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RESEARCH ARTICLE

Evaluation of protective effect of *Aegle marmelos* fruit (Bael) on Mycophenolate mofetil and Mycophenolate Sodium induced gastrointestinal toxicity in mice.

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Abstract

Mycophenolate mofetil (MMF) and Mycophenolate sodium (MPS) are the common allopathic drugs used in the prevention of graft rejection during transplantation and various autoimmune disorders. Clinically, MMF and MPS are known to cause various adverse effects, with diarrhea, weight loss and gastric ulcer as the most common events. This study aimed to minimize MMF / MPS associated diarrhea, weight loss and gastric ulcer upon co-administration of aq. extract of ripe fruit of *Aegle marmelos (*AM).Traditionally, the plant has been used for the improvement of GI function.

Methods: Ten groups of mice (n=6) were selected and dosed as: control (CMC 1%), MMF (210mg/kg), and MMF (210 mg/kg) followed by AM (100mg/kg), MMF (210 mg/kg) followed by AM (200mg/kg) and MMF (210 mg/kg) followed by Loperamide (3mg/kg). MPS (100mg/kg), and MPS (100 mg/kg) followed by AM (100mg/kg) and MPS (100 mg/kg) followed by AM (200mg/kg) and MPS (100 mg/kg) followed by Loperamide (3mg/kg). And standard: Loperamide (3mg/kg). Weight loss, diarrhea grade were monitored for six days. In second part of study, four group of mice (n=6) were selected and dosed as: control castor oil (1ml) followed by vehicle, Loperamide (3mg/kg) followed by castor oil (1ml), AM (100 & 200mg/kg) followed by castor oil (1ml) and diarrhea grade were monitored. In third part of study ten groups of mice (n=6) were selected and dosed as: control (CMC 1%), MMF (210mg/kg), and MMF (210 mg/kg) followed by AM (100mg/kg), MMF (210 mg/kg) followed by AM (200mg/kg) and MMF (210 mg/kg) followed by Ranitidine (10mg/kg). MPS (100mg/kg), and MPS (100 mg/kg) followed by AM (100mg/kg) and MPS (100 mg/kg) followed by AM (200mg/kg) and MPS (100 mg/kg) followed by Ranitidine (10mg/kg). And standard: Ranitidine (10mg/kg), after 4 hours of last dose of the treatment, the animals were sacrificed, and ulcer index evaluated. In fourth part of the study, eleven groups of mice (n=6) were selected for evaluation of antiulcer properties of AM using pylorus ligation technique. The animals were dosed as: Control (distilled water 2mL/kg), AM treated (200mg/kg), MMF (210mg/kg), MPS (100mg/kg), standard group Ranitidine (10mg/kg), and MMF (210mg/kg) followed by AM (100 and 200 mg/kg) and MMF (210mg/kg) followed by Ranitidine (10mg/kg), MPS (100mg/kg) followed by AM (100 & 200mg/kg) and MPS (100 mg/kg) followed by Ranitidine (10mg/kg). After 4 hours of last dose of the treatment, the animals were sacrificed, and ulcer index evaluated.

Result: MMF and MPS were found to induce diarrhea ($p<0.001$) and weight loss by 6^{th} day. Castor oil also induced diarrhea. MMF and MPS treated groups induced gastric ulcer ($p<0.001$). Pylorus ligation induced gastric ulcer in presence or absence of MMF and MPS ($p<0.001$). The groups treated with AM showed significant recovery, as found to have low or no diarrhea

(as indicated by diarrhea grade), reduced weight loss, and protection against gastric ulcer induced by MMF and MPS ($p<0.001$), in comparison to the respective control groups. In pylorus ligation study, the animals in the groups treated with AM, were found to have lower ulcer index ($p<0.001$) in comparison to the MMF treated group, MPS treated group or control group animals.

Conclusion: *Aegle marmelos* was found to provide significant protection against the MMF and MPS induced GI toxicity in mice as indicated by lower levels of diarrhea grade, weight loss and ulcer index.

Introduction:-

Gastrointestinal toxicity (peptic ulcer, diarrhea) and **weight loss** are commonly associated with the use of MMF and MPS, the widely used immunosuppressants for the prevention of graft rejection and various autoimmunity disorder treatments. Mycophenolic acid (MPA) is, the active metabolite of MMF / MPS, shows its pharmacological effect via inhibition of inosine monophosphate dehydrogenase (IMPDH), the rate-limiting enzyme in de novo purine synthesis **(Nema et al 2012)**. This ultimately leads to the selective inhibition of lymphocyte (T and B) cell division. MPA preferentially inhibits the de novo guanosine nucleotide synthesis in lymphocytes, and therapeutic dose of MPA also affect monocytes, as it significantly decreases guanosine triphosphate (GTP) pools in human peripheral blood monocytes, but not in neutrophils. MPA suppresses both cell mediated immune responses and antibody formations, which are major factors in both acute and chronic allograft rejection. Mechanisms responsible for GI side effects are not fully understood, but exposure of the intestinal epithelia to Mycophenolic acid during enterohepatic recycling may be involved. Enhanced exposure of the enterocytes to MPA, via enterohepatic recycling, is thought to play a role in one of MPA major side effects, gastrointestinal (GI) toxicity **[Stephan et al 2006].** ***Aegle marmelos*** is a medicinal plant belongs to the *Rutaceae* family and commonly known as wood, apple, bael, golden Bengal quince, apple, and stone apple, bili, traditionally used in treatments of various successful gastrointestinal complications **[Sharma et al 2011]**. There are various compounds found in *AM* which improve the gastrointestinal function and include Cineol, Marmesinin, Luvangetin, Marmin and Tannin **[Maity et al 2009]**. The aim of this study is to minimize the MMF / MPS associated diarrhea, weight loss and gastric ulcer, upon co-administration of aq. extract of ripe fruit of *Aegle marmelos* (AM). The co-administration of the protective herb will help to improve gastrointestinal function during treatment of various graft rejection and autoimmune disorders with MMF and MPS clinically.

Material & method:-

Drugs and chemicals:-

MMF was procured from BDS synthesis Limited, New Zealand, while MPS was obtained from Intas Pharmaceutical Pvt. India. NaOH and Phenolphthalein and Pentobarbitone sodium were obtained from Sigma-Aldrich Chemicals Pvt Limited, Bangalore India. Carboxymethyl cellulose was procured from Loba Chemie Pvt. Ltd. Mumbai India.

Aegle Marmelos fruit extract:-

The fresh ripe fruits of *AM* (family –Rutaceae) were collected from local market Hazratganj, near NBRI (National Botanical Research Institute), Lucknow, India. The aqueous extract of the fruits of AM was prepared according to the Das et al 2012 method at Ethno-pharmacology laboratories, NBRI Lucknow. The organoleptic/macroscopic characteristic were identified & authenticated by the scientist at NBRI.

Animals:-

One to five months old Swiss Albino mice (male and female; average weight 20-40g) were received from animal colony of National Laboratory Animal Centre, NBRI and housed separately at a temperature of 24-26 ^{0}C and humidity 30–35%, with 12-h light/dark cycles in plastic cages. The animals were provided with standard dry pellets diet (Amrut, India) and water *ad libitum*. Animals were allowed to acclimate to housing conditions for at least 1 week before initiation of experiments. All animal studies were carried out after approval of protocol (Reg. No. IAEC/NBRI/PH/6-6) by Institutional Animal Care Committee, N.B.R.I., Lucknow, India.

Preparation of suspension of MMF and MPS:-
The suspensions of 210 mg/kg MMF and 100mg/kg MPS were prepared fresh in 1% carboxy methyl cellulose in distilled water (10 mL/kg), and used as and when required.

Acute toxicity evaluation of Aegle marmelos:-
Swiss albino mice (male and female) selected for acute toxicity study. An acute oral toxicity study was carried as per OECD-423 guidelines. The aqueous extract of ripe fruit of *Aegle marmelos* was taken at various dose levels (5, 50, 300, 2000 mg/kg body weight) [Behera et al 2012], using 1 % carboxymethyl cellulose in distilled water (10 mL/kg), orally. The animals were observed incessantly for two hours and then for further four hours (occasionally) for any mortality. Behavior (feeding behavior, water intake, general motor activity, response to tail pinching, pupil size, fecal output, sedation, gross behavior, writhing, convulsion, *etc*.) of the animals and any other toxic symptoms were also observed for 72 hours and the animals were kept under observation up to 14 days [Ghosh, 1984].

Dose selection of Aegle marmelos:-
Acute toxicity, ripe fruit of Aegle *marmelos* studies were carried out using *Swiss* albino mice. No mortality was found and changes in the behavior were observed up to doses of 2000 mg/kg body wt. Therefore, 200 and 100 mg/kg doses were selected for screening of protective effect of AM on ulcer and toxicity induced by MMF andMPS.

Study I: Assessment of protective effect of *Aegle marmelos* fruit on Mycophenolate Mofetil (MMF) and Mycophenolate Sodium (MPS) induced diarrhea and weight loss:-
The sixty mice were divided into ten groups, each group has six mice (3 male and 3 female) (n= 6), and treatment given daily for six days through oral gavages, between 11:00am-1:00pm: I group (control) received 1% Carboxymethyl cellulose (CMC) in 10mL/kg distilled water, II and V groups (positive control) treated with MMF (210mg/kg) and MPS (100 mg/kg) and VIII groups (standard) treated with Loperamide (3mg/kg), III, IV, VI, VII, IX and XI (test control) received similarly MMF + AM (210 + 100mg/kg), MMF + AM (210 + 200mg/kg), MPS + AM (100 + 100mg/kg), MPS + AM (100 + 200mg/kg), MMF + Loperamide (210 + 3mg/kg) and MPS + Loperamide (100 + 3 mg/kg). Body weight and stools were monitored daily for 6 days, according to **Stephan et al 2007**. Diarrhea grade was defined according to **Takasuna et al 1996** described method as follows; 0= No diarrhea, 1= slight diarrhea, 2= moderate Diarrhea, 3= severe diarrhea.

Study II: Assessment of protective effect of *Aegle marmelos* on diarrhea induced by castor oil in presence of marketed anti-diarrheal drugs loperamide:-
Animals were fasted for 18h but allowed free access to water. They were randomized into four groups (n=6). All groups received castor oil at a dose of 1ml/animal orally. 30 min after castor oil administration, I group (control) received Castor oil (1ml) + Vehicle (0.5% Tween 80), II group (standard) received Loperamide (3mg/kg) + Castor oil (1ml), III and IV groups (test control) received similarly AM + castor oil (100mg/kg + 1ml) and AM + castor oil (200mg/kg + 1ml). After this administration, the animals were placed separately in metabolic cages with filter paper, which was changed every hour. The severity of diarrhea was assessed according to **Takasuna K. et al 1996**each hour for 6h, up to 24hr. **[Bairagi SM; et al 2014].**

Study III: Assessment of antiulcer properties of *Aegle marmelos* on MMF and MPS induced gastric ulcer:-
The sixty mice were divided into ten groups, each group has six mice (n=6). All mice were fasted for 24hr water *ad libitum*, at next day all mice was treated as: I group (control) received 1% Carboxymethyl cellulose (CMC) in 10mL/kg distilled water, II and V groups (positive control) treated with MMF (210mg/kg) and MPS (100 mg/kg) and VIII groups (standard) treated with Loperamide (3mg/kg), III, IV, VI, VII, IX and XI (test control) received similarly MMF + AM (210 + 100mg/kg), MMF + AM (210 + 200mg/kg), MPS + AM (100 + 100mg/kg), MPS + AM (100 + 200mg/kg), MMF + Loperamide (210 + 3mg/kg) and MPS + Loperamide (100 + 3 mg/kg). Four hr after mice was sacrificed, stomach was removed and cut and opened along greater curvature, and content of stomach was removed and the stomach was washed with warm saline and ulcer index was investigated. **[Goswami M; et al 2011].**

Study IV: Assessment of antiulcer properties of *Aegle marmelos* using pylorus ligation model in mice with or without MMF/ MPS:-
Sixty six male Swiss Albino mice were divided into 11 groups, each containing 6 mice. The animals were placed into individual cages according to the group division. The food was withdrawn 24 hour prior to the treatment, but water was allowed *ad libitum*.

The animals were anesthetized using pentobarbital (30 mg/kg body weight, intraperitoneally). The abdomen was opened by cut middle incision, and the pyloric end of stomach was ligated without causing any damage to its blood supply. The stomach was then replaced, and the abdominal wall was closed in two layers with sutures. The animals were allowed to recover partially from anaesthesia. Treatments were given by oral gavages: I group (control) received 2 ml/kg distilled water, II group (positive control) received suspension of AM (200mg/kg), III group (standard) received Ranitidine (10mg/kg), IV and VIII groups (positive control) received similarly MMF (210mg/kg) and MPS(100mg/kg), V, VI, VII, IX, X and XI groups (test control) received similarly MMF + AM (210 + 100mg/kg), MMF + AM (210 + 200mg/kg), MMF + Ranitidine (210 + 10mg/kg), MPS + AM (100 + 100mg/kg), MPS + AM (100 + 200mg/kg), MPS + Ranitidine (100 + 10mg/kg). Four hour later, the animals were sacrificed by an overdose of pentobarbital. Stomach was removed, cut and opened along greater curvature, and contents were removed for each animal. The stomach was washed with saline and ulcer index was calculated according to the method described by **Singh et al 2012, Prasad K et al 2014 and Wang et al 2011.**

Statistical analysis:-

All the data were expressed as Mean ± SEM and all data were analyzed by one way analysis of variation followed by Bonferroni's, P<0.001 was considered as level of significance.

Results:-

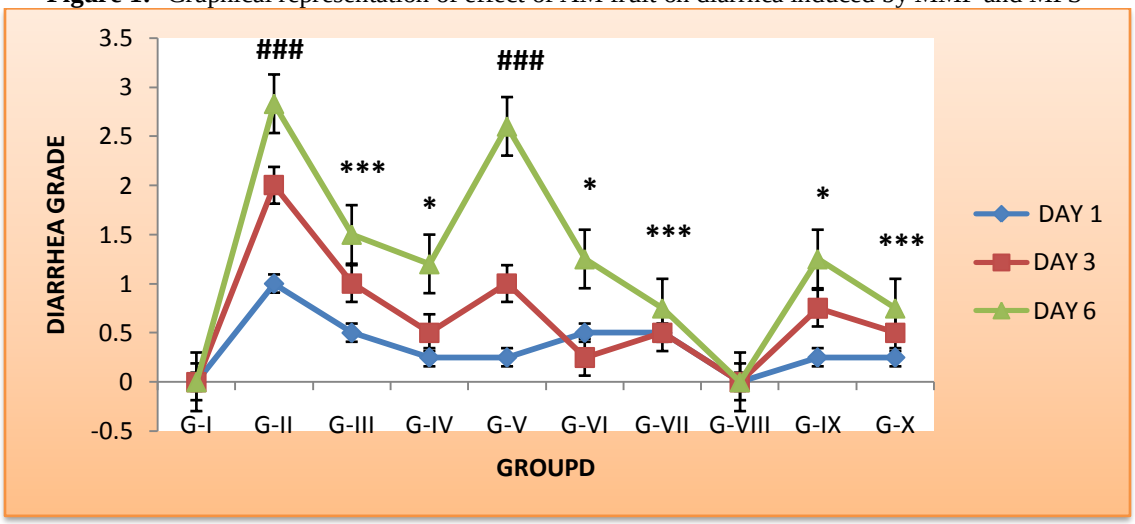
General behavior and acute toxicity study of *Aegle marmelos*:-

Aqueous extracts of *Aegle marmelos* up to 2000 mg/kg did not cause any mortality in *Swiss* albino mice. None of the doses tested produced any gross apparent effect on general motor activity, muscular weakness, fecal output, feeding behavior etc. during the period of observation.

Effect Of *Aegle Marmelos* Fruit On Diarrhea Induced By Mmf And Mps In Mice:-

MMF (210mg/kg) & MPS (100mg/kg) were induced significantly diarrhea in mice as compare to control group. AM (100mg/kg and 200mg/kg) reduced the diarrhea grade significantly which was caused by MMF and MPS as compare to positive control group. Loperamide (3mg/kg) reduced the diarrhea significantly which was caused by MMF and MPS as compare to positive control group. MMF caused more diarrhea to the MPS, both drug showed diarrhea as a GI toxicity in mice group significantly. The result indicated that the AM is effective against the diarrhea, when used MMF and MPS significantly in mice groups. Fig 1 represents the diarrhea cause by MMF and MPS and Protective effect when used AM.

Figure 1:- Graphical representation of effect of AM fruit on diarrhea induced by MMF and MPS

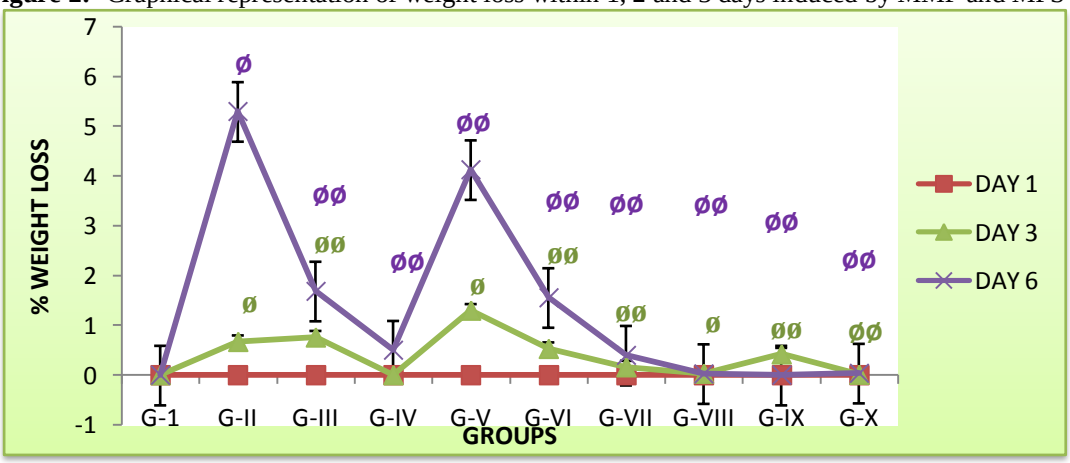


Values are significant different when compared with control group, p<0.001, p<0.05, ***Values are significant different when compared with treatment group, p<0.001, ** Values are significant different when compared with treatment group, p<0.01, * Value are significant different when compared with treatment group p<0.05

EFFECT OF *AEGLE MARMELOS* FRUIT ON WEIGHT LOSS INDUCED BY MMF AND MPS IN MICE

MMF and MMF caused weight loss not significantly as compare to control group. AM (100 & 200mg/kg) protected the weight loss, in the group which was treated with MMF & MPS (not significantly) as compare to positive control group. Loperamide alone caused slightly weight loss, and also with MMF and MPS caused weight loss, was not significantly as compare to positive control group. MMF caused more weight loss to MMF, however both (MMF & MPS) drug induced weight loss significantly in mice. AM was effective against weight loss caused by MMF and MPS significantly. Fig 2 represents the weight loss caused by MMF and MPS and protective effect of AM on weight loss.

Figure 2:- Graphical representation of weight loss within 1, 2 and 3 days induced by MMF and MPS



Values are mean ± SEM, n=6, Ø Values are not significant different when compared with control group, P > 0.05, ØØ Values are not significant different when compared with test group, P > 0.05

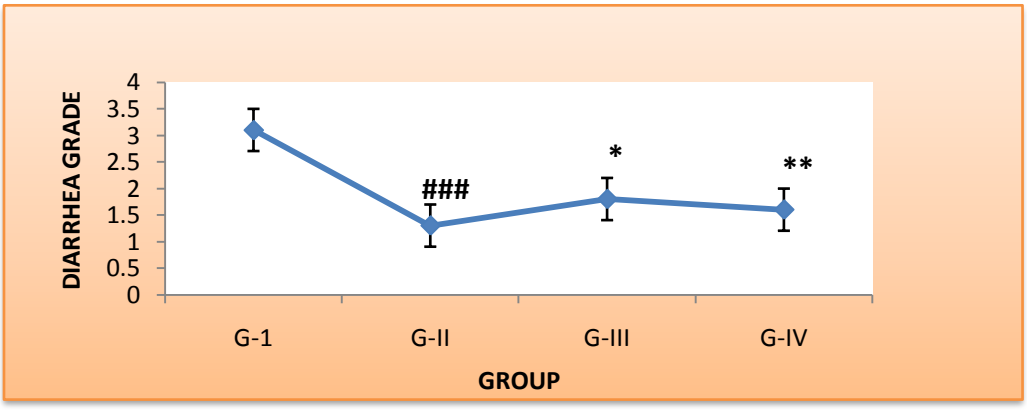
EFFECT OF *AEGLE MARMELOS* FRUIT ON CASTER OIL INDUCED DIARRHEA

Table1:- Effect of AM on Castor oil induced Diarrhea.

Group	Treatment	Diarrhea grade
G-I	Castor oil (1ml) +Vehicle (0.5% Tween 80)	3.1± 0.30
G-II	Loperamide (3mg/kg) + Castor oil (1ml)	1.3 ±0.20###
G-III	AM (100mg/kg) + Castor oil (1ml)	1.8± 0.30*
G-IV	AM (200mg/kg) + Castor oil (1ml)	1.6 ±0.22**
Values are mean ± SEM, *n=6*,### Values are significant different when compared with control group, p<0.001 ** Values are significant different when compared with treatment group, p<0.01, * Value are significant different when compared with treatment group p<0.05		

Loperamide reduced diarrhea significantly (p<0.001), which was induced by castor oil as compare to control group. When castor oil treated mice provided extract of AM (100 and 200mg/kg), reduced diarrhea significantly. Result indicated that AM have anti diarrheal properties in vivo, the anti diarrheal properties of AM represented in Fig 3

Figure 3:- Graphical representation of effect of AM fruit, on castor oil induced diarrhea



EFFECT OF *AEGLE MARMELOS* ON GASTRIC ULCER INDUCED BY MMF AND MPS

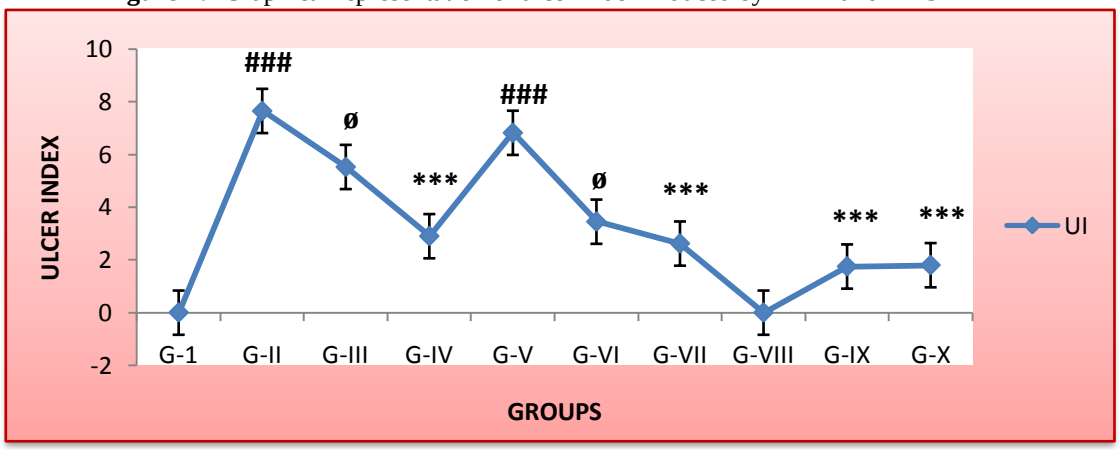
Table 2:- Effect of *Aegle marmelos* fruit extract on MMF & MPS induced gastric ulcer

Group	Treatment	Dose (mg/kg)	Ulcer index	% protection
G-I	Distilled water +CMC (1%)	10ml/kg	0±0	---
G-II	MMF	210	7.65±0.82###	---
G-III	MMF+AM	210+100	5.52±0.64ØØ	27.77
G-IV	MMF+AM	210+200	2.9±0.4***	62.09
G-V	MPS	100	6.2±0.55###	---
G-VI	MPS+AM	100+100	4.45±0.54ØØ	49.41
G-VII	MPS+AM	100+200	2.62±0.42***	61.58
G-VIII	Ranitidine	10	0	---
G-IX	MMF + Ranitidine	210 + 10	1.75±0.34***	75.72
G-X	MPS + Ranitidine	100 +10	1.8±0.44***	78.39

Values are mean ± SEM, n=6, ### Values are significant different when compared with control group, p<0.001,## Values are significant different when compared with control group, p<0.01, # Values are significant different when compared control with treatment group, p<0.05, Ø Values are not significant different when compared with control group, P > 0.05, ØØ Values are not significant different when compared with test group, P > 0.05, ***Values are significant different when compared with treatment group, p<0.001, ** Values are significant different when compared with treatment group, p<0.01, * Value are significant different when compared with treatment group p<0.05,% Protection= (UI Of Control Group - UI Of Test Group) / UI Of Control Group

MMF & MPS causes significantly gastric ulcer as compare to control group. AM (100 mg/kg) showed protective effect on gastric ulcer with MMF and MPS treated group was not significantly as compare to positive control group. AM (200 mg/kg) showed significantly protective effect against gastric ulcer caused by MMF and MPS as compare to positive control group. AM (100 mg/kg and 200 mg/kg) significantly protective on gastric ulcer induced by MPS as compare to positive control group. Ranitidine showed significantly protective effect on gastric ulcer caused by MMF and MPS as compare to positive control group. Percentage protection was found to be G-X>G-IX>G-IV>G-VII>G-VI>G-III respectively. However group III and VI was founded to insignificant. The result indicated, the MMF and MPS induced gastric ulcer significantly, and AM gives significant protective effect against, MMF and MPS induced gastric ulcer, and 200mg/kg (AM) was highly effective against gastric ulcer. Fig 4 represent ulcer index.

Figure 4:- Graphical representation of ulcer index induced by MMF and MPS



EFFECT OF *AEGLE MARMELOS* ON GASTRIC ULCER INDUCED BY PYLORUS LIGATION, IN WITH OR WITHOUT OF MMF AND & MPS

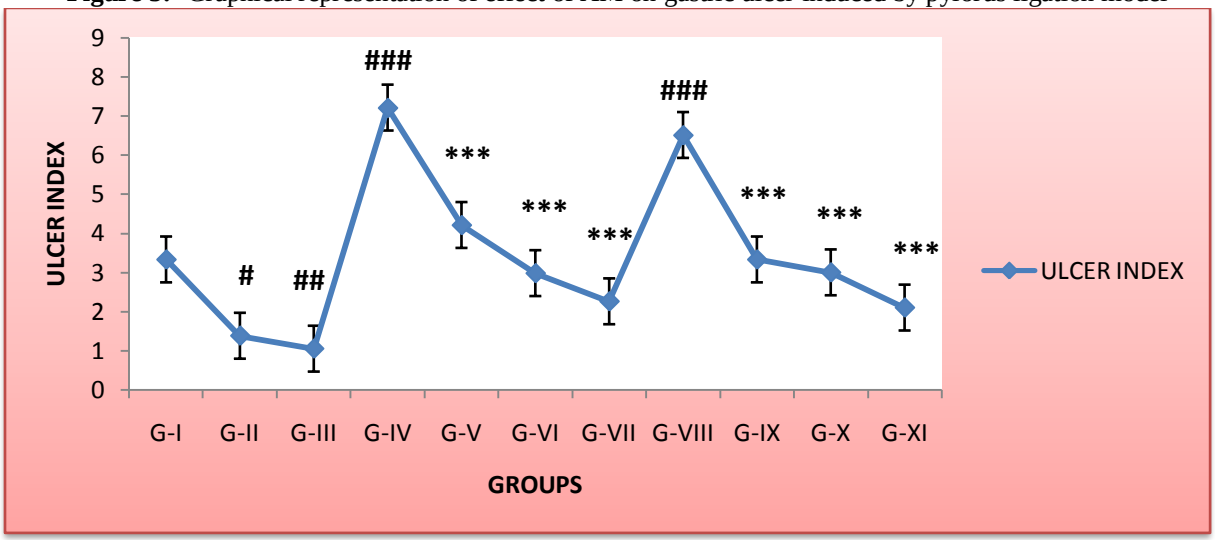
Table 3:- Effect of *Aegle marmelos* on gastric ulcer induced by pylorus ligation with or without MMF and MPS

Group	Treatment	Dose (mg/kg)	Ulcer index	% protection
G-I	D.WATER	2mL/kg	3.33±0.42	0
G-II	AM	200	1.38±0.52#	58.55
G-III	Ranitidine	10	1.05±0.33#	68.46
G-IV	MMF	210	7.21±0.57###	0
G-V	MMF+AM	210+100	4.21±0.26***	41.6
G-VI	MMF + AM	210+200	2.98±0.42***	58.66
G-VII	MMF+Ranitidine	210 + 10	2.26±0.16***	68.65
G-VIII	MPS	100	6.51±0.42###	0
G-IX	MPS + AM	100+100	3.31±0.42***	48.84
G-X	MPS+AM	100+200	3±0.34***	56.83
G-XI	MPS+Ranitidine	100 +10	2.1±0***	67.74

Values are mean ± SEM, n=6, ### Values are significant different when compared with control group, p<0.001, # Values are significant different when compared control with treatment group, p<0.05, ***Values are significant different when compared with treatment group, p<0.001

AM group alone showed significantly protective against pylorus ligation induced gastric ulcer as compare to control group. MMF and MPS group significantly causes ulcer in pylorus ligation group as compare to control group. AM (100 & 200mg/kg) showed significantly protective effect, which was caused by MMF and MPS as compare to positive control group. Marketed drugs Ranitidine also showed antiulcer effect, which was caused by pylorus ligation, in the presence of MMF and MPS as ulcer inducer as compare to positive control group, and it was confirmed that, the AM was significantly reduce the gastric ulcer caused by pylorus ligation/ or MMF and MPS. Percentage protection was found to be G-VII>G-XI>G-VI>G-II>G-IX>G-V respectively. The result indicated, the MMF and MPS induced gastric ulcer significantly in mice, and AM show significant antiulcer effect against pylorus ligation and MMF and MPS induced gastric ulcer. Fig 5 represent ulcer index

Figure 5:- Graphical representation of effect of AM on gastric ulcer induced by pylorus ligation model



Conclusion:-

The Present study was demonstrated the protective effects of AM on diarrhea, weight loss, and gastric ulcer induced by MMF and MPS in mice groups. . It was found that MMF and MPS caused GI toxicity (diarrhea, and gastric ulcer) and weight loss in mice, and *Aegle marmelos* (aq. Extract of ripe fruit) provided significant protection against the MMF and MPS induced GI toxicity in mice as indicated by lower levels of diarrhea grade, weight loss and Gastric ulcer induced by MMF, MPS and pylorus ligation, compared to control groups. Both drugs were having almost same toxicity profiles, but MPS showed less GI toxicity to MMF in mice. The 100mg/kg dose of AM was

less effective to 200 mg/kg dose of AM, against gastric ulcer and diarrhea grade, caused by MMF and MPS. Rao GHJ et al 2012 suggested that the antidiarrhoeal effect of the extract may be due to inhibition of prostaglandin synthesis. Anutiulcer activity due to increased hexose and sialic acid in the gastric mucosa after *Aegle marmelos* treatment may contribute, and cytoprotective effect by increasing the viscosity of the gastric mucus. AM results indicated decline of gastric acid, thus in a lesser degree of peptic erosion of the mucus gel layer [Singh P. and Guha D, 2012]. Body weight loss of mice may be due to decrease the fluid during the diarrhea, also gastric ulcer may involve. When gastric ulcer and diarrhea treated with AM the recovery was seeing, it was thought, those regions (diarrhea and gastric ulcer) may responsible for weight loss.

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