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In Vitro Evaluation of Anti-inflammatory and Anti-Cancer Activities of Flower Extracts of *Abelmoschus esculentus* L.

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Abstract: *Oxidative stress and inflammation are the predominant cause of chronic diseases, including multiple forms of cancers. Prevention of oxidative stress and inflammation is considered to be a target for preventing these disorders due to their significant roles in various degenerative diseases. Various natural products and plant extracts prevent the process of free radical- induced damages. The present study evaluated the biological properties of Abelmoschus esculentus L, which is a traditionally used Ayurvedic plant. Protein denaturation inhibition bioassay and Proteinase Inhibition assays were used for anti-inflammatory studies. Anticancer activity was evaluated using Brine shrimp lethality bioassay. The results of the present study anti-inflammatory properties were also evident in terms of Protein denaturation inhibition bioassay the Hydroalcoholic extract's IC_{50} value is 166.56 µg/ml, ethanolic extract's is 220.80 µg/ml, aqueous extract IC_{50} value is 234.01and Proteinase Inhibition assay the IC_{50} values for Hydroalcoholic, ethanolic and aqueous extracts were 131.54, 150.99 and 221.84 µg/mL, respectively. The benefit of a lethal dose (LD_{50}) is that it can be used to reduce significantly the number of animals used.(37) In Abelmoschus esculentus L the hydroalcoholic extract showed the lowest LC_{50} value (152.90 µg/ml) and in ethanol showed lowest LC_{50} Value (241.58µg/ml). According to the definition of LC_{50}, the high LC_{50} value means it has less toxicity and low LC_{50} value means it has more toxicity that means, hydroalcoholic extract from Abelmoschus esculentus L contained more toxic compounds (low LC_{50} value means high toxic). The study thus concludes that Abelmoschus esculentus L showed significant anti-inflammatory and anticancer properties. Further studies together with a bioassay-guided fractionation may identify possible bioactive compounds.*

Keywords: flavonoids; anti-inflammatory; anticancer; Abelmoschus esculentus L ; in vitro studies

I. INTRODUCTION

Numerous natural chemicals that have been extracted from various medicinal plants have been widely used to treat a wide range of chronic illnesses since ancient times. Flavonoids, phenolic acids, lignans, quinones, coumarins, and alkaloids are a few examples of secondary metabolites that have demonstrated significant antioxidant and other properties and have been crucial in the treatment of cancer. [(1)] The usage of plant-based natural chemicals has attracted a lot of attention because they have demonstrated enormous potential against cancer and are linked to few negative effects. An estimated 80% of people in poor nations rely on herbal or natural therapy to address a variety of illnesses. [(2)] The diverse pharmacological characteristics of plants are caused by the presence of a variety of secondary metabolites, including alkaloids, terpenoids, glycosides, steroids, flavonoids, and phenolic chemicals. [(3)] These bioactive compounds are reported to have various beneficial effects in decreasing the risk of diseases caused by reactive oxygen species (ROS). [(4)] By a variety of methods, including oxidative enzyme inhibition, ROS quenching, and free radical scavenging. Numerous biochemical characteristics, including antiallergic, anti-inflammatory, antioxidant, antibacterial, antifungal, antiviral, and anticancer effects, have been reported for plant extracts. [(5)]

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II. MATERIALS AND METHODS

2.1 Collection of plant material:

The fresh leaves of *Abelmoschus esculentus* L were collected from farm A/P Darganhalli Solapur (Tal) Maharashtra, India during October 2022. The flower of *Abelmoschus esculentus* L were shade dried at room temperature for 4 weeks. The dried parts were later coarsely powdered with help of electric grinder (mixer) after passed through sieve no. 20 to obtain coarse powder. Then this powder was stored in plastic container at room temperature to protect it from moisture.

2.2 Authentication of plant:

The taxonomical identification (authenticated) by Dr.S.D. Randive with the help of flora of D.B.F. Dayanand college of art and science Solapur District, Maharashtra, India and the voucher specimens were deposited at the

2.3 Extraction

2.3.1 Preparation of ethanolic extract:

The Soxhlet equipment was used during the extraction preparation phase. The extraction is done out using an ethanol solvent. The coarse powder (50g) was extracted in 250 mL of solvent ethanol using a Soxhlet device. The cycles repeated until a clear solution was achieved. The extractions were filtered using Whatman filter paper (No. 2). While hot, dry in an evaporator and then air dry. The retrieved materials were stored and used for future research.

2.3.2 Preparation of Aqueous extract:

A 500 ml conical flask containing about 50 gm of powdered drug was filled with 250 ml of water as a solvent. To prevent the growth of fungi, 5 ml of chloroform was added. The cold maceration method is used to extract the material in water. Following a 24-hour period, the extracts were filtered through Whatman filter paper (No. 2), dried in an evaporator, and then allowed to air dry. The materials that were recovered were stored and will subsequently be used in additional research.

2.3.3 Preparation of Hydroalcoholic extract:

A 500 ml conical flask containing about 50 gm of powdered drug was filled with 250 ml of water as a solvent. To prevent the growth of fungi, 5 ml of chloroform was added. The cold maceration method is used to extract the material in water. Following a 24-hour period, the extracts were filtered through Whatman filter paper (No. 2), dried in an evaporator, and then allowed to air dry. The materials that were recovered were stored and will subsequently be used in additional research.

2.4 Phytochemical Analysis:

Preliminary screening of Phytochemicals (Qualitative analysis): The presence/absence of secondary metabolites such as alkaloids, steroidal compounds, phenolic compounds, flavonoids, saponins, tannins, and anthraquinones was assessed in three extracts of *Abelmoschus esculentus* L using standard procedures. [6]

2.5 In-Vitro Anti-Inflammatory Activity:

For the current study of anti-inflammatory activity of *Abelmoschus esculentus* L leaves in vitro models are evaluated. [7]

2.5.1 Protein Denaturation Inhibition Bioassay:

i) Phosphate buffer saline of pH 6.3

Dissolve 1.79 gm of disodium hydrogen phosphate (Na_2HPO_4), 1.36 gm of potassium dihydrogen phosphate and 7.02 gm of NaCl in sufficient amount of distilled water to produce 1000 ml. Adjust the pH of saline (6.3) by adding 1N HCl.[8]

ii) Preparation of standard solution-

Diclofenac sodium used as standard stock solution of Diclofenac sodium in ethanol was prepared of 1000 µg/ml., from this stock solution 3 different concentrations of 100, 200 and 300 µg/ml were prepared.

iii) Preparation of test solution-

Stock solutions of the three leaves extract of 1000 µg/ml was prepared by using respective solvent. From this stock solution 3 different concentration of 100, 200 and 300 µg/ml were prepared.

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iv) Procedure

The assay procedure for the current study's protein denaturation inhibition test is outlined below. 0.9 ml of fresh egg albumin (5% aqueous solution) and 0.1 ml of a test solution containing an extract of *Abelmoschus esculentus* L flower produced in ethanol at three different concentrations (100 µg/ml, 200 µg/ml, and 300 µg/ml) were placed in separate test tubes for this experiment. The test solution was replaced with 0.1 ml of distilled water for the control. Additionally, the test solution was replaced with 0.1 ml of Diclofenac sodium for the standard. (Different concentrations created in ethanol: 100 µg/ml, 200 µg/ml, and 300 µg/ml). Mixture was incubated for five minutes at 37°C. After three minutes at 55°C, test tubes were cooled. Each test tube was filled with 2.5 ml of phosphate buffer saline pH 6.3 once they had cooled. Spectrophotometric measurements of the absorbance were made at 660 nm. [9]

v) Standardization-

Standardization of the in vitro protein denaturation inhibition bioassay was done by using standard Diclofenac sodium.

Calculation-

The percentage inhibition of protein denaturation calculated by,

$$\% \textbf{ Inhibition} = \frac{(\textbf{Absorbance of control} - \textbf{Absorbance of sample})}{\textbf{Absorbance of control}} \times \textbf{100}$$

2.5.2 Proteinase Inhibition:

i) Chemicals used for the activity:

Disodium hydrogen phosphate, potassium dihydrogen phosphate NaCl, Casein, Tri– chloracetic acid.

ii) Preparation of standard stock solution: Aspirin used as standard stock solution in (Hydroalcoholic (50:50), ethanol, aqueous) was prepared of 1000 µg/ml. The stock solution was serially diluted at increasing concentrations (100, 200, 300 µg/ml) using the appropriate solvent.

iii) Preparation of test solution: A 1 gm flower extract (Hydroalcoholic (50:50), ethanol, aqueous) was diluted in ethanol and distilled water, yielding a 1000 µg/ml concentrated stock solution. The stock solution was serially diluted at increasing concentrations (100, 200, 300 µg/ml) using the appropriate solvent.

iv) Procedure: The (2 ml) reaction mixture contained 0.06 ml trypsin, 1 ml of mM tris buffer (pH 7.4), and 2 ml of plant extract solution (ethanol, aqueous) serially diluted in each test tube, as well as a control test tube containing 2 ml solvent with the equivalent extract instead of the plant extract itself. The samples were incubated at 37℃ in a BOD incubator for 10 minutes before adding 1 ml of 0.65% w/v casein. The samples were re-incubated for 20 minutes following which 2 ml of HClO4 was added to terminate the reaction. The cloudy suspension rotated at 7830 rpm for 15 minutes. A spectrophotometer was used to measure the absorbance of the supernatant at 280 nm against a blank of Tris-HCl buffer. Performed in triplicate, with inhibitory concentration. The IC_{50} value was determined. [10]

Percentage of Proteinase inhibition can be calculated by following formula.

Calculation:

The percentage inhibition of protein denaturation calculated by,

$$\% \textbf{ Inhibition} = \frac{(\textbf{Absorbance of control} - \textbf{Absorbance of sample})}{\textbf{Absorbance of control}} \times \textbf{100}$$

2.6. In Vitro Anti-Cancer Activity:

i) **Preparation of sea- water**

38 gm of sea salt (without iodine) and 6 mg of dried yeast was weighed, and diluted in 1 litre of distilled water and filtered off to get a clear solution.

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ii) Hatching of Brine Shrimp

50 mg of Artemia salina leach (Brine shrimp eggs) was added to one side of container which contain the 1 litre of artificial sea water. Then cover the container with the help of aluminium foil and placed in dark area for 48 hours for the hatching of the brine shrimp eggs. The continuous oxygen supply was provided during the hatching time.

iii) Preparation of stock & test solution

To make a 1000 µg/ml concentrated stock solution, 1 gm of flower extract (hydroalcoholic ethanol and aqueous) was dissolved in 200 µl of pure DMSO and sea water. From this stock solution, varying concentrations (**10, 20, 30, 40, 50 µg/ml**) were serially diluted with sea water. The same procedure was used for the standard drug 5-flurouracil with varying concentration (**0.5, 1, 1.5, 2, 2.5 µg/ml**).

iv) Experimental procedure:

10 live Brine shrimp nauplii were carefully taken by micropipette with 2.5 ml simulated sea water, then 2.5 ml plant extract solution (hydroalcoholic, ethanol and aqueous) was added to each test tube, which was serially diluted. The process also included a control test tube containing 10 live nauplii with 5 ml simulated sea water, which was placed in a dark area. After 24 hours, the test tube was inspected with a magnifying glass, and the number of surviving nauplii was counted. The lethality concentration LC_{50} value was determined. [11]

Calculation-

The percentage mortality can be calculated by,

$$\% \textbf{ Mortality} = \frac{\textbf{No. of Nau paliitaken} - \textbf{Live}}{\textbf{No. of nau paliitaken}} \times 100$$

III. RESULTS AND DISCUSSION

3.1 Phytochemical Analysis.

Using solvents, phytochemical tests are carried out to find the optional metabolites, such as alkaloids, glycosides, flavonoids, steroids, triterpenoids, and phenolic compounds, in the concentrated Flower of *Abelmoschus esculentus* L.

Table no.1: Result of the Qualitative phytochemical test for the *Abelmoschus esculentus* L Flower Extracts

Sr. No.	Phytoconstituents	Test	Ethanol	Aqueous	Hydroalcoholic
1	Carbohydrate	Molish test	++	-	++
		Fehling's test	++	++	+++
2	Alkaloids	Dragendorff's test	+	+	++
		Mayer's test	+	+	+
		Hanger's test	++	++	++
		Wagner's test	+	+	++
3	Flavonoids	Shinoda's test	--	--	--
		Lead acetate test.	++	++	+++
		Alkaline test	+	+	+
		Hydrochloric acid zinc reduction test: Ferric chloride test	+ +		+ + +
4	Glycosides	Legal's test	-	+	-
		Killer – Killani's test	++	++	+++
		Modified Bontrager's test	++	+	++
		Coumarin glycoside	+	-	+
			+	+	++

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		Foam test	++ +	- +	++ ++
5	Phenols	Ferric Chloride Test	++	-	+
6	Steroids	Salkowski's test Liebermann Burchard test	++	+	
7	Test for Tannins	Ferric chloride test: Gelatin test:	+	+	++
8	Test for Terpenoids		+	+	+
9	Test for Saponins	Foam test: Haemolysis test: Froth test:	- ++ +	+ + +	+ ++ +
10	Test for Phytosterols	Lieberman Burchard's test for phytosterols: Salkowski test:	++ +	+ -	++ +

(- absent, +present, ++ Moderately present, +++ strongly present)

The Flower of ***Abelmoschus esculentus* L** were extracted using ethanol, water and Hydroethanol, and the results of these extracts were complete positives for Phytosteroids, alkaloids, flavonoids, glycosides, saponin, tannins, and phenolic compounds.

Thus, alkaloids, flavonoids, glycosides, phenolic compounds, saponin, steroids, and coumarins are present in the Flower extracts of ***Abelmoschus esculentus* L** as secondary metabolites.

3.2 Anti-Inflammatory Activity:

3.2.1 In-Vitro Protein Denaturation Inhibition Bioassay

In-vitro percentage (%) inhibition of the protein denaturation induced by the heat of hydroalcoholic, ethanol and aqueous extraction of *Abelmoschus esculentus* L Flower at different concentration are shown in table no.2.

Table no.2: *In-vitro* protein denaturation inhibition bioassay of hydroalcoholic, ethanol and aqueous extract of *Abelmoschus esculentus* L, Diclofenac sodium (standard drug)

Sr. No.	Sample	Concentration (µg/ml)	Percent inhibition (Mean±SEM)	IC_{50} value (µg/ml)
1	Hydroalcoholic	100	34.21 ± 0.55	**166.56**
2		200	59.73 ± 1.12**	
3		300	78.49± 1.80***	
1	Ethanol	100	22.2±0.56	**220.80**
2		200	47.69 ±1.12	
3		300	66.44 ±1.80**	
1	Aqueous	100	20.21±0.53	**234.01**
2		200	41.07 ±0.34	
3		300	62.24±1.89**	
1	Standard (Diclofenac sodium)	100	24.21 ± 0.56	**159.08**
2		200	65.61 ± 1.40	
3		300	84.40 ± 0.68	

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Values are represented as (Mean±SEM) (n=3),where (*p<0.05, **p<0.01, ***p<0.001).
Bioassay did in triplicate manner; each concentration was performed for 3 replicate.
The Hydroalcoholic, ethanol and aqueous extracts' prevention of heat-induced protein denaturation was how the in vitro anti-inflammatory action was achieved. At 100 µg/ml, 200 µg/ml, and 300 µg/ml, respectively, the % inhibition with SEM of the hydroalcoholic extract is 34.21 ± 0.55, 59.73 ± 1.12**, and 78.49± 1.80***. At 100 µg/ml, 200 µg/ml, and 300 µg/ml, respectively, the % inhibition of ethanolic extract is 22.2±0.56, 47.69 ±1.12, and 66.44 ±1.80**. At 100 µg/ml, 200 µg/ml, and 300 µg/ml, respectively, the % inhibition of the reference drug (Diclofenac sodium) is 24.21±0.56, 65.61±1.40, and 84.40 ±0.68. The percentage inhibition of three the extracts was compared with the standard drug Diclofenac sodium. Hydroalcoholic extract's IC_{50} value is 166.56 µg/ml, ethanolic extract's is 220.80 µg/ml, aqueous extract IC_{50} value is 234.01 and standard drug IC_{50} value is 159.08 µg/ml. As mentioned in Table No.2

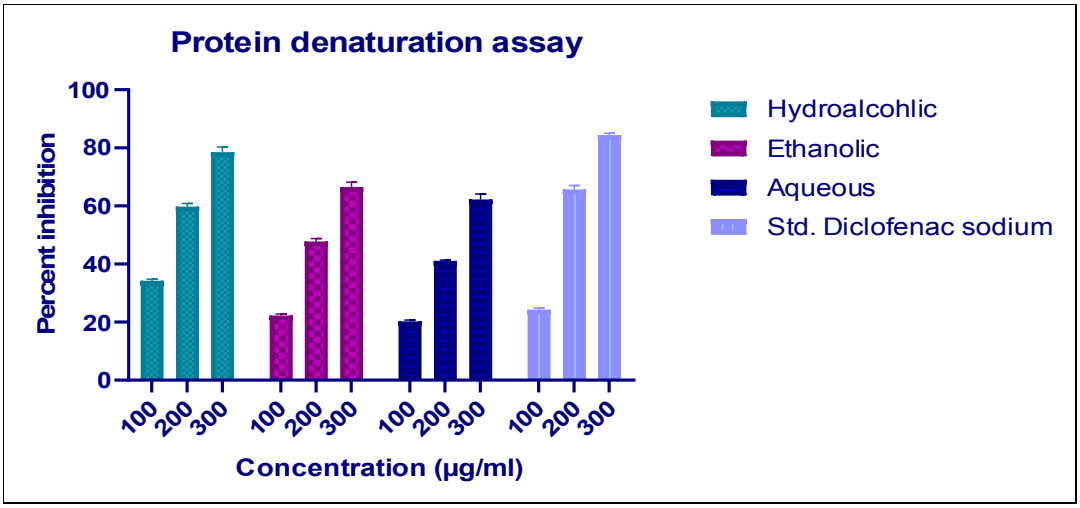

Figure no 1. Effect of hydroalcoholic, aqueous & ethanolic extracts of *Abelmoschus esculentus* L on Protein denaturation.

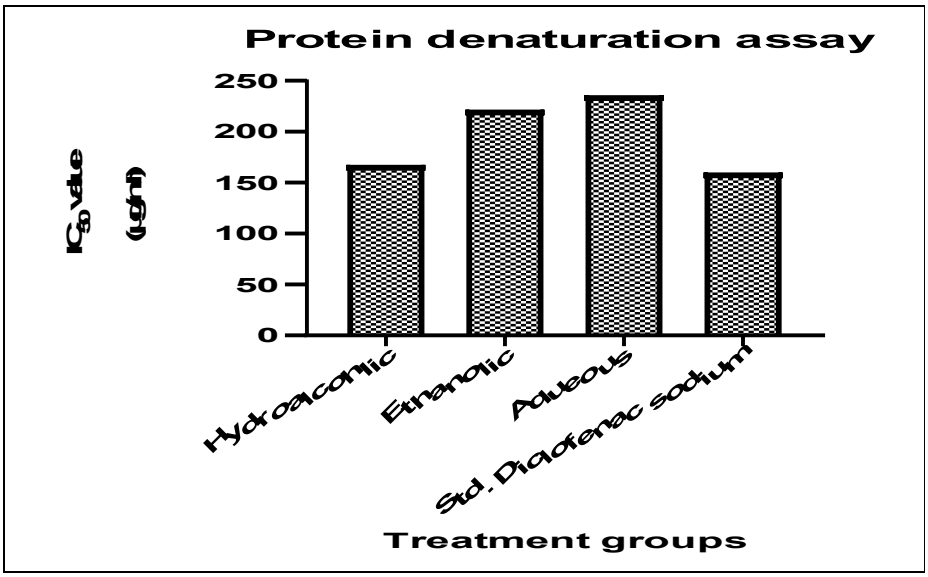

Figure no 2. Comparison of IC_{50} Value of hydroalcoholic, aqueous & ethanolic extracts of *Abelmoschus esculentus* L on Protein denaturation.

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3.2.2. Proteinase Inhibition

Bioassay percentage inhibition of the proteinase enzyme by hydroalcoholic, ethanol and aqueous extracts of *Abelmoschus esculentus* L. flower at different concentration are shown in table below compared with standard drug Diclofenac sodium.

Table no 3. Anti-inflammatory activity of hydroalcoholic, ethanol and aqueous extracts of *Abelmoschus esculentus* L. by proteinase inhibition.

Sr. No.	Sample	Concentration (µg/ml)	Percent inhibition (Mean±SEM)	IC_{50} value (µg/ml)
1	Hydroalcoholic	100	45.43 ± 1.56	131.54
2		200	60.12 ± 0.98**	
3		300	70.19 ± 1.30***	
1	Ethanol	100	43.35 ± 0.65	150.99
2		200	56.12 ± 1.02**	
3		300	71.69± 1.80***	
1	Aqueous	100	22.31 ±0.53	221.84
2		200	44.12 ± 0.84	
3		300	67.14±0.89**	
1	Standard (Diclofenac sodium)	100	19.31 ± 1.26	99.50
2		200	52.51 ± 1.20**	
3		300	88.39 ± 1.68***	

Data is expressed as Mean±SEM (n=3) using graph pad prism 8.0.1 and analysed by ANOVA followed by Dunnett's multiple comparison test, where * p≤0.05, **p≤ 0.01, ***p≤0.001.

IC_{50} value calculated by equation y= mx + c.

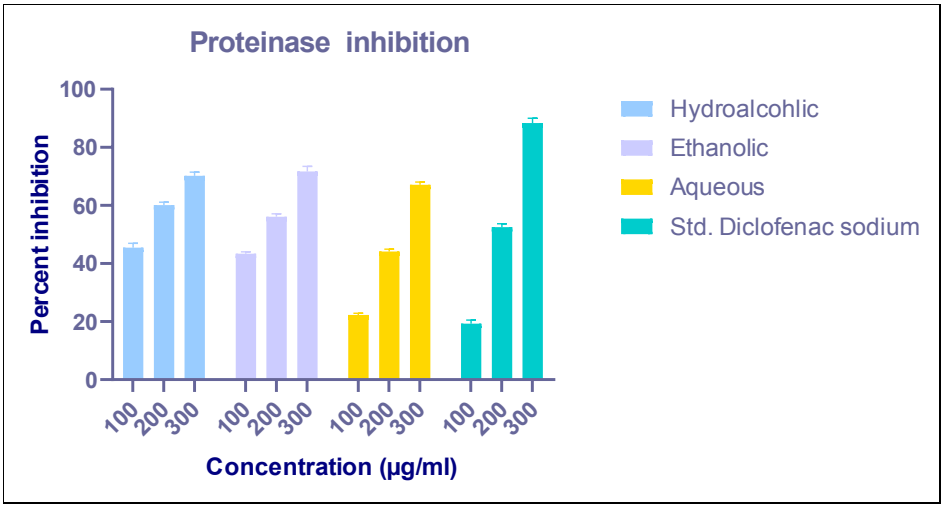

Figure no 3. Effect of hydroalcoholic, aqueous & ethanolic extracts of *Abelmoschus esculentus* L on Proteinase inhibition.

Neutrophils live in lysosomal granules and are a major source of serine proteinase. Leukocyte proteinase contribute to tissue damage during inflammatory reactions, and proteinase inhibitors protect against this impact. Proteinase inhibition action can reduce inflammation by inhibiting neutral proteinase. The IC_{50} values for Hydroalcoholic, ethanolic and aqueous extracts were 131.54, 150.99 and 221.84 µg/mL, respectively. In contrast, the reference standard had an IC_{50} value of 99.50 µg/mL. The results reveal that the Hydroalcoholic extract inhibits proteinase most when compared to the aqueous and ethanolic extract.

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3.3. In-Vitro Anticancer Activity

3.3.1 Brine shrimp lethality bioassay

In-vitro Brine shrimp lethality bioassay of hydroalcoholic, ethanolic and aqueous extract of *Abelmoschus esculentus* L flower at different concentration are shown in table no.4

Table no. 4: Effect of hydroalcoholic, ethanolic and aqueous extract of *Abelmoschus esculentus* L & 5-Fluorouracil (standard Drug) on percentage mortality using Brine Shrimp Lethality bioassay.

Plant extracts	Conc. (µg/ml)	Mean % Mortality (Mean ± SEM)	LC_{50} (µg/ml)
5-FU (Std.)	20	30.12±1.16	**3.76 (µg/ml)**
	50	46.58±1.13	
	100	84.57±0.58**	
	200	90.25±1.18**	
	500	98.58±0.19***	
Hydroalcoholic	20	27.55±1.15	**152.90 (µg/ml)**
	50	34.25±1.14	
	100	50.14±1.12	
	200	63.88±1.10**	
	500	87 ± 0.98***	
Ethanol	20	11.12±1.12	**241.58 (µg/ml)**
	50	29.25± 1.14	
	100	42.15± 1.15	
	200	49.25±1.11	
	500	77.89 ± 1.12**	
Aqueous	20	12±1.07	**257.18 (µg/ml)**
	50	21.11±1.12	
	100	37.77±1.54	
	200	48±1.03	
	500	78±1.73	

Data is expressed as Mean±SEM (n=3) using graph pad prism 8.0.1 and analysed by ANOVA followed by Dunnett's multiple comparison test, where * p≤0.05, **p≤ 0.01, ***p≤0.001.

LC_{50} value calculated by equation y= mx + c

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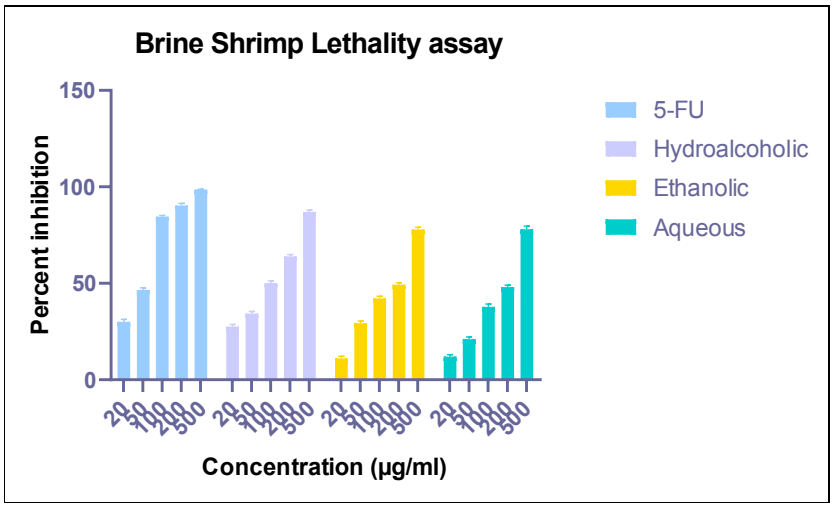

Figure no 4. Effect of hydroalcoholic, aqueous & ethanolic extracts of *Abelmoschus esculentus* L on Brine Shrimp Lethality bioassay.

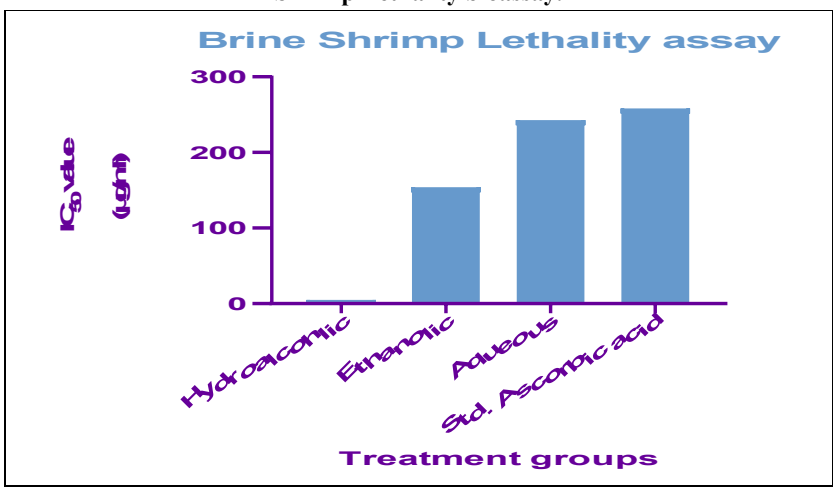

Figure no 5. Comparison of IC_{50} Value of hydroalcoholic, aqueous & ethanolic extracts of *Abelmoschus esculentus* L on Brine Shrimp Lethality bioassay.

The mortality percentages of the shrimp larvae exposed to different flower extracts of *Abelmoschus esculentus* L are shown in Table 4. The order of activity was Hydroalcoholic >Ethanolic> Aqueous extract shown in Figure 4.

Among the three prepared different polarities of flower extracts hydroalcoholic extracts have displayed significant activity in plant against the brine shrimp larvae. Moreover, there is an increase in the mean percentage of mortality with increase in concentration of leaves extract. The toxicity of the extract is generally investigated using LC_{50}.

The benefit of a lethal dose (LD_{50}) is that it can be used to reduce significantly the number of animals used. In *Abelmoschus esculentus* L the hydroalcoholic extract showed the lowest LC_{50} value (152.90 µg/ml) and in ethanol showed lowest LC_{50} Value (241.58 µg/ml). According to the definition of LC_{50}, the high LC_{50} value means it has less toxicity and low LC_{50} value means it has more toxicity that means, hydroalcoholic extract from *Abelmoschus esculentus* L contained more toxic compounds (low LC_{50} value means high toxic). It is probable that the highest toxicity shown in above both extract may be due to the presence of semi polar bioactive compounds. So we conclude that hydroalcoholic extract of *Abelmoschus esculentus* L Produces *in-vitro* anti-cancer activity. [12-15]

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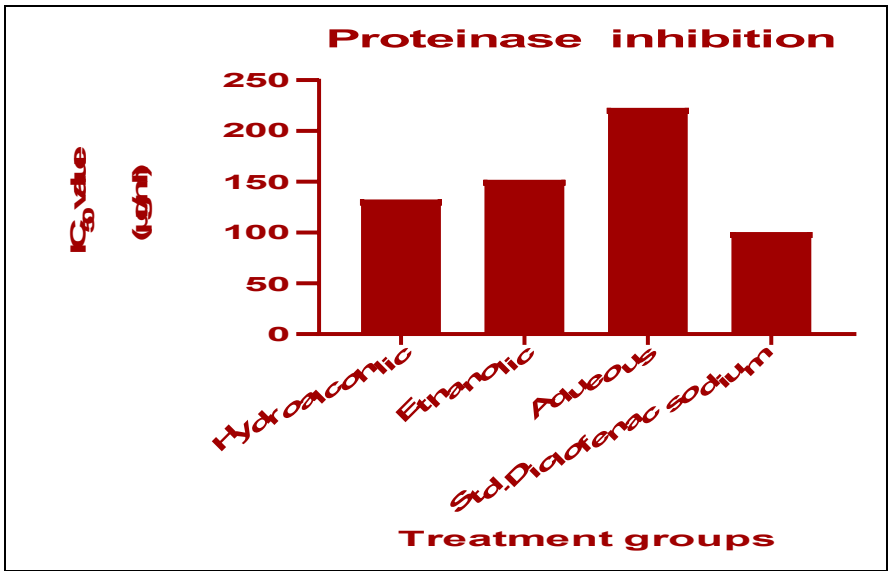

Figure no 4. Comparison of IC_{50} Value of hydroalcoholic, aqueous & ethanolic extracts of *Abelmoschus esculentus* L on Proteinase inhibition.

By Comparison of IC_{50} Value of hydroalcoholic, aqueous & ethanolic extracts of *Abelmoschus esculentus* L the results reveal that the Hydroalcoholic extract inhibits proteinase most when compared to the aqueous and ethanolic extract see Figure no 4.

IV. CONCLUSION

In the present work, phenol, flavonoid and condensed tannin contents of *Abelmoschus esculentus* L and their related biological activities are demonstrated for the first time in three different solvents. These plant extracts possess an interesting *in vitro* activities and have a very appreciable anti-inflammatory and anticancer activity suggesting it's potentially use as a source of natural anti-inflammatory and anticancer agent. The Hydroalcoholic exctract shown significant activity then Ethanolic then Aqueous extract Moreover, *Abelmoschus esculentus* L extracts were found to be active against carcinoma which support its ethno pharmacological use.

V. ACKNOWLEDGEMENTS

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