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ANTIULCER ACTIVITY OF THE METHANOLIC *AZADIRACHTA INDICA* (NEEM) LEAVES EXTRACT ON INDUCED GASTRIC ULCERS

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Keywords:

Neem, MTT assay, Animal testing, Gastric inflammation, Induced gastric ulcers, Cell viability, Ulcer index

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ABSTRACT: Gastric inflammation and ulceration are directly associated with accumulating reactive oxygen species, interleukins, TNF-α, and depletion of antioxidants. In addition to the frequent use of non-steroidal anti-inflammatory drugs, gastric lesions are also provoked by stress, alcohol consumption and *Helicobacter pylori.* Among other NSAIDs, Indomethacin has been very effective compound to generate a gastric ulcer in rats' gastric tissues. Ulcer of stomach generated by using Indomethacin involves the production of reactive oxygen species and upregulation of the activity as well as expression of matrix-metalloproteinase (MMPs). Various laboratory studies reveal that compounds from natural origin modify various metabolic disorders by changing pathological and physiological process. It can be a preventive method for various diseases including gastric ulcers. This project aimed to discover the anti-ulcerative effect of methanolic crude leaves extract on gastric ulcers induced by Indomethacin and cold stress in rodent's model. Crude Neem leaves extract significantly inhibited gastric cancer cells survival in the cell culture model, protection in Indomethacin and stress induced gastric ulcers.

INTRODUCTION: Gastric ulcers are due to the erosion of the stomach linings, front section of the small intestine, or maybe the lower part of esophagus [1, 2]. Ulcers can be peptic or duodenal depending on their location in the stomach [3, 4]. The pain may be in the form of like burning sensation or it may be dull ache. Some of the fewer common symptoms are belching, vomiting, decrease in body weight, and loss of appetite. The main complications of ulcer include hemorrhage, rupture and blockage of the stomach.15% of people identified with bleeding [5, 6]. Some of the common factors responsible for this condition include the bacteria *H. pylori* and NSAID's.

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Other factors that contribute less to the condition include Zollinger Ellison disease, tobacco smoking, Crohn disease, stress caused by severe illness, Behcet disease, liver cirrhosis [1, 6]. The general procedure followed to cure the ulcers is to reduce consumption of tobacco products, alcohol and non-steroidal anti-inflammatory drugs. Treatment of gastric ulcers is done by lowering gastric secretions using PPI and H2 blocker.

H. pylori-produced ulcers involve combined therapy of amoxicillin, clarithromycin, and a proton pump inhibitor. Due to resistance to antibiotics, many drugs may be in effective. Ulcer that is bleeding may be treated by endoscopy [6]. 4% of the world's population gets affected by gastric ulcer [1]. Globally there have 25% increase in people with gastric ulcer. Approximately half of the world's population caught with gastrointestinal problems due to *H. pylori* infection that results in gastric cancer [7, 8]. Symptoms of duodenal ulcer include the pain arising after three hours of consumption meal.

Feeling of bloatingness and abdominal fullness are signs of gastric ulcer. Nausea, vomiting, reduced appetite, anxiety, loss of body weight and vomiting containing blood, this can be because of bleeding in gastric ulceration which results in hematemesis. Foul smell in faces is caused by removing oxidized iron from hemoglobin, known as melena. Sometimes peptic ulcer can cause gastric or duodenal perforation, resulting in sudden peritonitis and serious pain, which needs instant surgery [9, 10].

The non-steroid anti-inflammatory drugs that inhibit cyclo-oxygenase, glucocorticoids, are majorly involves in causing gastric ulcers. The cycle of pain in the abdomen after taking food easily differentiates the ulcer i.e., gastric and duodenal ulcers. When food enters the stomach, pain may start, indicating peptic ulcer. If pain intensity reduces when the food enters the stomach, the duodenal ulcer may be present. Pain caused by duodenal ulcer may start after 2-3 hours of meal. Gastric ulcer symptoms may vary depending on patient's age and location of the ulcer in the stomach [11]. Perforated and haemorrhagic gastric ulcers are a major factor contributing rise in mortality rates. These conditions worsened when gastric complications converted into cancer, contributing to elevation in morbidity and mortalities [12, 13].

Bleeding may occur due to erosion of artery by ulcer. A destruction in the surface of the gastro-intestinal tract may produce catastrophic outcome due to absence of treatment. This causes bacterial peritonitis. The symptoms involve immediate severe pain in abdomen. Infiltration is a type of rupture that characterized by the dent leads to further progression of ulcer to the nearby organs like liver and pancreas. Cancer involves diagnosis by biopsy, *H. pylori* is the major mediator that is responsible for development of stomach cancer from the ulcer by many folds [11]. In Western countries the people get peptic ulcer or stomach ulcer with *Helicobacter pylori* infections and are roughly unpredictable. They have shown very diverse reports on ulcer at different ages like 20% people at age of 20, 30% people at age of 30, 80% people at age of 80. There is a huge difference in the prevalence of gastric ulcers between developed and developing countries. Developed countries are more prone to *Helicobacter pylori* infection. Generally, the transitions are through the infected food, contaminated water, and saliva [14]. Aspirin in low doses accelerates the bleeding from ulcers caused by *H. pylori*. They both commonly have proved to exert synergistic effects in manifested patients [15].

Almost each part of *Azadirachta indica* has been noted to be involved with many medical characteristics. Some of them are like antimicrobial activity, storage property, treatment of liver damage due to paracetamol, a modulator of hepatic glutathione-dependent enzymes and glutathione; it shows antiviral activity *in-vitro* studies, also have insecticidal and antibacterial activity. Chemo-preventive effects of *Azadirachta indica* have been evaluated recently on 7, 12-dimethyl Benzanthracene persuade hamster oral pouch carcinogenesis, also, against IBD virus in broilers [16, 17, 18]. In India, farmers consider the Neem tree for its insecticidal properties from long ago. Branches of Neem tree are used to protect the grains from pest attach. It is reveals that from ancient time's Neem tree are used in India for its curative and cosmetic properties. Because of its antimicrobial and insect-repellant effects, neem tree seeds are employed to extract the Neem oil in soap, wax, glidant, and lubricant manufacturing units.

It has been reported that it used contraceptives due to its spermicidal properties. Dose concentration of the extract is considered to inhibit gastriculcer, free and total acidic volume of gastric acid secretions. Nimbidin isolated from Neem is a bitter compound of Neem that shows strong anti-inflammatory activity and antioxidant activity, therefore reducing the production of free radicals. The chemical compound from the Neem tree known as Nimbidin is responsible for ulcer inhibition activity [18]. The aq. extract from bark of the tree can reduce gastric ulcer, this is due to the glycosides present in the Neem tree bark. Bhunimbadi Ghanasar is a medical preparation containing Neem leaves extract; it is very effective against the acid dyspeptic disease and has no complications. Nimbatiktam a crude Neem seed oil extract that contains mainly Nimbidin about 1.2% w/w as the major constituent and it has greater potential of ulcer healing capacity. Limonoids, i.e. Salannin is a bitter chemical constituent isolated from Neem seed oil, show an ulcer healing effect. Salannin also showed

protective action against aspirin-induced gastric lesions when given orally to experimental animals. Recent studies showed evidence regarding potential therapeutic efficacy for reducing the gastric hyper-acidity and healing gastric ulcer. The Neem leaves extract is very effective in the different doses against the ulcers produced by stress due to cold exposure and drug Indomethacin. The crude extract of Neem tree bark and leaves showed the equal response as showed by Ranitidine, Omeprazole [19, 20, 21].

Gastric ulcers are related to inflammation which may include many complications. Many factors are responsible for this condition. Other available drugs are very costly. Presently there is not any promising drug from natural origin. Also the gastric lesions are given rise to gastric cancer. This makes it very important topic to study. Since gastric ulcers are the major problem to the world. Treatments available are very costly and less effective. Drugs available also cause various side effects. The objective of this study is to provide the drugs from natural origin. This may be cheap and easily available.

This may show the preventive and curative effect of Neem leaves on various models of gastric ulcers. Gastric ulcers are due to many causes like NASAID's, H. pylori, ROS, oxidative stress. If gastric ulcer is untreated for long time, it may result in development of gastric cancer. This may cause life threatening situation. This can be avoided by treating the ulcers. So rationale of the study is to evaluate an effective treatment for gastric ulcer. Neem leaves has shown its protective action on many different models of ulcers. We also evaluate protective action of Neem leaves on gastric ulcers.

MATERIALS AND METHOD:

Preparation of Methanolic Neem Leaves Extract for Comparative Study: Fresh Neem leaves was collected from Patna (Bihar), Krishna Nagar-Kolkata and Jadavpur (IACS), Kolkata, West Bengal and dried in shade. Neem leaves were collected by Indian Association for the Cultivation of Science, Kolkata, in 2014 and verified (DKY-01). Leaves were powdered with mixer grinder. 10-20g of powdered Neem leaves was dissolved in 50-60ml of methanol and was kept for 2days at 37°C followed by continuous shaking. The supernatant collected was filtered and entire process repeated for 3 times for complete extraction. The filtrate was evaporated on water bath to concentrate the extract. The concentrated extract was run through TLC in TLC chamber, whose solvent system comprises of methanol (2ml), chloroform (up to 20ml) and few drops of water (5 µl). After the completion of run, the TLC plate was dried under hot air and checked under UV light at (354nm).

Lieberman-Burchard Test: The concentrated extract was run through TLC in TLC chamber, whose solvent system comprises of methanol (2ml), chloroform (up to 20ml) and few drops of water (5 µl). After the completion of run, the TLC plate was dried under hot air and then sprayed with Lieberman-Burchard's reagent and heated for few minutes. The deep green or dark spot confirms the presence of terpenoids or steroids.

Isolation of Band of Interest: The concentrated extract was run through TLC in TLC chamber, whose solvent system comprises of methanol (2ml), chloroform (up to 20ml) and few drops of water (5 µl). After the completion of run, the TLC plate was dried under hot air and checked under UV light at (354nm). The crude Neem leaves extract that shows good results were used to prepare the crude extract. The band of interest was scrapped out of TLC and further extracted with methanol for 3days and then filtered. The filtrates were dried on rotary evaporator at 50-60 °C for 1-2 hours. After complete drying, the extracts were collected in eppendorf and weighed, wrapped in aluminum foil and kept in refrigerator for further study.

Mass Spectroscopy: The fractions of different band of interest and crude extract were given for Mass analysis to identify purity of samples.

Cell Viability assay: AGS and K562 cells were used for cell viability assay. AGS are the gastric cell lines and K562 are the leukemia cancer cell lines. AGS cell lines are the adherent cell lines while the K562 cells are non-adherent type.

Maintenance of Cell Culture: AGS and K562 are maintained in complete medium (RPMI) in incubator at 37 ˚C with 95% air and 5% CO_2.

The cells were examined daily under phase contrast microscope for the presences of any contamination. The medium of the cells was changed on every alternate day. Cells may take 1-2 days to confluent.

Cell Plating: Cells were counted by cell counter. Then they were seeded as 10 million cells per well in 96 wells plate. Cell plates were then incubated at 37°C and supplied with 5% CO_2 pressure for two days to confluent.

Dose Preparation: Bands of respective fractions were weighing accurately and dissolved in DMSO (10%). Doses of each band are calculated by following method:

$$V_1S_1=V_2S_2$$

MTT Assay: Cells were seeded 10^4 cells per well. Confluent cells for 2 days in incubator. The complete media was removed and 100µL experimental media (RPMI-1640) was added. The stock solution of drug was prepared by using DMSO at a dose of (1mg/ml). Drug was then introduced to every single well which followed by incubation for 4 hours and 100µL of MTT was added.

The working concentration used for the assay was 0.5mg/ml per well. MTT dye was diluted in PBS solution (1mL in 9ml). The cells in wells were incubated for 3 hours at 37°C, air 95% and5% CO_2. After that the reagent was discarded very carefully (without disturbing the formazone complex formed at the bottom). The formazone was dissolved by using same concentration of DMSO (5µl) per well. The cell plates were subjected to shaking for 30mins. Absorbance was taken at 595nm. Assay was performed at different incubation hours.

Indomethacin Induced Gastric Ulcers:

Animals used: Male Sprague-Dawley rats (180-200 g), which bred in animal house cage by giving continuous supply of food and water was used throughout the project. Further the rats were kept in 12 hours light and dark periods that are housed at 25 °C. Group 1 (n = 4) this group was treated as control and kept on normal diet. Group 2 (n = 4) this group was treated with Indomethacin by oral gavaging (10mg/kg.bw.) for seven days. Group 3 (n = 4) this group was treated with Indomethacin by oral gavaging at a dose of (10mg per kg, b.w.) and after 30 minutes with crude Neem leaves extract at a dose of (50mg per kg, b.w.) for seven days. Group 4 (n = 4) these group was treated with crude Neem leaves extract (50mg per kg, b.w.) for seven days. On eighth day of experiment all animals were sacrificed. The stomach was collected and washed by using 0.9% saline. The stomach collected was stored at -80 °C for further studies.

Measurement of gastric lesions: Gastric lesions were counted under the magnifying glass and different scoring system was used to calculate ulcer index. The ulcer scores were given based on Martin *et.al.* as; 0 for no lesions; 10 for less than 5 minor lesions; 20 for more than 5 minor lesions; 30 for 1 to 5 blood-stained bands; 40 for 1 to 5 blood-stained bands of length >5mm; 50 for 5 to 10 blood-stained bands having length >5mm; and 60 given for entire lesions of the mucosal with bleeding. The sum of the total ulcer counted was divided by the number of rats to get the average ulcer index.

Tissue Histology (Hematoxylin and Eosin Staining): Tissues were minced into 2-3 mm pieces. The tissue samples were soaked in 4% p-formaldehyde, dehydrated and immersed in paraffin wax. Nearly about 5µm thick uniform sections were re-hydrated in decline alcohol series followed by staining with hematoxylin and eosin.

This process of fixing, absorption, and staining were carried out in exact parallel manner to ensure comparative significance among all groups. All the pictures or Images were recorded with an Olympus microscope using Camedia software (Chicago, IL, USA). It was processed by using Adobe Photoshop version 7.0.

Tissue Extraction: Gastric tissues were hanged in phosphate-buffer (10mM), protease inhibitors, cut up at 4 °C. This suspension of gastric tissues was centrifuged at 12000g for fifteen minutes. The supernatant thus formed was captured as phosphate-buffer extracts. The pellet was also separated in lysis buffer (composed of 10 mM Tris-HCl, pH about 8.0, 150 mM of NaCl, 1%of Triton X-100, protease inhibitors) and centrifuged at12000gfor 15 minutes to obtain Triton X-100 extracts (Swarnakar *et al* 2005).

Estimation of Protein Concentrations by Folin-Lowry Method: BSA standard solution prepare with 1 mg per ml of BSA which was recognized as working standard. The various dilutions of BSA solutions were prepared by using stock BSA solution (1 mg/ ml) dissolved in water. The total final volume considered for each sample is 1.2ml and the BSA concentration is 0 to 90mg per ml.

The volume of 1ml distilled water was used as blank and 10µl of sample added whose concentration is to be estimated, each sample mixed with 4.5 ml of reagent I and incubated for 10 min. After 10 min of incubation 0.5 ml of reagent II was added and further incubated for 30 min in dark place at room temperature. The absorbance was checked at 660 nm and the standard graph was plotted. The concentration of isolated protein present in our sample was calculated from the standard graph.

SDS-PAGE: SDS-PAGE gels was prepared on a casting assembly by adding resolving gel mixture in to the gel plate assembly, polymerized and then the stacking gel solution was added from top of the resolving gel. Before polymerization of the stacking gel, the comb was inserted carefully to prepare the sample slots.

Samples (PBS and TX extracts) were mixed with 1X sample loading buffer and electrophoresis under non-reducing conditions. The gels were generally run at 60 volts for stacking gel followed by 90 volts for resolving gels. Gels were stained with Coomassie blue stain followed by destining.

Stress Induced Gastric Ulcers:

Animals used: Random-bred Bulb C mice aged 7-8 week's b.w. about 25-30g were employed for this project study. They were kept in suitable air-conditioned animal facility at (IICB, Kolkata) followed by 12 hours light and dark periods, and feeded with standard food pellets and tap water. All mice were housed according to the "Principles of Laboratory Animal care" of the national Institute of Health (NIH, USA) and Indian Animal Ethic Committee(IAEC), protocol number 2015/SSN-10 (IICB/AEC/Meeting/July 2015). The mice were kept for fast for 24hours with providing only water before the experiment.Group1 (*n* =4): mice were treated as control and feeded with PBS. Group 2 (*n*=4): Animals were treated as positive control they were forced to swim for 4-5 hours in water maintained at 25 °C. Group 3 (*n* = 4): mice were treated as standard. Omeprazole was used as standard drug. The mice were feeded with (20mg per kg, b.w.) of Omeprazole by oral gavaging one hour prior to forced swim test.

Group 4 (*n*=4): mice were treated with crude Neem leaves extract at a dose of (25mg per kg, b.w.) by oral gavaging one hour forced swim test. Group 5(*n*=4): mice were treated with crude Neem leaves extract at a dose of (50mg per kg, b.w.) by oral gavaging one hour forced swim test. Group 6 (*n*=4): mice were treated with methanolic crude extract prepared from Neem leaves at a dose of (75mg per kg, b.w.) by oral gavaging one hour forced swim test. After 4-5 hours mice were sacrificed, stomach was removed and washed by using with 0.9% of saline solution and stored at -80 °C for project studies.

RESULTS AND DISCUSSION:

Chromatographic study of Neem Leaves Collected from Different Locations by TLC Method: Neem leaves collected from different locations were used to prepare the methanolic Neem leaves extract. The Neem leaves were brought from Patna (Bihar), Jadavpur (Kolkata) and Krishna Nagar (Nadia, W.B.). Leaves were dried on bloating paper under the shade. Methanolic extract was prepared from each lot of Neem leaves. Chromatographic separation of methanolic extract was done using thin layer chromatography.

Extract was applied on TLC plate using capillary. TLC plates were then dried for 5-10 minutes and kept into solvent system. The solvent system used was 2 ml methanol; 5µlwater and chloroform to make volume up to 20ml. TLC plates were kept in above solvent system. Detection was done under UV light at wavelength 254 nm. Experiments were performed several times to check the accuracy of the results. Some of the results are shown in **Fig. 1**. TLC data indicates that methanolic Neem leaves extract contains several constituents. The separation of bands in methanolic Neem leaves extract of Krishna Nagar Neem leaves is better as compared with Patna and Jadavpur Neem leaves.

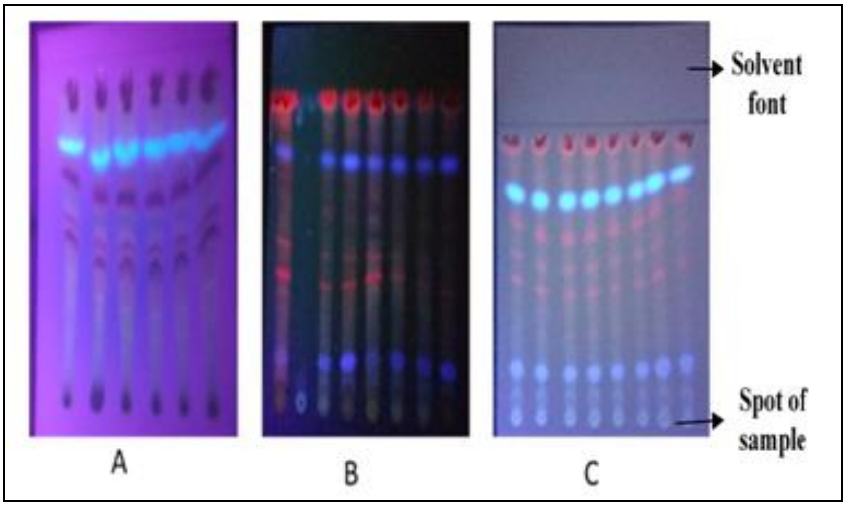

FIG. 1: TLC PLATES OF METHANOLIC NEEM LEAVES. A: TLC OF PATNA NEEM LEAVES. B: TLC OF NEEM LEAVES COLLECTED FROM JADAVPUR AREA (W.B.). C: TLC OF NEEM LEAVES COLLECTED FROM KRISHNA NAGAR (KOLKATA)

From above TLC plates, the crude Neem leaves extract prepared from Krishna Nagar Neem leaves are showing very intense and greater number of bands as compared with the other extracts from different locations.

Also, the UV bands are more visible in Krishnanagar leaves extract as compared to other Neem leaves collected from different geographical regions suggesting variation in their chemical constituent's content. Thus, environmental conditions play important role on growth and overall chemical composition in leaf of the plant.

Liebermann-Burchard Spray test: The Liebermann-Burchard or acetic anhydride spray test was used for the detection of sterols, terpenoids, and alkaloids. The formation of a dark green or brownish color after a few minutes signified positive.

Since this test uses acetic anhydride and sulphuric acid as reagents, caution must be exercised so as not to receive severe burns. The test was confirmed by appearance of dark green and brownish color.

TLC was performed and detection was done using Liebermann-Burchard reagent and the chromatographic separation pattern was shown in **Fig. 2: A, B, C**. It shows that all the extracts may have nearly same content of compounds. The dark green and yellow bands of Krishna Nagar leaf extract TLC were better than others that repeated several times.

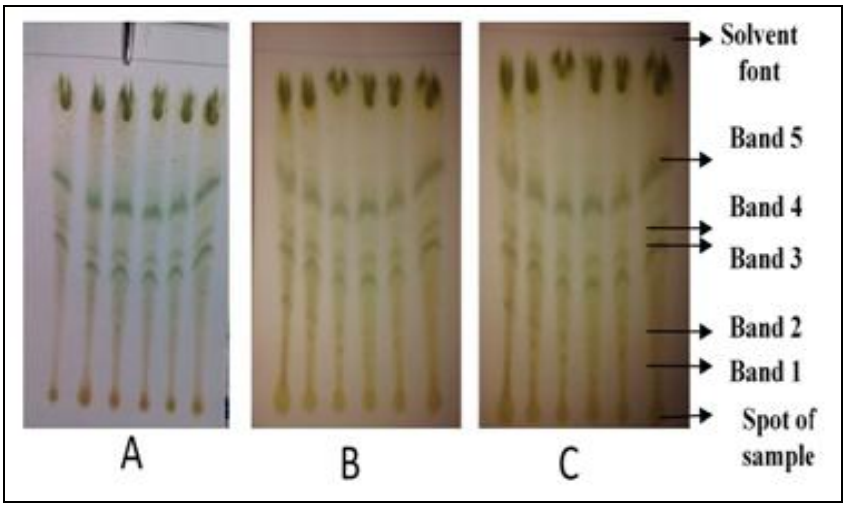

FIG. 2: LIEBERMANN-BURCHARD TEST. (A) PATNA NEEM LEAVES. (B) JADAVPUR NEEM LEAVES. (C) KRISHNA NAGAR NEEM LEAVES. THE BANDS PLATES ARE COMPARED VISUALLY BY WIDTH AND INTENSITY OF THE BANDS

This study shows that methanolic extract prepared by using Krishna Nagar Neem leaves shows a greater number of bands with distinct color. Furthermore, methanolic Neem leaves extract is more than then aqueous extract.

Mass Spectroscopy: As from previous study we found that the methanolic extract from Krishna Nagar Neem leaves shows better separation as compared to others, mass spectroscopy of the extract was performed. The spectrum observed was depicted in **Fig. 3.** Above mass data shows number of peaks, which confirms Neem contains many compounds. Whereas large peaks show that these may be the compounds having greater solubility in methanol. Based on TLC separation, bands were distinguished as B1, B2, B3, B4, B5, and B6. The bands of interest (B5 and B6) are scrapped, and their mass spectra were also done. It is also shown in **Fig. 4** and in **Fig. 5.**

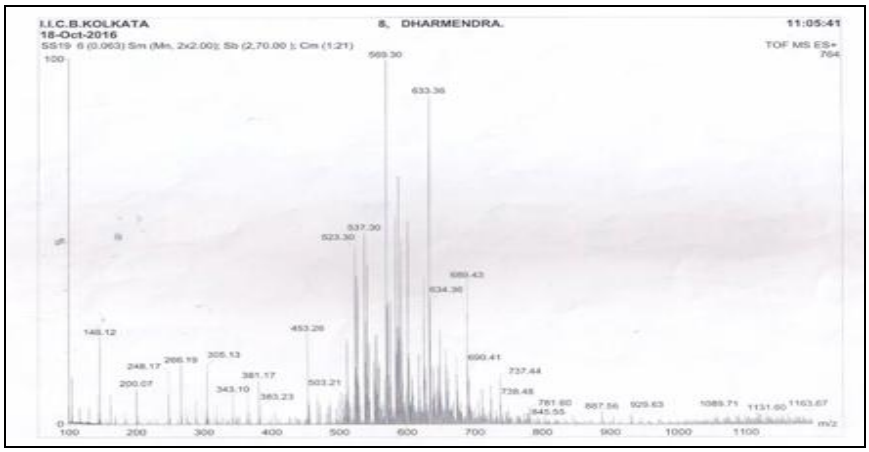

FIG. 3: MASS SPECTRUM OF CRUDE NEEM LEAVES EXTRACT OF KRISHNA NAGAR LEAVES

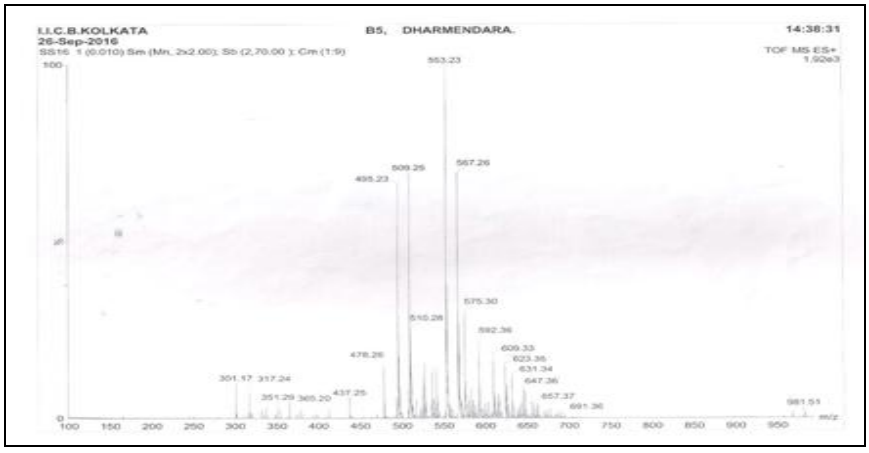

FIG. 4: KRISHNA NAGAR NEEM LEAVES, MASS SPECTRUM OF BAND B5

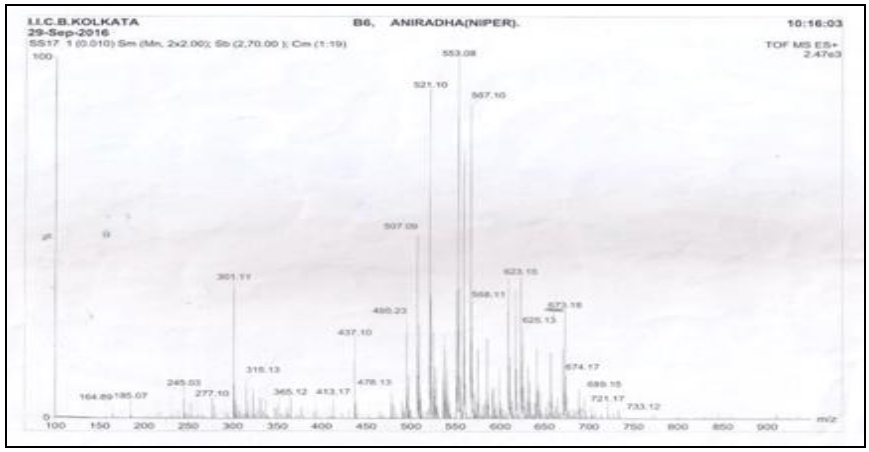

FIG. 5: KRISHNANAGARNEEMLEAVES, MASS SPECTRUM OF BAND B6

The mass spectra of B5 band show 2-3 larger peaks with more intensity. It shows that there were 2-3 major compounds present on the extract. After comparison of bands B5 and B6 mass spectrum, it is observed that some peaks are same as in both band whereas some are different showing that there may be presence of some different compounds.

Cell Viability Assay:

MTT Assay: After the preliminary information from TLC and mass spectroscopy study, we next checked the effect of methanolic Neem leaves extracted two compounds *viz.* compound B5 (obtained from band B5) and compound B6 (obtained from band B6) on the cancer cell lines for their apoptotic effect. AGS and K562 Cell were cultured. Cells were seeded at the density of 10^4 cells per well. They were passaged on confluence for two days in the presence of 5% FBS. After reaching 70–80% confluence (kept 2 days for confluent), in the presence of 5% FBS, cells were treated with compound B5 and B6 at various concentrations for 4 hours. Extract (1mg/ml) was dissolved in DMSO, and control was treated with only DMSO. Cell viability determination was done by MTT (MTT 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. After that, MTT reagent was added to the cells treated with the compound B5 and B6 and incubated for 3 hours. After 3 hours, medium from cells was removed and DMSO was added. The absorbance was measured at 595nm. Cell culture was done under strict aseptic conditions to avoid any contamination.

Results of MTT Assay on AGS Cell Lines using Compound from Band B5: Upon treatment of compound B5 on the gastric carcinoma cells lines (AGS), cell viability decreases suggest compound B5 inhibiting the cancer cell survival.

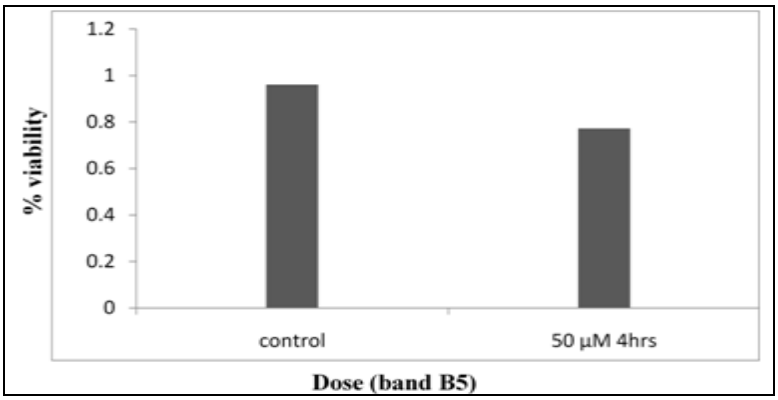

FIG. 6: BAND B5 NEEM LEAVES EXTRACT DECREASED VIABILITY OF AGS CELLS WHEN TREATED WITH DOSE OF 50MM FOR 4 HOURS

Results of MTT Assay on AGS Cell Lines using Compound from Band B6: Upon treatment of compound B6 on the gastric carcinoma cells lines (AGS), cell viability decreased suggests that compound B6 inhibiting the cancer cell survival. The potency of B6 was high as compared to B5 regarding cell mortality.

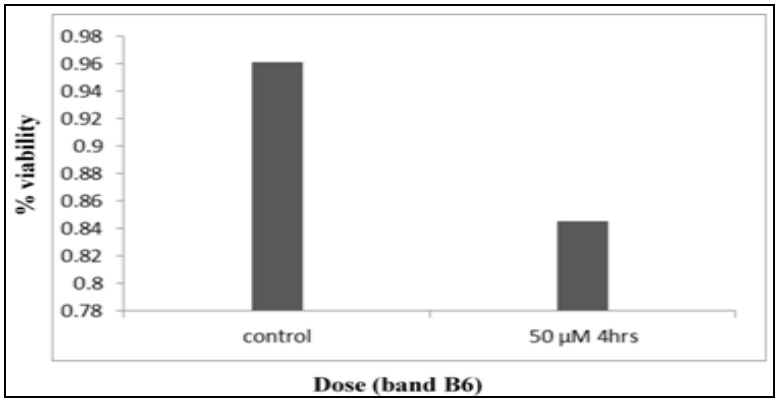

FIG. 7: BAND B6 OF NEEM LEAVES EXTRACT DECREASED VIABILITY OF GASTRIC CARCINOMA CELLS WHEN TREATED WITH DOSE OF 50MM FOR 4 HOURS

Results of MTT Assay on K562 Cell Lines using Compound from Band B5: Similar experiment was performed using K562 cancer cell lines at different concentrations. It was observed that on increasing the concentration of the compound from Band B5, the cell survival of cancer cell lines decreases.

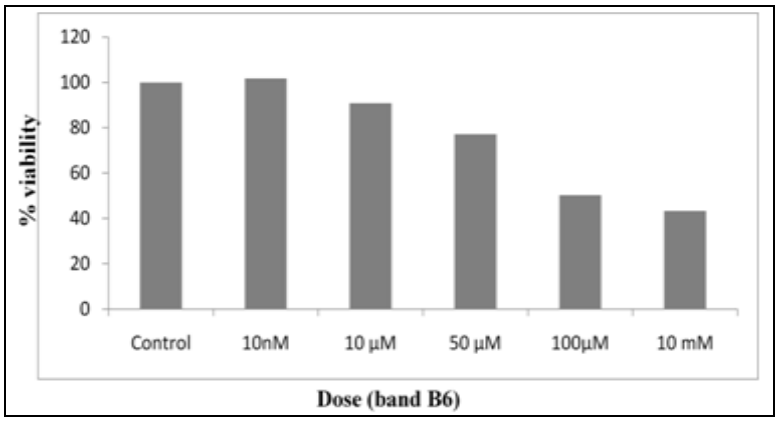

FIG. 8: DOSE DEPENDENT EFFECT OF COMPOUND FROM BAND B5. AT THE DOSE OF 10MM MAXIMUM CELL DEATH OCCURRED

Results of MTT Assay on K562 Cell Lines using Compound from Band B6: K562 cells were treated with different doses of compound from Band B6. It also shows that, as the concentration of extract increases, cancer cell survival decreases.

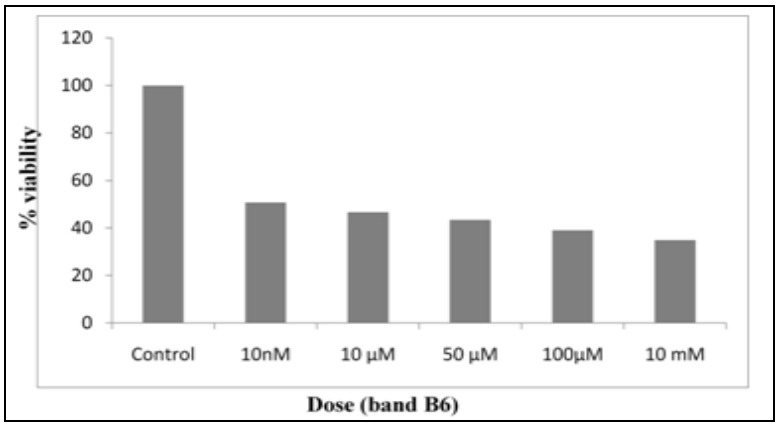

FIG. 9: COMPOUND B6 DECREASED CELL VIABILITY WITH NCREASEIN DOSE. THE ACTIVITY OF COMPOUND B6 ARE LITTLE MORE AS COMPARED WITH THE B5. THEY ARE ALSO INCUBATED FOR 4 HOURS AFTER DOSE TREATMENT

Effect of Compound B5 and B6 on MMPs Level on AGS Cell Supernatant as Judged by Gelatin Zymography: Our next aim is to check the level of MPP and level of inhibition by the compounds B5 andB6. Gelatin zymography has been done to check the release of MMPs in the supernatant culture. MMPs are involved in the inflammation and cancer progression. We found that MMPs are secreted in the AGS cell culture media. Samples were taken from the AGS cell suspension treated with both compounds *viz*. B5 and B6 that incubated for 4hrs. The results show that the intensity of MMPs produced differed upon treatment with the compound B5 and B6.

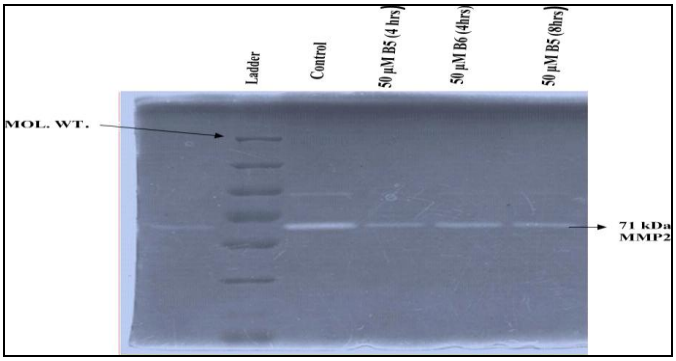

FIG. 10: IT IS CLEAR FROM THE FIG THAT THE MMP9 AND MMP2 ARE RELEASED FROM THE CELL SUSPENSION OF AGS CELLS.THE INTENSITY OF BAND DECREASES IN DRUG TREATED SUSPENSION

Macroscopic View of Stomach of Indomethacin Induced Gastric Ulceration: Indomethacin had high ulcerogenic potential. Because of this reason it is used to induce a stomach ulcer in experimental model. In this test, we have divided the animals in to four groups *viz*. A) Control (Untreated), B) Indomethacin (10mg per kg, b.w.) treated, C) Indomethacin plus Neem leaves extract (50mg per kg, b.w.) and D) Neem compound treated. Then they were given treatment accordingly. Crude Neem leaves extract was given to animals 30 mint prior to Indomethacin. Crude Neem leaves extract shows protective action on the Indomethacin induced gastric ulcer are inhibited in extract treated animals (group C and D). Severity of the ulcers has been reduced (observed based on morphology of stomach isolated from the treated mice) as compared with the animals treated with Indomethacin.

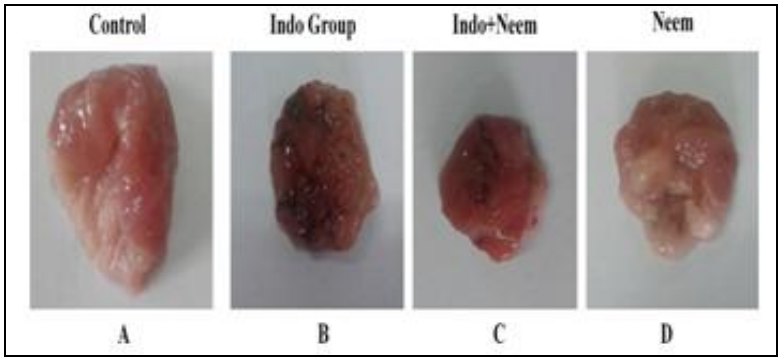

FIG. 11: CRUDE NEEM LEAVES EXTRACT TREATED RATS HAVE SHOWN LESS LESIONS ON STOMACH AS COMPARED TO THE INDOMETHACIN INDUCED ULCERS ENSURING THAT THE NEEM LEAF HAVE PROTECTIVE ACTION AGAINST GASTRIC ULCERS. THE RATS TREATED WITH ONLY CRUDE NEEM LEAVES EXTRACT HAS HEALTHY STOMACH THAN OTHERS

Ulcer Index of Indomethacin Induced Gastric Ulcers: The ulcer index (quantitative measurement done by counting number of ulcers) was also calculated. Ulcer index of Indomethacin treated group is higher. While the animals treated with Indo + Neem leaves extract have lower ulcer index. From this study we came to know that the crude Neem leaves extract obtained from Krishna Nagar leaves can inhibit the gastric ulcer.

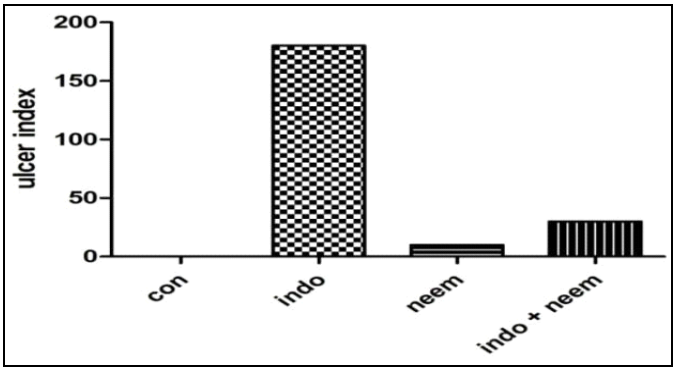

FIG. 12: ULCER INDEX OF RAT STOMACH FOLLOWING TREATMENT WITH INDOMETHACIN AND NEEM LEAF EXTRACT. FEWER LESIONS DECREASED IN STOMACH OF ANIMALS OF ANIMALS WHICH ARE TREATED WITH CRUDE NEEM LEAVES EXTRACT COMPARED TO INDOMETHACIN TREATMENT ONLY

Estimation of protein concentrations by Folin-Lowry method: Standard curve: Absorbance at 660nm: Next, we have estimated the amount of protein present in the sample by Folin-Lowry method. This protein concentration is used further to run samples in SDS-PAGE.

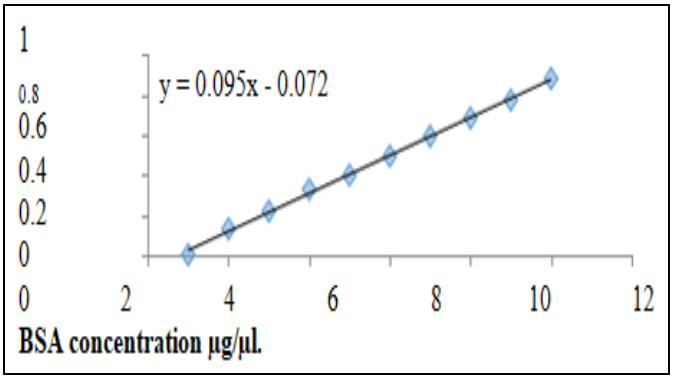

FIG. 13: STANDARD CURVE BSA

SADS-PAGE of Tissue Extracts of Stomach from Indomethacin Induced Gastric Ulcers: This is performed to check the expression of IL-17 and TNF-α which is involved in the inflammation during gastric ulcers. IL-17 expression in the Neem extract treated samples is less where as it is highly expressed in the Indomethacin treated group.

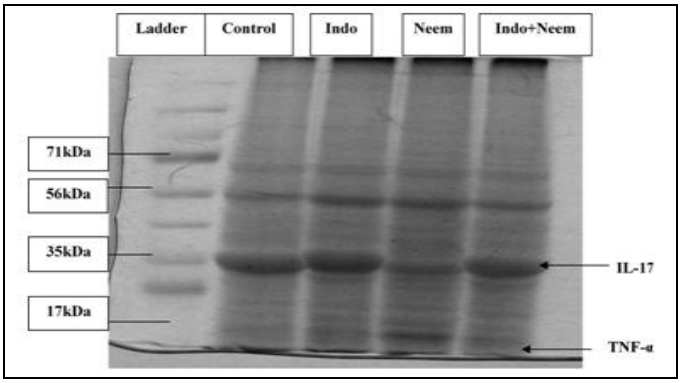

FIG. 14: SDS PAGE OF INDOMETHACIN INDUCED GASTRIC ULCER OF PBS EXTRACT

Macroscopic View of Stomach of Mice Upon Stress Induced Gastric Ulceration and Tissue Histology of Stomach: Stress induced ulcer is a common acute gastric lesion of the stomach. In this context, we have investigated the effect of Neem crude extract on the stress induced gastric ulcers. Mice were made to perform forced swim test to generate stress. Then they were treated with the varying doses (E25, E50 and E75mg per kg, b.w.) of crude Neem extract. Crude Neem leaves extract administered is orally to animals. The difference in the morphology of stomach isolated from mice. Crude Neem leaves extract shows its protective action on the stress induced gastric ulcers. Crude Neem leaves extract administered orally to animals in different doses. Study on stress induced gastric ulcer shows that the anti-ulcer and protective action of extract was dose dependent. Ulcer index or lesions are reduced as we increase the dose.

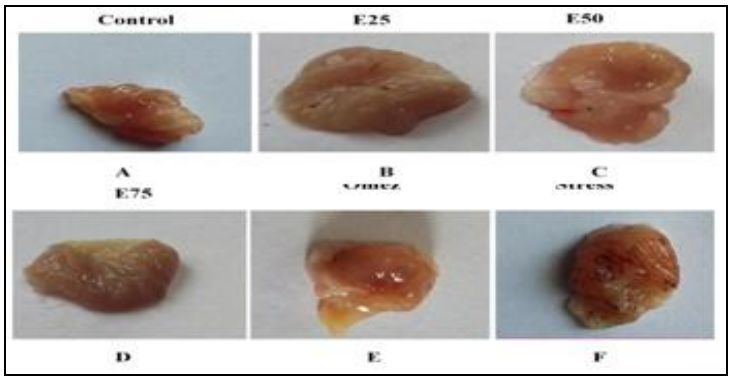

FIG. 15: STRESS INDUCED GASTRIC ULCER IN BULB C MICE SHOWN IN FIG. 15 (A-F). BULB C MICE TREATED WITH DIFFERENT DOSES TO SEE RESPONSE. WE OBSERVE THAT WHEN DOSE HAS BEEN INCREASED CONSEQUENTLYTHE RESPONSES ARE MORE POTENT. IT HAS BEEN NOTED THAT AT HIGHER DOSE (75MG/KG, B.W.) THERE WAS NO ULCER

Ulcer index of Stress Induced Gastric Ulcers:

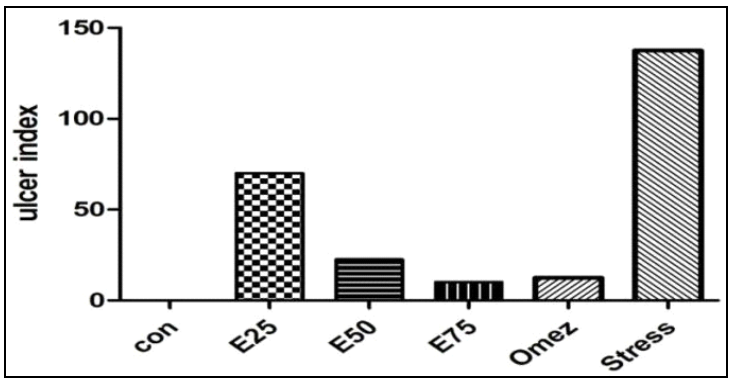

FIG. 16: CRUDE EXTRACT SHOWS GOOD PROTECTIVE ACTION ON STRESS INDUCED GASTRIC ULCERS. ULCER INDEXES ARE DIRECTLY RELATED TO DOSE RESPONSE I.E., WITH INCREASE IN THE DOSE OF CRUDE EXTRACT PROTECTION ARE MAXIMUM

Ulcer index of stress induced gastric ulcer. We found that the crude Neem leaves extract shows protective action on gastric ulcers. The effective action of crude extract is dose dependent.

% of Inhibition in Stress Induced Gastric Ulcers: Ulcer inhibition study is shown in figure below. It represents that the crude Neem leaves extract treated groups have maximum inhibition as compared with the others.

Percentage of ulcer inhibition of crude extract at dose 75mg per kg, b.w. is equal to Omeprazole.

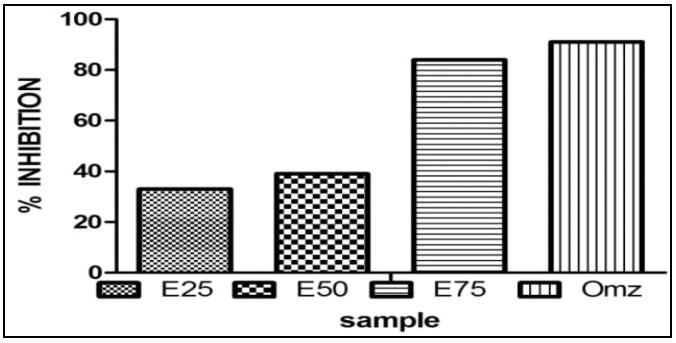

FIG. 17: IT SHOWS THAT THE PERCENTAGE OF INHIBITION IS INCREASES WITH INCREASE OF DOSE. E75 (75MG/KG) DOSE OF CRUDE EXTRACT THE INHIBITION ARE MAXIMUM

Tissue Histology of Stress Induced Gastric Ulcer (Hematoxylin and Eosin Staining):

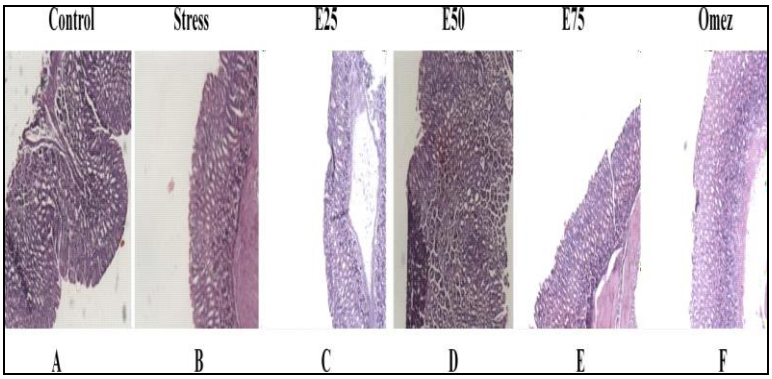

FIG. 18: TISSUE HISTOLOGY PICTURES REPRESENT THE STATUS OF SURFACE EPITHELIUM, GASTRIC PITS ANDLEVEL OF INFLAMMATION OF THE RESPECTIVE GROUPS (A, B, C, D, E, AND F). GROUPS TREATED WITHETHANOL SHOWED DENUDATION OF SURFACE EPITHELIUM AND LOSS OF GASTRIC PITS, WHERE AS BLACK TEA PRE-TREATED PREVENTED THE GASTRIC EPITHELIAL DENUDATION

CONCLUSION: Crude methanolic extract was prepared from two geographical locations for comparative study on its biological function. Crude Neem leaves extract inhibited AGS and K562 cells growth *in-vitro*. The mechanism involves apoptosis-induced cell loss for both AGS and K562 cells. In Indomethacin-induced gastric ulcers methanolic crude Neem leaf extract (at a dose of 50mg per kg b.w.) inhibited gastric ulcers in rodents. Also, the inhibitory effect of crude Neem leaves extract in stress induced gastric ulcers was prominent at doses (25mg per kg, 50mg per kg, and 75mg per kg, b.w.).

Our study suggests that the results are dose dependent inhibitory activity of Neem leaf on stress as well as chemical induced gastric ulcers. Methanolic crude Neem leaves extract (suburban) showed potent effect on the gastric ulcers compared to leaves from city are. This study initiative of use of methanolic Neem leaves extract as source of the “drug hunters” and potential agent for human health management gastric ulcer. In future novel compound/compounds could be isolated from Neem leaves having potent anti-ulcer activity.

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