Evolution Of Anti-Ulcer and Anti-Inflammatory Activity of *Citrus Limon* Seeds in Rats

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***Abstract*—H. pylori is the most prevalent infection that causes peptic ulcers. Spicy foods, prolonged NSAID usage, and stress are the main causes of peptic ulcers. Numerous microorganisms and injury from foreign objects can cause inflammation. Many synthetic medications are available to treat Peptic ulcers and inflammation, but they often have more adverse effects than benefits. Fortunately, many natural medications also have anti-ulcer and anti-inflammatory properties. Among them citrus limon seeds' hydralcoholic extraction has anti-ulcer and anti-inflammatory properties.**

***Index Terms*—Anti-ulcer, anti-inflammatory and Citrus limon seeds.**

I. INTRODUCTION

Plants will be being one of the important sources of medicine since dawn of human civilization, there are the gifts of nature to mankind for treating different types of diseases. Almost from prehistoric period herbal medicine are used for alleviation of suffering caused by different disease in human.

Herbal therapy provides rational means for the treatment of many diseases which are considered to be obstinate and incurable in other system of medicine. It lays a great deal of emphasis upon the maintenance of positive health of an individual and thus aims at both the prevention and cure if diseases.

Peptic ulcers is one of the most common gastrointestinal condition worldwide, peptic ulcers impact 10% of the global population [1]. Duodenal peptic ulcers account for about 19 of every 20 cases. An estimated 15,000 fatalities are attributed to peptic ulcers annually. Hemorrhage and perforation from peptic ulcers were estimated to occur annually in 19.4–57 and 3.8–14 cases per 100,000 people, respectively. The average long-term recurrence of perforation was 12.2%, while the average 7-day recurrence of bleeding was 13.9%. [2]. Antacids and antiulcer medications share 6.2 billion rupees and account for 4.3% of the Indian pharmaceutical market share [3]. Even in now a day, 75–80% of people worldwide still receive their primary medical care from herbal medicine, mostly in underdeveloped nations because of its greater cultural tolerance and compatibility with the human body, and lesser side effects [4].

A skin or mucous membrane ulcer is an open sore that is characterized by the sloughing off of inflammatory dead tissue [5]. Lesions on the skin's surface or in a mucous membrane that are defined by an upper surface loss of tissue are called ulcers. Although they can occur practically anywhere, ulcers are most frequently seen on the skin of the lower limbs and in the gastrointestinal tract. There are numerous varieties of ulcers, including peptic ulcers, vaginal ulcers, esophageal ulcers, and mouth ulcers. Peptic ulcers are common in many individuals. The duodenum or stomach lining erodes as a result of peptic ulcers [6]. "Gastric ulcer" and "duodenal ulcer" are the two most prevalent forms of peptic ulcer. The term alludes to the ulceration place. It is possible for a person to develop duodenal and stomach ulcers simultaneously. The stomach is the site of gastric ulcers, which are painful and typically affect elderly adults. Pain relief may not always come from eating. Nausea, vomiting, and weight loss are possible additional symptoms. Even when patients with stomach ulcers produce normal or reduced amounts of acid, ulcers can still develop in the partial

absence of acid [7]. Patients with duodenal ulcers awaken from sleep due to intense pain and burning sensation in the upper abdomen, which is caused by the ulcer located at the beginning of the small intestine. Pain typically starts when the stomach is empty and goes away after eating. Duodenal ulcers primarily affect men and are more common in younger people. There could be ulcers in the duodenum which may appear on both the anterior and posterior walls [8]. Peptic ulcer symptoms, such as bloody stool, excruciating abdominal pain, cramps, and blood in the vomit, can occasionally be fatal [9].

Damage to living tissues brought on by bacterial, viral, or fungal infections, physical agents, or a compromised immune response are the usual causes of inflammation. The first objective of the inflammatory response is to identify and eradicate the pathogenic agents; subsequently, it aims to eliminate damaged tissue components, ultimately leading to the restoration of the impacted tissues, organs, or system [10–12]. Macrophages and neutrophils are known to release several mediators during an inflammatory response, which are in charge of the beginning, intensification, duration, control, and final resolution of the acute state of inflammation [13–14].

For our current study, we have chosen the Citrus limon plant because it has been associated with a number of pharmacological activities at different plant sections. The literature states that citrus limon seeds have traditionally been advised for the treatment of inflammatory and ulcerative disorders. However, the plant's seeds have not been shown to have any known anti-inflammatory or anti-ulcer qualities. However, we decided to investigate the plant's anti-inflammatory and anti-ulcer properties in a variety of experimental animal models using seed extracts due to the plant's therapeutic capabilities as reported in Ayurveda scriptures [15].

II. MATERIAL AND METHODS:

Wister albino rats (180-250g) of either sex were procured from VAB biosciences 1-6-194/45/D, Bapujinagar, Musheerabad, Hyderabad - 500020. The animals were acclimatized for seven days and housed under standard well aerated condition of temperature 22ºC (+ 3ºC) and relative humidity (30%) with a 12:12 light-dark cycle. The animals were fed with standard pellet and water and libitum. and the animal studies were performed in accordance to guidelines of CPCSEA.

The dried seeds of *Citrus limon* plant were collected from Bidar area, Karnataka. The plant seeds were authenticated at Botany department of Smt. Veeramma Ganga Siri college for Women, Kalaburagi. The seeds were dried in shade, powered and stored in air tight containers for the study.

Equipment like plethysmometer, oral feeding needle, syringe, magnifying lens and dissection kit are used for the study.

Preparation of extract:

Hydroalcoholic extract of *Citrus limon* seeds (HECL) was prepared: Using a mixer, coarsely crush the dried seeds. The powder is then placed into a Soxhlet column and extracted using hydro alcohol (ethanol and water, 70:30) as the solvent. The extraction process is maintained until the solvent is colorless.

Chemicals: Rabeprazole, Ethanol, acacia, Ether, Phenolphthalein, Indomethacin, Carragenan suspension, Declofenacsodium, Formalin and Histamine.

Toxicity studies:

LD_{50} studies were conducted in albino mice by using OECD guidelines No- 423 for *Citrus limon* seeds extracts.

Anti-Ulcer activity:

Pylorus ligation induced ulcer model [`19]:

Albino wistar rats of either sex weighing between 180-220 gm were divided into 4 groups of 6 animals each.

Group A

(Control Group) Received Vehicle control (1% acacia) throughout the Experiment.

Group B

(Standard Group) Received Standard drug Rabeprazole (30mg/kg, ip)

Group C

(Test Group) Hydro alcoholic extraction of *Citrus limon* seeds (500mg/kg,ip)

For five days, the medications were given every day. The rats were starved for 24 hours on the fifth day prior to the pyloric ligation. Coprophagy was being avoided with great care. The rats were put to sleep with anesthetic ether after a 24-hour period. A midline incision was made to reveal the abdomen. With the stomach's blood supply intact, a ligature was inserted at the pyloric sphincter and the stomach was raised out. The abdomen wall is sutured in two layers after the stomach was carefully restored. The rats were slain after six hours with an overdose of anesthetic ether, and their stomachs were removed. The amount of gastric juice was measured after it was collected, emptied into test tubes, and centrifuged for 10 minutes at 1000 rpm. Using a pH meter, the stomach juice's pH was determined. After that, the contents' free and total acidity were examined. In order to check for ulcers in the glandular region of the stomach, the stomachs were opened along their larger curvature and then cleaned under running water. The number of ulcers on each stomach was counted, and using a hand lens (10x), microscopical scoring was performed. Mean ulcer score for each animal is expressed as ulcer index and the percentage protection was calculated by using the formula [19].
%Protection = [(UI control – UI treated) / UI control] x 100 [20-21]
The results were expressed as mean with the standard deviation and subjected to statistical analysis using student ‘t’ test. The results are tabulated in Table No. 2

Indomethacin Induced Ulcers in Rats: [22]
Twenty rats of comparable weight (200±25g) used for this study. They were fed standard diet and water *add libitum.* They were housed under standard environmental conditions in a well-ventilated room under 12/12 hours’ light/dark cycle and allowed two weeks of acclimatization. The animals were divided into four groups;
Group A
Control Group) Received Vehicle control (1% acacia) throughout the Experiment.

Group B
(Induced Group) Inducing Indomethacin (20mg/kg).

Group C
(Standard group) Received Indomethacin (20mg/kg) and standard drug rabeprazole (30mg/kg)

Group D
(Test Group) Received Indomethacin(20mg/kg) and Hydroalcoholic extraction of *Citrus limon* seeds.

Procedure: All of the animals were permitted to fast for 48 hours following their acclimation. There was no therapy or ulcer induction given to Group A. But animals in group B were given indomethacin orally, while those in group C received indomethacin plus a conventional medication and those in group D received indomethacin plus a hydroalcoholic extract of citrus limon seeds. Every suspension was made and given orally 30 minutes prior to use. The animals were left without food or drink for eight hours following the induction of ulcers with indomethacin.

Duration of Study: The study was lasted for four weeks. However, the actual animal experiment was lasted for 72 hours based on the fact that gastric epithelium is renewed every 2 to 4 days

Sample Collection: At the end of the experimental periods, animals were mildly anesthetized with anesthetic agent and the stomach obtained following standard laboratory procedures. The stomachs were examined macroscopically

Sample Analysis: The stomachs were washed with saline water and examined for macroscopically mucosal lesions using dissecting microscope [22]

Swim stress induced ulcer [23]:
Wistar albino rats were selected and divided into 3 groups each containing five animals.
Group A
(Control Group) Received Vehicle control (1% acacia) throughout the Experiment.

Group B
(Standard Group) Received standard drug Rabeprazole (30mg/kg, ip)

Group C
(Test Group) Received Hydroalcoholic extraction of *Citrus limon* seeds

This strategy involved creating stress in order to evaluate ulcers. The animals were fasted for a whole day. The animals received specific medication administration after the 24-hour period. The rats were given drugs for two hours, and then they were made to swim for five hours at intervals in a glass cylinder that measured 45 cm in height and 25 cm in diameter. The water in the cylinder was kept at 230 degrees for the entire five hours. The animals were taken out and slaughtered after five hours of swimming, and the stomach was removed. In order to check for ulcers in the glandular section of the stomach, the stomachs were opened along their larger curvature and then cleaned with water. Scoring was done under a microscope with the help of hand lens (10X), and the number of ulcers on each stomach was recorded.

Anti-Inflammatory activity:

Carrageenan induced paw edema model in rats:

Albino rats of either sex weighing 150 – 200 g are selected and marked, they are maintained on standard pellet diet and free access to water *at labitum*. They were starved overnight prior to the experiment. The animals were divided into 4 groups of each containing six animals. The various groups treated will be as follows:

Group A

(Control Group) Received Vehicle control (1% acacia) throughout the Experiment.

Group B

(Induced Group) Inducing Carrgeenan.

Group C

(n=6) Received Carrageenan and standard drug Diclofenac (50mg/kg p.o)

Group D

(n=6) Received Carrageenan and Hydroalcoholic extraction of *Citrus limon* seeds.

To maintain constant paw volume, the rear paws (both left and right) were marked just beyond the tibio-tarsal junction. Each time the paw was dipped in the mercury column, it was dipped up to the set mark. Using the mercury displacement method, the initial paw volume of each rat—both left and right—was recorded. Thirty minutes prior to the injection of 0.1 ml of 1% carrageenan suspension in normal saline at the subplantar region of the left hind paw—the right hind paw serving as reference—the rats will receive the normal control, Diclofenac sodium, and test extracts. Following that, the injected paws' oedema volume was measured plethysmographically using the mercury displacement method.[24]. The volume of oedema was measured at different predefined time intervals (0,1,2,3, 4 hours) for comparison's sake. The mean oedema volume was computed by measuring the difference in the paw volumes of the treated animals. Therefore, the oedema volume in the groups treated with test substances (Vt) and the control group (Vc) was computed. The percentage inhibition was calculated by using Formula: [25]

$\%Inhibition = Vc- Vt/ Vc \times 100$

Where,Vc = Oedema Value of Control

Vt = Oedema Volume of Test

Results were analysed using student ‘T’ test and tabulated.

Figure.1: Plethysmometer

Formalin induced paw edema: 0.1 ml of 2% formalin will be injected into the sub plantar area of right hind paw of anaesthetized rat. All drugs will be given orally one hour prior to formalin injection and continued for 7 consecutive days. Degree of inflammation will be measured plethysmometrically on days 1 and 7[26].

Group A

(Control Group) Received Vehicle control (1% acacia) throughout the experiment

Group B
(Induced Group) Inducing Formalin (0.1ml of 2%)

Group C
(Standard Group) Received Formalin and Standard Drug Diclofenac (50mg/kg p.o)

Group D
(Test Group): Received Formalin and hydroalcoholic extraction of *Citrus limon* seeds.

Statistical analysis:
It was performed using students unpaired 't' test and P values less than 0.05 were considered significant. Data are represented as mean + SEM.

Histamine induced paw edema:
Procedure: With a few minor adjustments, the experiment will be carried out utilizing the Winter et al. approach. Wistar rats will be randomly assigned to four groups (n = 5/group) following a 12-hour fast. While the second group will be given histamine treatment, the first group will be given water to drink. Histamine with standard medication will be administered to the third group, and histamine plus hydroalcoholic extract of citrus limon seeds will be administered to the fourth. About one hour following the treatments, histamine (0.1 percent w/v, 0.1 mL) was injected into the subplantar area of the right hind paw to cause edema. The paw's volume differential before and after 0.5, 1, 2, 3, and 4 hours of edema induction will be used to determine the outcomes. Plethysmography will be used to determine the paw volume. [27,29].
Group A
(Control Group) Received Vehicle control (1% acacia) throughout the experiment

Group B
(Induced Group) Inducing Histamine (0.1% w/v, 0.1 ml)

Group C
(Standard Group) Received Histamine and Standard Drug Diclofenac (50mg/kg p.o)

Group D
(Test Group) Received Histamine and Hydroalcoholic extraction of *Citrus limon* seeds.

Statistical analysis:
Results were expressed as mean +SEM. Differences between means were determined by using one-way analysis of variance followed by Turkey's multiple comparison test.

III.RESULT AND DISCUSSION

Successive solvent extraction
The *Citrus limon* seeds powder was subjected to batch extraction in Soxhlet apparatus. The percentage yield, color, consistency and solubility in water of different solvents are noted in table

Table 1: Data showing the Extractive Values of *Citrus limon* seeds extract.

Plant part used	Extract	% yield	Color	Consistency	Solubility in water
Citrus limon seeds	Ethanolic	12 %	light green	Sticky	Insoluble

Acute toxicity study
LD_{50} studies were conducted in albino mice by using OECD guidelines No- 423 for *Citrus limon* seeds extracts. It was found that the extract even at 2000 mg/kg dose had not shown any mortality confirming its practically non-toxic in nature and it falls in GHS category 5.

Anti - ulcer Activity
Pylorus ligation induced ulcer model
Mucus production increased significantly with gastric secretion, rising from 21.17+81 mg in the PL control to 42.82+3.24 mg at the 500 mg/kg dose ($P<0.001$). In a similar manner, rabeprazole (30 mg/kg) completely inhibited the formation of lesions and significantly decreased the stomach acid level. [Table 2]
As we can see, the test group, where rabeprazole is standard and has an ulcer protection of 81.2%, whereas the control group shows 69.22% ulcer

protection

Table 2: Effect of Hydroalcoholic extraction of *Citrus limon* seeds on Ulcer Index and their % protection in pylorus ligation induced ulceration in rats

Gr.no.	Treatment	Dose	Ulcer index	% Protection
1.	Control	-	12.03+0.64	-
2.	Rabeprazole	0.30mg/kg	2.75+0.42***	81.02 %
3.	HECL	500mg/kg	4.47 ± 0.40***	69.22 %

All Values are expressed as mean±SEM; n=6. One-way ANOVA with a significance threshold of ***P<0.001, **P<0.01, *P<0.05, ns= not significant, when compared with control group, P<0.01 when compared with disease control group, was used to analyze all data statistically.

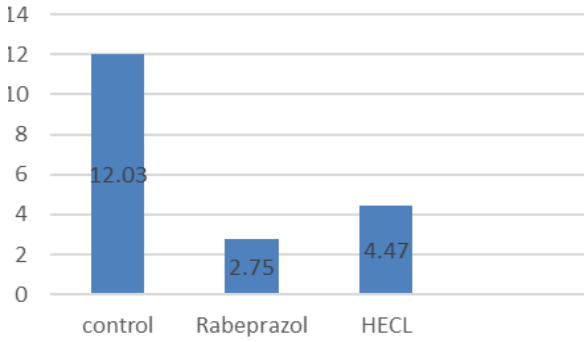

Figure.2: Effect of Hydroalcoholic extraction of *Citrus limon* seeds on Ulcer Index and their % protection in Pylorus ligation induced ulceration in rats.

Indomethacin induced ulcers:

The percentage protection produced at a dose 0.30mg/kg of Rabeprazole, 500mg/kg of Hydroalcoholic extract of *Citrus limon* seeds were 71.73% and 44.78% respectively.

The ulcer protective action at 500mg/kg dose of ethanolic extract of *Citrus limonseeds* was found to be closer to reference standard drug Rabeprazole. The results are tabulated in Table no.3

Table 3: Effect of Hydroalcoholic extraction of *Citrus limon* seeds on Ulcer Index and their % protection in Indomethacin induced ulceration in rats

Gr.no.	Treatment	Dose	Ulcer index	% Protection
1.	Control	-	6.90	-
2.	Indomethacin	0.20mg/kg	7.29+0.43***	-
3.	Rabeprazole	30mg/kg	1.95 ± 0.236**	71.73%
4.	HECL	500mg/kg	3.81 ± 0.401***	44.78%

All Values are expressed as mean±SEM; n=6. One-way ANOVA with a significance threshold of ***P<0.001, **P<0.01, *P<0.05, ns= not significant, when compared with control group, P<0.01 when compared with disease control group, was used to analyze all data statistically.

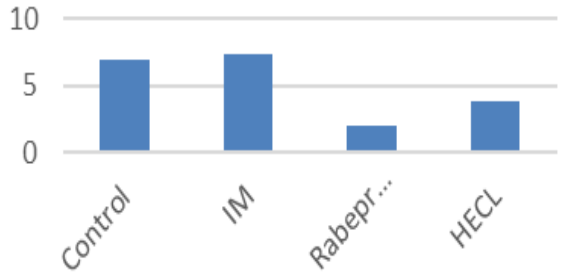

Figure.3: Effect of Hydroalcoholic extraction of *Citrus limon* seeds on Ulcer Index and their % protection in Indomethacin induced ulceration in rats.

Swim stress induced ulcers:

At a dose of 0.30 mg/kg of Rabeprazole and 500 mg/kg of Citrus limon seed ethanolic extract, the percentage protection generated was 70.5% and 55.5%, respectively. The Hydroalcoholic extract of Citrus limon seeds was found to provide ulcer-preventive effects at a dose of 500 mg/kg that was more comparable to the reference standard medication Rabeprazole. The outcomes are listed in Table No.4

Table 4: Effect of Hydroalcoholic extraction of *Citrus limon* seeds on Ulcer Index and their % protection in Swim Stress induced ulceration in rats

Gr. no.	Treatment	Dose	Ulcer index	% Protection
1.	Control	-	7.2	-
2.	Rabeprazole	0.30mg/kg	1.08 ± 0.301 ***	70.75 %
3.	HECL	500mg/kg	3.2 ± 0.443 ***	55.5%

All Values are expressed as mean±SEM; n=6. One-way ANOVA with a significance threshold of ***P<0.001, **P<0.01, *P<0.05, ns= not significant,when compared with conrol group, P<0.01 when compared with disease control group, was used to analyze all data statistically.

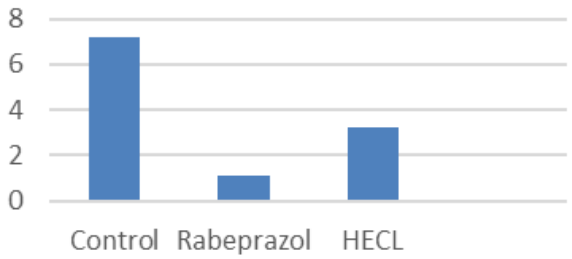

Figure.4: Effect of Hydroalcoholic extraction of *Citrus limon* seeds on Ulcer Index and their % protection in Swim Stress induced ulceration in rats

Anti-inflammatory activity:

Carrageenan induced rat paw oedema method in rats

When applied to acute paw oedema caused by carrageenan, the anti-inflammatory properties of diclofenac sodium and HECL were assessed at doses of 50 mg/kg and 500 mg/kg b.w., respectively. Significant (P<0.05) anti-inflammatory action was achieved by the HECL, and the outcomes were similar to those of standard anti-inflammatory medication diclofenac sodium (50 mg/kg). According to table 4, Significant inhibition was observed at 500 mg/kg of HECL at 2 hours, while diclofenac sodium demonstrated protection against acute carrageen-induced paw oedema at 1 hour when compared to the control group.

Table-5: Effect of HECL on carrageenan induced rat paw oedema

Treatment	Increase in paw size (ml) Different time intervals (hr)			
	0.5	1	2	3
Control	0.283 ±0.016	0.376 ±0.021	0.483± 0.016	0.353 ±0.016
Carrageenan induced.	0.300 ±0.006	0.383 ±0.016	0.483± 0.016	0.363 ±0.016
Standard drug (Diclofenac sodium)	0.283 ±0.016^{ns}	0.233 ±0.016**	0.210± 0.002** *	0.200 ±0.016**
Test drug (HECL)	0.283 ±0.016^{n}	0.263 ±0.016**	0.230± 0.003**	0.216 ±0.016*

All Values are expressed as mean±SEM; n=6. One-way ANOVA with a significance threshold of ***P<0.001, **P<0.01, *P<0.05, ns= not significant, when compared with conrol group, P<0.01 when compared with disease control group, was used to analyze all data statistically.

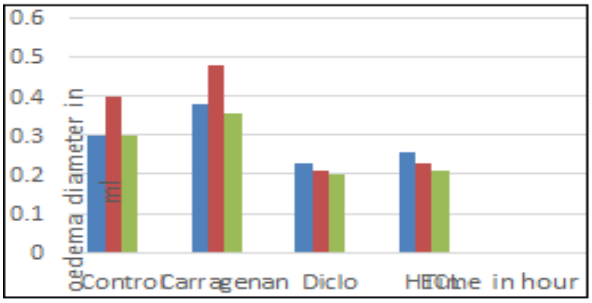

Figure.5: Effect of Ethanolic extracts of *Citrus limon* seeds on carrageenan induced paw oedema in rats.

Formalin induced paw oedema:

Anti-inflammatory effect of HECL by Formalin induced paw oedema:

When applied to acute paw oedema caused by formalin, the anti-inflammatory properties of diclofenac sodium and HECL were assessed at doses of 50 mg/kg and 500 mg/kg b.w.,

respectively. Significant (P<0.05) anti-inflammatory action was achieved by the HECL, and the outcomes were similar to those of standard anti-inflammatory medication diclofenac sodium (50 mg/kg). Table 6 displays the effect of HECL on formalin-induced paw oedema.

Table 6. Effect of HECL in formalin induced rat hind paw oedema

Gr.no.	Treatment	Dose	Paw vol increase on day 7 (ml)	% Protection
1.	Control	-	1.13+0.12	-
2.	Formalin	125ml/kg	1.50+0.43***	-
3.	Diclofenac	50mg/kg	0.46 ± 0.12**	59%
4.	HECL	500mg/kg	0.60 ± 0.40***	44.78%

All Values are expressed as mean±SEM; n=6. One-way ANOVA with a significance threshold of ***P<0.001, **P<0.01, *P<0.05, ns= not significant, when compared with conrol group, P<0.01 when compared with disease control group, was used to analyze all data statistically.

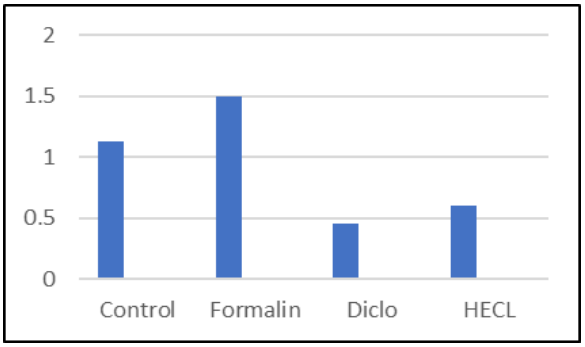

Figure.6: Effect of Hydroalcoholic extraction of *Citrus limon* seeds on paw volume increased and their % protection in Formalin induced paw oedema inrats

Histamine induced paw oedema:

Anti-inflammatory effect of HECL by Histamine induced paw oedema:

When tested against acute paw oedema caused by histamine, the anti-inflammatory properties of diclofenac sodium and HECL were determined at doses of 50 mg/kg and 500 mg/kg b.w., respectively. Significant (P<0.05) anti-inflammatory action was achieved by the HECL, and the outcomes were similar to those of standard anti-inflammatory medication diclofenac sodium (50 mg/kg). Table 7 displays the efficacy of EECL on histamine-induced paw oedema.

Table 7. Effect of HECL in Histamine induced rat hind paw oedema

Treatment	Increase in paw size (ml)			
	Different time intervals (hr)			
	0.5	1	2	3
Control	0.26±0.02	0.62±0.03	0.47±0.02	0.44±0.03
Histamine induced.	0.28±0.03	0.67±0.02	0.51±0.03	0.48±0.02
Standard drug (Diclofenac sodium)	0.20±0.01	0.27±0.01	0.15±0.02	0.10±0.01
Test drug (HECL)	0.22±0.02	0.32±0.03	0.24±0.03	0.18±0.02

All Values are expressed as mean±SEM; n=6. One-way ANOVA with a significance threshold of ***P<0.001, **P<0.01, *P<0.05, ns= not significant, when compared with conrol group, P<0.01 when compared with disease control group, was used to analyze all data statistically

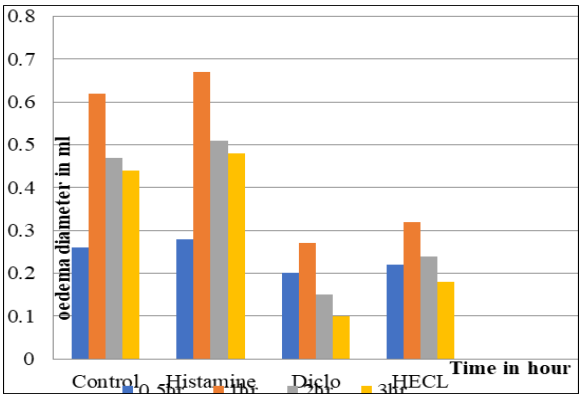

Figure.7: Effect of Hydroalcoholic extraction of *Citrus limon* seeds on Histamine induced paw oedema in rats.

IV. CONCLUSION

Citrus lime seeds are rich in antioxidants, and they also help decrease diabetes, increase immunity, and are beneficial for skin health since they contain vitamin C and have antimicrobial and anti-parasitic properties. The current studies' findings provide clear information about citrus limon seeds' anti-inflammatory and anti-ulcer properties.

With the increasing danger that manufactured drugs cause, most studies are concentrating on naturally occurring plants, seeds, peels, and other materials. Here, we used a hydroalcoholic extraction method for citrus seeds. Lemon seeds have anti-inflammatory and peptic ulcer-reducing properties. An investigation is carried out to check the toxicity of extraction of limon seeds; at high concentrations, the extract shows no negative effects when compared to our standard substance; on the other hand, the synthetic medication that was selected as the benchmark drug is more damaging than our test drug. The test medication is not exhibiting any harmful effects even at 2000 mg/kg.

In summary, the strong activity of citrus limon seeds can be attributed to a variety of processes mediated by phytoconstituents, as revealed by the hydroalcoholic extraction process. The study will be helpful in understanding the chemical makeup of the seeds, which would be a highly economical way to prevent disease in its early stages without resorting to synthetic medications.

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