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RESEARCH ARTICLE

Isolation, Fractionation and Evaluation of the Anti-Inflammatory Properties of *Citrullus lanatus* Thumb

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ABSTRACT:

The main objective of this experiment was to investigate the anti-inflammatory activity of *Citrullus lanatus* methanol extract (ME) and its fractions in adult male albino rats. *Citrullus lanatus* has been traditionally used for various disease and disorders. The phytochemical evaluation of methanolic seeds extract showed the presence of carbohydrate, alkaloids, steroids, saponins, flavonoids, tannins and phenolic compounds. The present work revealed that the inflammatory tissue damage was due to liberation of ROS from phagocytes invading inflammation sites. By this it proved that, oral methanolic extract of *Citrullus lanatus* was found to have anti-inflammatory activity & it may be due to its free radical scavenging activity. 200 mg/kg of methanolic extract of *Citrullus lanatus* had shown significant ($p<0.05$) anti-inflammatory activity during 30min, 1 hr & 3 h. On the basis of the results obtained in present investigation after fractionation, it is possible to conclude that amongst all the fractions non-aqueous fraction (200 mg/kg) of methanolic seeds extract of *Citrullus lanatus* has most significant ($p<0.01$, $p<0.001$) anti-inflammatory activity after 30 mins,1h, 2h, 3h and 5h.

Keywords: Citrullus lanatus, isolation, fractionation, anti-inflammatory activity.

1. INTRODUCTION

Inflammation is defined as the local response of living mammalian tissue to injury due to any agent. It is a normal protective response possessed by living tissue against injury caused by physical trauma, noxious chemicals or microbiological agents that removes pathogens or other stimuli and further help to restore cells to normal state or replace damaged tissue with scar [1].

The discovery that certain natural agents produce marked anti-inflammatory effects presents an opportunity and effective adjunct to the management of these cases. As such, a review of the physiological action and clinical studies, involving the use of proven natural anti-inflammatory herbal agents, enables practitioners to use these substances in a safe and responsible way, and thereby help patients eliminate or minimize their reliance upon more dangerous NSAIDs and other synthetic anti-inflammatory drugs. Experimental research reveals that the efficacy of many natural anti-inflammatory agents stems from their ability to modulate the activity of the enzymes, cyclooxygenase and/or 5-lipoxygenase. These natural substances have been shown to reduce inflammation and pain associated with various types of arthritis and traumatic joint injuries. Unlike their synthetic counterparts, they have not been shown to cause erosion injury to the intestinal tract, accelerate cartilage destruction or produce liver and kidney toxicity. For these reasons, herbal agents can be considered viable alternatives to conventional anti-inflammatory drugs in a large percentage of arthritic patients and those suffering from other joint inflammatory conditions &anti- aging, Alzheimer's disease, rheumatism, acne skin allergy and ulcers [2].

A lot of active compounds for various diseases have been isolated from herbal. These isolated compounds serve as drug or as a model for preparing drugs. Bioassay directed fractionation technique is a useful method to isolate

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bioactive compound from herbal drugs through fractionation. It is a separation in which a certain quantity of a mixture (solid, liquid, solute or suspension) is divided up in large number of smaller quantity (fraction) in which the composition changes according to a gradient. Fractions are collected based on difference in a specific property of individual components. Common trait in fractionation is the need to find an optimum between the amount of fractionation collected and the desired purity in each fraction. Fractionation makes it possible to isolate more than two components in a mixture in single run.

Different technique may be used in order to obtain fraction (simple mixture) and isolate (pure) components from natural source. Broadly, the following steps can be executed for investigation on a sample:-

- Qualitative chemical analysis i.e. determination of nature of constituents in an extract or its fraction or mixture of fraction which lead to isolation of active lead compound.
- Quantitative chemical analysis i.e. determination of the purity of an isolated substance or the concentration of single substance or group of substance in a mixture by fingerprinting & different other techniques.
- Bioassay i.e. determination of biological or pharmacological activity of substance and the dose range over which they exert their effects.

Fractionation is widely employed in many branches of science and technology. Mixture of liquid and gas are separated by fraction distillation by difference in boiling point. Fractionation of component also takes place in column chromatography by a different affinity between stationary phase and mobile phase. In fractional crystallization and fractional freezing chemical substance are fractionated based on difference in solubility at given temperature. In cell fractionation, cell component are separated by difference in mass.

The process of fractionation is carried out in various ways in which each group of compound having one or more particulars features. Thus solubility, shape, size, electrical charge and several other features may influence grouping. Initial fractionation may be based on solubility difference, while second may utilize molecular size.

The amount of fraction available is the most important factor in making decision about its future treatment and analysis. Investigative method can be either non destructive or destructive. Destructive methods are those where the sample can be recovered and used for the other tests. Some physiochemical procedures, e.g. chemical test, analytical chromatography, mass spectrometry and all biological testing procedures are destructive, i.e., the used sample cannot be recovered.

The active compounds are usually discovered by several cycle of fractionation of the extract linked with testing of each fraction, until pure compound was isolated from active fraction, process known as bioassay-guided fractionation.

The different methods of active fractionation are precipitation, solvent-solvent extraction, distillation, dialysis, column procedure, and electrophoresis.

So isolated compounds could be used as such or could be used as a model for synthesizing new anti-inflammatory medicine.

The whole plant (*Citrullus lanatus* Thumb) is well known for its anti-inflammatory & analgesic activity. It is an annual herb (10m and 32.8ft) long stems lying or creeping on the ground, with curly tendrils. It is largely cultivated throughout India and in all warm countries, indigenous in tropical & South Africa. *Citrullus lanatus* Thumb. *(Cucurbitaceae)* is a vine-like (scrambler and trailer) flowering plant originally from Southern Africa. Its fruit, which is also called as *watermelon,* is a special kind referred to by botanists as pepo, a berry which has a thick rind (exocarp) and fleshy center (mesocrap and endocarp). Pepos are derived from an inferior ovary, and are characteristic of the *Cucurbitaceae*. It is loosely considered a type of melon, has smooth exterior rind (green, yellow & sometimes white) and a juicy, sweet interior flesh (usually pink, but sometimes orange, yellow, red and sometimes green if not ripe.

Citrullus lanatus Thumb is used as antioxidant, antimicrobial agent, and anthelmintic, anticancer, in urinary infections, as diuretic, as antibacterial, as demulcent, and purgative.

2. MATERIALS AND METHODS

The plant material (Seeds) of *Citrullus lanatus* Thumb were collected from grain market, Karnataka. The authentification was done by Dr. C.K. Nighwal, Mandsaur and the voucher specimen no. BRNCOP / C/012/ 2011/*Citrullus lanatus* Thumb was submitted in department.

Healthy wistar albino rats (160-180 g) of either sex with no prior drug treatment were used. Animal study was performed in division of pharmacology B R N C O P, Mandsaur, animal house. They were kept to standard animal house conditions such as temperature (24.0 ± 1.0^{o}C), relative humidity (55-65%) and 12 hrs light/dark cycles. They were fed with commercial pelleted rat feed and had free access to water throughout the course of study (Gill et al, 2011). Permission was taken from institutional animal ethical committee B R N C O P (181/ MPh / 2011/ IAEC/ BRNCP/ 11-12/ Mandsaur). All the reagents used were obtained from central store house of BRNCOP Mandsaur.

The seeds were cleaned, washed, shade dried and carefully powdered in grinder. All samples were kept in air tight light - protected containers. Hexane was used for defatting the drug.

The extraction was carried out by using methanol by simple maceration process for 24 hrs. 1 kg of the drug was macerated using Methanol as solvent. The solvent was completely removed by rotary evaporator. The % yield was calculated for extract after drying.

The percentage yield of extract was calculated by using following formula:

Percentage yield =Weight of extract/ Weight of powder drug× 100

Pharmacological studies:

The acute toxicity study was carried out according to OECD (Organization for Economic Co-operation and Development) Guideline No.420 in adult male albino rats. Fixed dose method as in Annex 2d: Test procedure with a starting dose of 2000 mg/ kg body weight was adopted.

After the sighting study, starting dose of 2000 mg / kg of each extract was given to 5 animals, and animals were observed for 14 days for any behavioral change and death. The study was repeated with low dose if animal death is observed [3].

Oral Anti-inflammatory activity: Carrageenan – induced paw edema method.

The animals are grouped for *Citrullus lanatus* Thumb.

1. Group I (Control): Carrageenan (1%, p.o)
2. Group II (Standard): Diclofenac sodium (100mg/kg, p.o)
3. Group III: Methanolic extract of *C. lanatus* (MECL), (200mg/kg, p.o)

The extracts were administered orally, one hr before the sub-plantar injection of carrageenan (0.1ml of 1% solution). Paw volumes were measured plethysmometrically at 0min, 30 mins, 1, 2, 3, 4, 5 h after carrageenan & compared with the diclofenac treated and control groups.

Descriptive statistics and comparison of differences between each data set were calculated by the use of sigma stat 3.5 trial version software. The data were expressed as mean ± SEM, and analyzed by one way ANOVA in each experiment. Statistical significance was accepted at the level of $p<0.05$. In the case of significant variation ($p<0.05$), the values were compared by Dunnet test.

Fractionation of active crude extract with different solvents:

First methanolic extract was solubilized in water. Few amount of extract was soluble & few are not soluble. So two fractions (aqueous soluble & aqueous insoluble) were obtained. Aqueous soluble fraction was further fractionated by partition techniques. The solvents were selected on the basis of their polarity. Solvent system was selected from non-polar to polar. Solvents that gave more separation in the form of separate spot on the TLC plate were selected for fractionation [4].

Procedure for obtaining aqueous soluble fraction (A), n-Butanol fraction (B) and chloroform fraction (C):

About 13.47 g portion of the concentrated extract was measured separately into a 500 ml separating funnel and diluted in 150 ml of water. As it was not completely soluble, it was filtered non-aqueous portion was obtained. Then to the filtrate 200 ml of n-Butanol was added. Solution was shaken vigorously; separating funnel was hanged for several minutes for separation to occur. Two portions were obtained, n-Butanol & aqueous fraction. n-Butanol fraction was separated. The aqueous fraction was then extracted with chloroform, to get chloroform fraction.

The aqueous, n-Butanol, chloroform, non- aqueous fraction was then concentrated and dried.

Screening the fraction A, B, C, D, for Anti-inflammatory activity:

The animals are grouped into 5 types

Group I (Control): Treated with Carrageenan (1%, p.o)

Group II : Treated with Aqueous fraction (fraction A) (200 mg/kg, p.o)

Group III :Treated with n-Butanol fraction (fraction B) (200 mg/kg, p.o)

Group IV: Treated with Chloroform fraction (fraction C) (200 mg/kg, p.o)

Group V: Treated with Non - Aqueous fraction (fraction D) (200 mg/kg, p.o)

Descriptive statistics and comparison of differences between each data set were calculated by the use of sigma stat 3.5 trial version software. The data were expressed as mean ± SEM, and analyzed by one way ANOVA in each experiment. Statistical significance was accepted at the level of $p<0.05$. In the case of significant variation ($p<0.05$), the values were compared by Dunnet test.

Non-Aqueous fraction was insoluble in water and it was soluble in ethanol. For better separation different solvents systems were selected, ethanol: methanol, ethanol: water, ethanol: chloroform, methanol: water, methanol: chloroform, water: chloroform in different ratios. The solvent system which gave better separation using TLC was selected for further fractionation. TLC of non-aqueous fraction was done with selected solvent systems.

3. RESULTS:

S.no	Extract	Weight of extract	Weight of powder	% yield	Characteristics
1	Methanolic	24.0524	1 kg	2.4	Waxy, Intense brown

Table 1: Percentage yield of methanolic extract of *Citrullus lanatus* Thumb.

Determination of percentage yield of crude extracts:

Phytochemical screening of crude extract:

S.no	Experiment	*Citrullus lanatus* (Methanolic extract)
1.	Carbohydrate	+
2.	Alkaloids	+
3.	Steroids	+
4.	Saponins	+
5.	Flavonoids	+
6.	Tannins & phenolics	+

+ sign indicates presence whereas – sign indicates absence of constituents.

Table 2: Qualitative chemical analysis of crude extract

Pharmacological studies:

Acute toxicity studies on female rats shows one mortality at a dose of 2000 mg/kg, during a time period of 14 days. No skin allergic symptoms were seen during study. The behavioral, neurological and autonomic responses were studied for a time period of 6 hrs of toxicity study. During the study no noticeable responses were seen in the rats.

Treatment Methanolic Extract	Dose (mg/kg)	No. of animals	Mortality			Toxicity profile
			After 24 h	After 7 days	After 14 days	
Citrullus lanatus	2000	5	1	0	0	Safe

Table 3: Mortality in acute toxicity study

Anti-inflammatory studies:

Carragenan induced paw edema method (for *Citrullus lanatus* extract).

Sub-plantar injection of carragenan provoked marked, time related increases in the hind paw volume of untreated control rats. 200 mg/kg of methanolic extract of seeds of *Citrullus lanatus* had shown significant ($p<0.05$) anti-inflammatory activity during 30 min, 1 h and 3 h.

Groups	Treatment	Average volume of mercury displaced (ml.)						
		0 min	30 min	1 h	2 h	3 h	4 h	5 h
1	Control	1.10 ± 0.24	2.60 ± 0.48	3.10 ± 0.40	2.60 ± 0.24	4.20 ± 0.37	4.80 ± 0.48	2.00 ± 0.0
2	*Citrullus lanatus* (200 mg/kg)	1.20 ± 0.20	1.2 ± 0.20*	1.80 ± 0.20*	2.40 ± 0.24 ns	3.00 ± 0.31*	4.00 ± 0.54 ns	1.60 ± 0.24 ns
3	Standard (100 mg/kg)	1.20 ± 0.20	2.0 ± 0.31ns	1.20 ± 0.20***	1.04 ± 0.04***	1.08 ± 0.0***	1.08 ± 0.04***	1.00 ± 0.0***

Values are expressed as mean ± SEM. Number of animals used were five in each groups.*$p<0.05$, **$p<0.01$, ***$p<0.001$, ns - non-significant *vs*. control (Dunnet's test)

Table 4: Anti-inflammatory activity of methanolic extract of *Citrullus lanatus* Thumb.

Groups	Treatment	% inhibition of paw volume						
		0 min	30 min	1 h	2 h	3 h	4 h	5 h
1	Control	-	-	-	-	-	-	-
2	*Citrullus lanatus* (200mg/kg)	- 9.09	53.84	41.93	7.69	28.57	16.6	20
3	Standard (100 mg/kg)	- 9.09	23.07	61.29	60	74.28	77.5	50

Table 5: % inhibition of paw volume of methanolic extract of *Citrullus lanatus* Thumb.

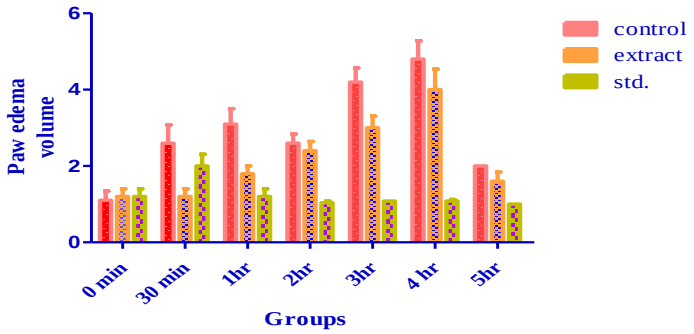

Fig: 1 - Anti-inflammatory activity of *Citrullus lanatus* extract

Fractionation of active extract with different solvent:

Selection of solvent system:

The solvent for fractionation was selected with the help of the TLC.

Table 6: Solvents selected for TLC

Solvent	Polarity index	Solubility in Water(% W/W)
n-Butanol	3.9	7.810
Chloroform	4.1	0.815

Chromatography of extract using different solvents:

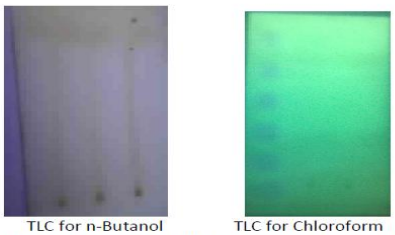

Fig: 2 - TLC of fractions using n-Butanol & chloroform as solvent.

TLC of fractions using n-Butanol as solvent.

Solvent system - n-Butanol

No. of spots - 5 spots.

R_f value of fractions - 0.045, 0.151, 0.30, 0.575, 0.727

TLC of fractions using Chloroform as solvent.

Solvent system - Chloroform

No. of spots - 5 spots.

R_f value of fractions - 0.176, 0.36, 0.57, 0.79, 0.98

So, on this basis n - Butanol & chloroform were selected for fractionation.

Fractionation of crude extract with n-Butanol, chloroform as solvents.

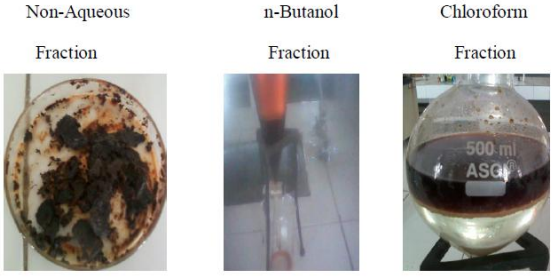

Fig: 3- Separation of crude extract by using selected solvents

Determination of percentage yield (% w/w) of fractions:

Drug taken	Colour	Consistency	Yield (g)
Fraction A (Aqueous)	Dark brown	solid	6 g
Fraction B (n-Butanol)	Dark brown	Semisolid	0.0637 g
Fraction C (Chloroform)	Dark brown	Semisolid	0.7116 g
Fraction D (Non-Aqueous)	Dark brown	Semisolid	6.2 g

Table 7: Calculation of percentage yield of fractions

Phytochemical screening of fractions:

Table 8: Chemical examination of various fractions

S.no	Experiment	Non-Aqueous	n-Butanol	Chloroform	Aqueous
1.	Carbohydrate	-	+	-	+
2.	Alkaloids	+	+	+	+
3.	Steroids	+	+	-	+
4.	Saponins	-	-	-	-
5.	Flavonoids	-	+	-	+
6.	Glycosides	-	-	-	+
7.	Tannins & phenolics	+	-	-	+

Acute toxicity studies-fixed dose procedure for fractions:

Acute toxicity studies on male rats showed no mortality at a dose of 2000 mg/kg, during a time period of 14 days. No skin allergic symptoms were seen during study. The behavioral, neurological and autonomic responses were studied for a time period of 6 hrs of toxicity study. During the study no noticeable responses were seen in the rats.

Treatment fractions	Dose (mg/kg)	No. of animals	Mortality			Toxicity profile
			After 24 h	After 7 days	After 14 days	
Fraction (A) Aqueous	2000	5	0	0	0	Safe
Fraction (B) n-Butanol	2000	5	0	0	0	Safe
Fraction (C) Chloroform	2000	5	0	0	0	Safe
Fraction (D) Non-Aqueous	2000	5	0	0	0	Safe

Table 9: Observation of acute toxicity study of fractions

Screening the fractions (A, B, C, D) for Anti-inflammatory activity:

The anti-inflammatory activity of various fractions were performed and found that not all the fraction showed anti-inflammatory activity but fractions D (Non-Aqueous fraction) showed more potent activity so fraction D (Non-Aqueous fraction) was selected for further study.

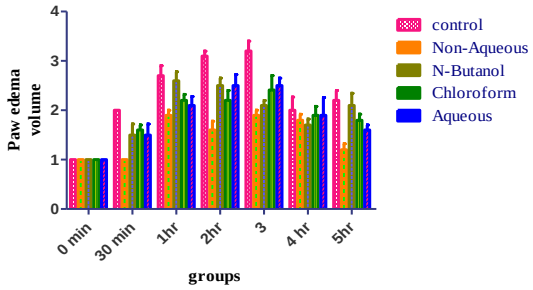

Fig: 4 - Anti-inflammatory activity of fractions

Groups	Treatment	Average volume of mercury displaced (ml.)						
		0 min	30 min	1 h	2 h	3 h	4 h	5 h
1	Control	1 ± 0.0	2 ± 0.0	2.7 ± 0.20	3.1± 0.10	3.2 ± 0.20	2 ± 0.27	2.2 ± 0.20
2	Non-Aqueous (D) (200mg/kg)	1 ± 0.0	1 ± 0.0***	1.9 ± 0.10**	1.6 ± 0.18***	1.9 ± 0.10***	1.8 ± 0.12^{ns}	1.2 ± 0.122**
3	n-Butanol (B) (200 mg/kg)	1 ± 0.0	1.5 ± 0.22^{ns}	2.6 ± 0.18^{ns}	2.5 ± 0.15^{ns}	2.1 ± 0.10^{ns}	1.7 ± 0.12^{ns}	2.10 ± 0.244^{ns}
4	Chloroform (C) (200 mg/kg)	1 ± 0.0	1.6 ± 0.10^{ns}	2.2 ± 0.122^{ns}	2.2 ±0.20**	2.41 ± 0.29*	1.9 ± 0.18^{ns}	1.8 ± 0.122^{ns}
5	Aqueous (A) (200 mg/kg)	1 ± 0.0	1.5 ± 0.22^{ns}	2.10 ± 0.18^{ns}	2.5 ± 0.22^{ns}	2.5 ± 0.15*	1.9 ± 0.36^{ns}	1.6 ± 0.10^{ns}

Values are expressed as mean ± SEM. Number of animals used were five in each grous.*p<0.05, **p<0.01, ***p<0.001, ns - non-significant *vs*. control (Dunnet's test)

Table10: Anti-inflammatory activity of fractions

Groups	Treatment	% inhibition of paw volume						
		0 min	30 min	1 h	2 h	3 h	4 h	5 h
1	Control	-	-	-	-	-	-	-
2	Non-Aqueous (200mg/kg)	0	50	29.62	48.38	40.62	10	45.45
3	n-Butanol (200 mg/kg)	0	25	3.70	19.35	34.37	15	4.54
4	Chloroform (200 mg/kg)	0	20	18.51	29.03	32.78	5	18.18
5	Aqueous (200mg/kg)	0	25	22.22	19.35	21.87	5	27.27

Table 11: % inhibition of paw volume of fractions

Chromatography of Non-Aqueous fraction:

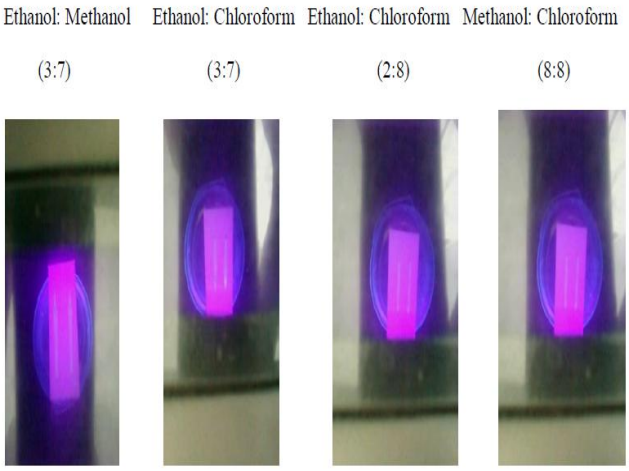

Fig: 5 -TLC of most potent (Non-aqueous fraction)

TLC of Non-Aqueous fraction (D):

S.no	Solvent system	Ratio	No.of spots	Rf value
I.	**Ethanol: Methanol**			
1		5:5	1	0.58
2		4:5	1	0.26
3		3:7	3	0.15, 0.7, 0.82
4		2:8	1	0.5
5		8:2	2	0.17, 0.6
II.	**Ethanol: Water**			
1		5:5	-	-
2		4:4	1	0.26
3		3:3	1	0.75
4		2:2	1	0.72
5		8:8	1	0.76
III.	**Ethanol: Chloroform**			
1		5:5	-	-
2		4:5	-	-
3		3:7	4	0.18, 0.30, 0.69, 0.84
4		2:8	4	0.17, 0.5, 0.71, 0.85
5		8:2	1	
IV	**Methanol: Water**			
1		5:5	-	-
2		5:4	1	0.62
3		7:3	-	-
4		8:2	-	-
5		2:8	-	-
V.	**Methanol: Chloroform**			
1		5:5	2	0.72, 0.81
2		5:5	2	0.51, 0.59
3		7:7	2	0.51, 0.72
4		8:8	3	0.41, 0.54, 0.67
5		2:2	2	0.5, 0.6
VI.	**Water: Chloroform**			
1		5:5	1	0.18
2		4:5	-	-
3		3:7	-	-
4		2:8	-	-
5		8:2	-	-

Table 12: TLC of Non-aqueous fraction

4. DISCUSSION:

Citrullus lanatus is been traditionally used for various disease and disorders. The phytochemical evaluation of methanolic seeds extract showed the presence of carbohydrate, alkaloids, steroids, saponins, flavonoids, tannins & phenolic compounds.

The methanolic extract showed significant activity on edema in all the three phases. This edematous response was also significantly reduced in rats pretreated with diclofenac, a known cyclooxygenase inhibitor. Carrageenan is a strong chemical for the release of inflammatory & pro- inflammatory mediators. Several ROS are released during such inflammation. The present work reveals that the inflammatory tissue damage is due to liberation of ROS from phagocytes invading inflammation sites. By this it proves that, oral MECL was found to have anti-inflammatory activity & it may be due to its free radical scavenging activity. 200 mg/kg of methanolic extract of *Citrullus lanatus* had shown significant ($p<0.05$) anti-inflammatory activity during 30min, 1 hr & 3 h.

The result in this study indicates that the seeds extract of *C. lanatus* posses anti-inflammatory activity against the phlogistic agent used. Inflammation induced by carragenan involves three distinct phases of the release of the mediator, including serotonin & histamine in the first phase (0-2h), kinins in the second phase (3h), & prostaglandin in the third phase (>4h). NSAID such as the reference diclofenac used in this study are known to inhibit cyclooxygenase enzymes I & II which are implicated in the production of inflammation-mediating agent prostaglandin E_2 (PGE_2) from arachidonic acid.

On the basis of the results obtained in present investigation after fractionation, it is possible to conclude that amongst all the fractions non-aqueous fraction (200 mg/kg) of methanolic seeds extract of *Citrullus lanatus* has most significant ($p<0.01$, $p<0.001$) anti-inflammatory activity after 30 mins,1h, 2h, 3h and 5h. The above findings justify the anti-inflammatory activity of *Citrullus lanatus* as suggested in the literatures.

The anti-inflammatory activity of *Citrullus lanatus* appears due to the presence of its active principles, which accelerates the anti-inflammatory process and decrease the inflammation

The study reveals that both *Citrullus lanatus* & non-aqueous fraction treated groups posses good anti-inflammatory properties which may be due to individual or combined action of phytoconstituents like alkaloids, steroids, tannin, phenolic, amino acids. Further investigations are necessary to determine the bioactive constituents present in non-aqueous fraction used in this studies.

5.CONCLUSION

The present study was aimed to fractionate the methanolic extract of *Citrullus lanatus* Thumb & to identify the constituents responsible for anti-inflammatory activity. The anti-inflammatory activity of *Citrullus* lanatus appears due to the presence of active principles, which accelerates the anti-inflammatory process and reduces the inflammation. Further, anti-inflammatory activity by non-aqueous fraction was found to be better than *Citrullus lanatus* treated groups in rat.

Further studies are required to identify the active constituent present in the non-aqueous fraction of MECL.

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Conflict of Interest: None Declared

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