engaged with gastro-protective action of A. scholaris. This is evident that gastro-protective activity of A. scholaris doesn't follow this pathway.

The results of the study manifested the gastro-protective activity of A. scholaris. This gastroprotection is mediated by involvement of Histamine and ATP-dependent K^+ channel pathway. Further examination will give about the job of dynamic flavonoids present in A. scholaris on the gastric protection.

5. CONCLUSION:

The current study explored the pathways of gastro-protective action of aqueous extract of A. scholaris in ethanol-induced ulcer models in rats. Gastro-protective activity was due to stimulation of K_{ATP} channels and blockade of Histamine receptors most probably H_2 receptors. It has been concluded from the data that A. scholaris has tendency to modify proton pump in a way to be protective for gastric mucosa. The gastro-protective activity may belong to the saponins, glycosides and flavonoids which have earlier been reported in leaves of the plant. However, further investigation is needed to isolate the active components singularly and investigation their involvement in various pathophysiological animal models.

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