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EVALUATION OF *IN-VIVO* & *IN-VITRO* ANTI-INFLAMMATORY ACTIVITY OF ETHANOLIC LEAF EXTRACT OF *PIPER ATTENUATUM* B. HAM

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Keywords:

Piper attenuatum B. Ham, Leaves, Carrageenan, Egg albumin, Acetyl salicylic acid

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ABSTRACT: Inflammation is initiated as a healing process by the tissue in response to an injury by pathogens, irritants or cell damage. *Piper attenuatum* B. Ham is a rare piper species found in the tropical, sub-tropical region and mainly found in the southern part of India. A plant has constituents like alkaloids, amides, glycosides, tannins *etc*., which are responsible for its therapeutic efficacy. This research focuses on the use of leaves of *Piper attenuatum* B. Ham as anti-inflammatory agents. The phytochemical and analytical evaluation was done for ethanolic leaf extract. Various part of the plant is used for treating fever, malaria, diuretic, and cancer. Piperine is a major alkaloid found in almost all piper species & responsible for anti-inflammatory activity. In the present study, ethanolic leaf extract of *Piper attenuatum* B. Ham at doses 200mg/kg and 400mg/kg showed significant *in-vivo* anti-inflammatory activity in carrageenan-induced paw edema in rats and *in-vitro* anti-inflammatory activity by protein denaturation method for dose 500, 400, 300, 200 & 100 µg/mL.

INTRODUCTION: Inflammation is a normal protective response to tissue injury, and it involves a complex array of enzyme activation, mediator release, fluid extravasations, cell migration, tissue breakdown, an increase of protein denaturation and repair. It is a complex process that is frequently associated with pain [1]. The fundamental aim of inflammatory response is to localize and eliminate the harmful agents; secondarily, to remove damaged tissue components to culminate in healing the affected tissues, organs, or system.

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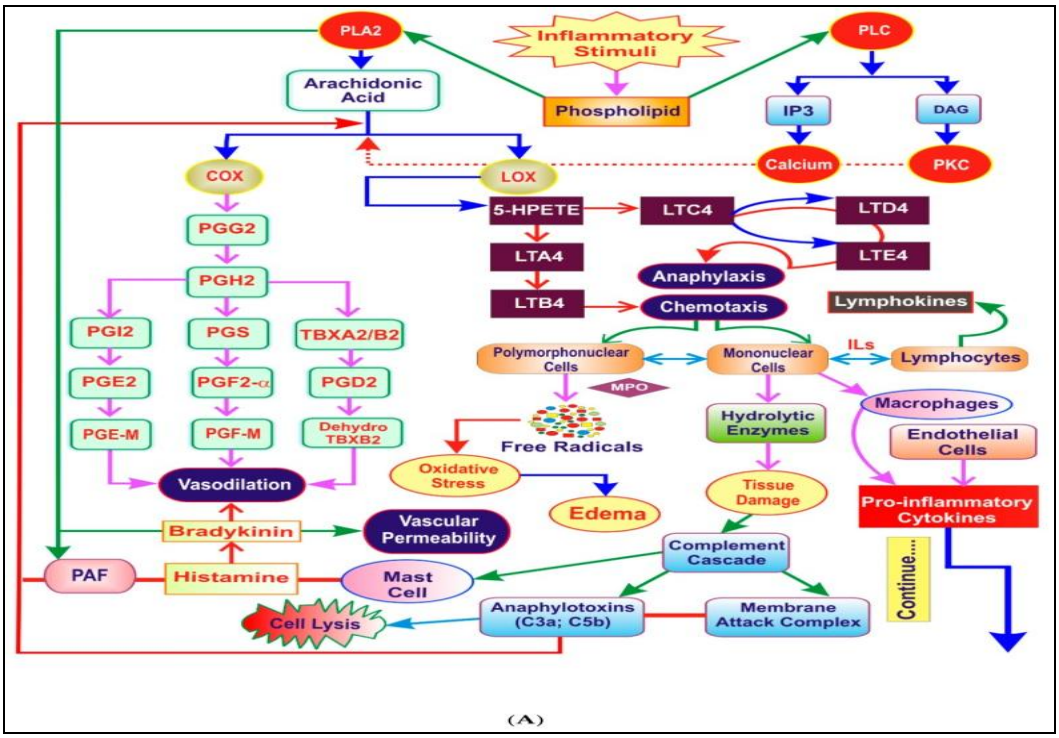
The inflammatory response involves macrophages and neutrophils known to secrete different mediators that are responsible for the initiation, progression, persistence, regulation, and eventual resolution of the acute state of inflammation [2]. Both the innate immune response and the adaptive immune response are involved in the formation of inflammation [3, 4, 5].

The innate immune system is the defense mechanism against invading microorganisms and cancer cells, involving the activity of various cells, including macrophages, mast cells, and dendritic cells. The adaptive immune systems involve the activity of more specialized cells such as B and T cells responsible for eradicating invading pathogens and cancer cells by producing specific receptors and antibodies [6].

The uncontrolled inflammatory response is the main cause of a vast continuum of disorders, including allergies, cardiovascular dysfunctions, metabolic syndrome, cancer and autoimmune diseases [7]. Non-steroidal anti-inflammatory drugs are most commonly prescribed for the treatment of pain and inflammatory conditions such as rheumatoid arthritis, osteoporosis, and Alzheimer's disease [8]. Unfortunately, besides the excellent anti-inflammatory potential of the NSAIDs, the severe side effects such as gastrointestinal ulceration, perforation, obstruction and bleeding has limited the therapeutic usage of NSAIDs [9]. Medicinal plants have long been recognised as important sources of therapeutically active compounds. Evidence-based research supports the medical and pharmacological benefits of plant-derived compounds, increasing interest in identifying and categorizing bioactive compounds from natural sources [8]. *Piper attenuatum* B. Ham belongs to the family Piperaceae is found in Vishakhapatnam, Madurai, and Tirunnelveli. Plant is used in the treatment of muscular pain, throat pain, headache and used as rubefacient [10]. *Piper attenuatum* B. Ham also having Hypolipidemic [11], anti-diabetic [12], anti-oxidant [13], anti-cancer [14], anti-trypsomonal [15], anti-ulcer [16], hepatoprotective [17], anti-pyretic [18] and antimicrobial activities [19]. *Piper attenuatum* B. Ham contain Alkaloids, Amides, Aristolactam, Cepharadione, Guineensine, Piperine, Piperlonguminine and Piperolactam [20]. Piperine was the first amide isolated from piper species. Piperine was reported that it display central nervous system depressant, analgesic and anti-inflammatory activities [20]. Various studies showed that Piperine has antimicrobial activity [21]. Piperine is helpful in reducing inflammation, improving digestion, and relieving pain and asthma. It is reported that Piperine increases serotonin production, which may help relieve stomach ulcerations. It improves the bioavailability of other nutritional substances, including beta carotene, curcumin, selenium, pyroxidine, glucose, and amino acids [22]. Piperine has anti-cancer property, it inhibits/suppresses the Wnt/β-catenin pathway & has anti-cancer effect on colorectal cancer cells [23]. Piperine is also used to treat Alzheimer's disease or other cognitive dysfunction disorder [24]. Piperlonguminine is also an alkaloid that is used to treat several disorders. Piperine and Piperlonguminine are responsible for the anti-diabetic activity [12]; they are also used against rotenone-induced neuronal injury [25]. Piperine, Piperlon guminine, and various analogues of Piperlonguminine inhibit prostaglandin and leukotriene biosynthesis *in-vitro* [26]. There is a massive need to explore new anti-inflammatory agents with selective action and lesser toxicity. Plants and isolated phytoconstituents are promising and interesting sources of new anti-inflammatories.



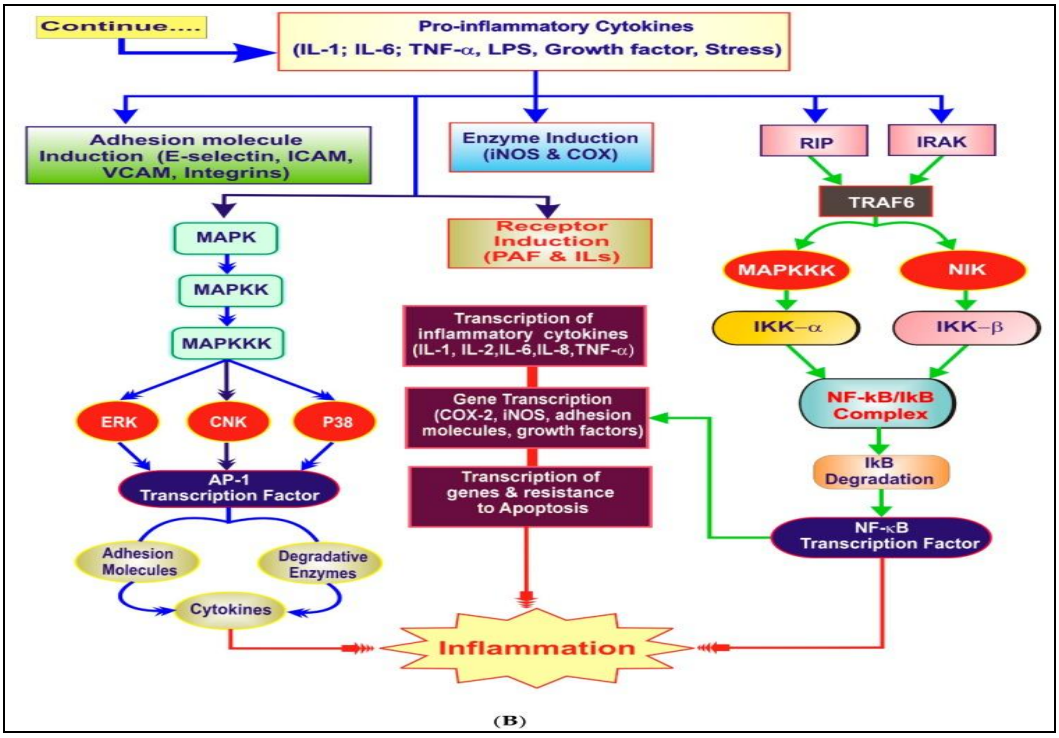


FIG. 1: THE INFLAMMATORY CASCADE. THE ARROWS IN THE FIGURE REPRESENT PROCESS [27]

MATERIAL AND METHODS:

Material: Leaves of plant *piper attenuatum* B.Ham was procured from Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI) Palode, Thiruvananthapuram, Kerala, India and authenticated.

The voucher specimens are submitted and preserved as Institute Herbarium Nos: APC/2021/PCOG/9-10. All the chemicals and drugs were procured from commercial sources.

Animals: Healthy Wistar albino male rats weighing between 150-200 gm were taken for the study. They were housed under controlled conditions of temperature (22±3 °C); the relative humidity should be at least 30% but not exceed 70% (other than during room cleaning) it was 55±5%. Lighting was artificial it was 12 h light and 12 h dark cycles according to OECD Guideline 423. Standard pellet diet and water are given to all animals. The present work was carried out with prior permission by IAEC of Alwar Pharmacy College with CSPSEA registration number 963/c/06/CPCSEA.

Methods:

Ethanolic Leaf Extraction of *Piper attenuatum* B. Ham: The leaf of *Piper attenuatum* B. Ham was shade dried and powdered. The total quantity of powdered material was about 300 gm. This powdered material was subjected to defat with Petroleum Ether for 72 h in a Soxhlet apparatus. Then after 72 h this defatted material is subjected to extraction with ethanol (99.99%) in a Soxhlet apparatus for 48 h. The extracts were concentrated to dryness under reduced pressure and controlled temperature (40-50°C) using a flash evaporator [12].

Phytochemical & Analytical Evaluation of Ethanolic Extract of *Piper attenuatum* B. Ham: Ethanolic extract of *Piper attenuatum* B. Ham was subjected to phytochemical evaluation for various phytoconstituents present in it using standard methods, while analytical evaluation is done by thin-layer chromatography, column chromatography, HPTLC, and mass spectroscopy.

***In-vitro* Anti-inflammatory Activity:** The reaction mixture (5 mL) consisted of 0.2 mL of egg albumin (from fresh hen's egg), 2.8 mL of phosphate-buffered saline (PBS, pH 6.4) and 2 mL of varying concentrations of ethanolic extract so that final concentrations become 100, 200, 300, 400, 500 µg/mL. A similar volume of double-distilled water served as control. Then the mixtures were incubated at (37 °C ± 2) in a BOD incubator for 15 minutes and then heated at 70 °C for 5 minutes. After cooling, their absorbance was measured at

660 nm (SHIMADZU, UV 1800) by using the vehicle as a blank. Acetylsalicylic acid at the final concentration of (100, 200, 300, 400, 500µg/mL) was used as a reference drug and treated similarly for determination of absorbance.

The percentage inhibition of protein denaturation was calculated by using the following formula.

$$\% \text{ Inhibition} = \text{Abs}_{\text{control}} - \text{Abs}_{\text{test}} / \text{Abs}_{\text{control}} \times 100$$ [28]

***In-vivo* Anti-inflammatory Activity:** Carrageenan was prepared as a 1% W/V solution in 0.9% saline no more than 24-h before use. The lambda form does not contain gel strongly at room temperature and is injectable to induce an inflammatory response. Anti-inflammation assay in inflamed rodents induced by carrageenan was done according to the procedure.

Procedure: Animals were weighed and randomized into 4 groups (n = 6). Before medication, the volume of the left paw of every animal was determined using a Plethysmometer. All of the animals were starved overnight before the experiment, and water used to be furnished ad libitum to ensure uniform hydration. Group, I served as control and did not receive any drugs. Group II bought the normal Indomethacin (10 mg/kg, p.o), and Group III and IV got crude extract in two different doses (200mg/kg, 400mg/kg p.o) accordingly. Thirty minutes following the subcutaneous injection of 0.1 ml of 1% w/v freshly ready λ-carrageenan solution into the plantar facet of the left hind paw. The paw was marked with ink on the stage of the lateral malleolus and immersed in the water reservoir of digital Plethysmometer as much as that mark to measure the paw volume.

The paw volume was measured at 0, 30, 60, 90, and 120 minutes immediately after carrageenan injection in the control and other treated group (V_t). The percentage of inhibition of each group is determined through the following formula.

$$\% \text{ Inhibition of Paw edema} = V_c - V_t / V_c \times 100$$

Where V_c and V_t represent average Paw volume of control and treatment animal respectively [29].

Statistical Analysis: Results interpretation was done after subjecting the data obtained from various studies. Statistical analysis was performed using Graph pad Prism 9.0.2 version, which included one-way ANOVA followed by a test like Dunnett and t-test. $P<0.05$ is considered statistically significant.

RESULT: Phytochemical evaluation of ethanolic leaf extract of plant *piper attenuatum* B. Ham shows the presence of alkaloids, steroids, flavonoids, tannins, and cardiac glycosides. Ethanolic extracts were soluble in ethanol. Analytical evaluation of ethanolic leaf extract of *piper attenuatum* B. Ham Isolation of ethanolic leaf extract of *Piper attenuatum* B. Ham by TLC.

TABLE 1: PHYTOCHEMICAL SCREENING OF PLANT *PIPER ATTENUATUM* B. HAM

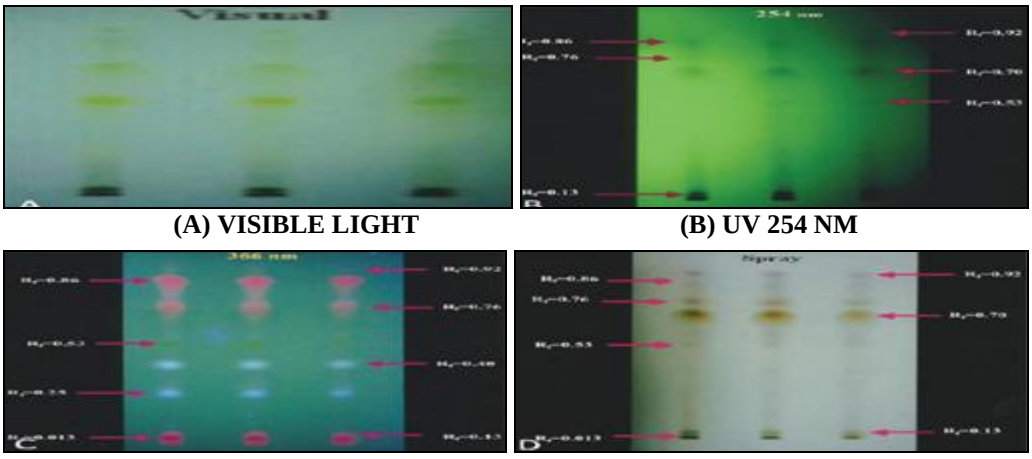
S. no.	Test	Observation	Ethanolic Extract	Confirmed Compound
1	Test for Alkaloids Hager's Test Mayer's	All tests were Positive	+	Alkaloid
2	Test for Steroids Salkowski reaction Liebermann-Burchard	All tests were Positive	+	Steroid
3	Test for flavonoids Ferric Chloride test Lead acetate test	All tests were Positive	+	Flavonoid
4	Test for Tannins Lead acetate test 5% Fe Cl_3 test	All tests were Positive	+	Tannin
5	Test for Cardiac Glycoside Legal's test Libermann test	All tests were Positive	+	Cardiac Glycoside
6	Solubility	Soluble	In ethanol	

TABLE 2: TLC OF ETHANOLIC LEAF EXTRACT OF *PIPER ATTENUATUM* B. HAM VISUALIZED UNDER VISIBLE LIGHT, UV – 254NM, UV – 366NM & VANILLIN – SULFURIC ACID SPRAY

S. no.	*Rf* value	Visual Light	UV-254 nm	UV-366 nm	Vanillin-sulfuric acid spray
1	0.92	Light yellow	Faint dark	Faint red	Violet spot
2	0.86	Dark Grey	Dark gray	Pinkish red	Light brown
3	0.76	----------	Faint dark	Red	Brown
4	0.70	Green	Dark gray	----------	Green

5	0.53	Yellow	Light dark	Green	Light green
6	0.40	----------	---------	Light blue	----------
7	0.25	----------	----------	Blue	----------
8	0.13	Dark Green	Dark gray	Pink	Green
9	0.013	----------	----------	Red	Light green

Isolation of Ethanolic Leaf Extract of *Piper attenuatum* B. Ham by TLC: To isolate and identify the bioactive compounds of the ethanolic leaf extract of *Piper attenuatum* B. Ham, TLC was initially performed. TLC of ethanolic leaf extract *Piper attenuatum* B. Ham revealed a maximum of 9 compounds in the order of decreasing Rf values, as shown in **Table 2**. The best resolutions were obtained when examined under UV light and after derivatization with the vanillin-sulfuric acid spray. Compounds with Rf values of 0.92, 0.86, 0.53, and 0.13 were visualized in all TLC chromatograms.



(A) VISIBLE LIGHT **(B) UV 254 NM**

(C) UV 366N **(D) VANILLIN - SULFURIC ACID SPRAY**

FIG. 2: TLC OF ETHANOLIC LEAF EXTRACT OF *PIPER ATTENUATUM* B. HAM

Isolation of Ethanolic Leaf Extract of *Piper attenuatum* B. Ham by Column Chromatography: Ethylacetate = EA, Methanol = Met EEPAa (S1) - Ethanolic extract *Piper attenuatum* B. Ham EEPAb (S2) - Ethanolic extract *Piper attenuatum* B.Ham EEPAc (S3) - Ethanolic extract *Piper attenuatum* B. Ham.

TABLE 3: DIFFERENT MAIN FRACTION OBTAINED FROM ETHANOLIC EXTRACT OF *PIPER ATTENUATUM* B. HAM BY COLUMN CHROMATOGRAPHY. N - HEXANE = NH

S. no.	Solvent Used	Ratio	Fraction Colour	Fraction Name	Selected Crystal for Phytochemical study
1	n – H : EA	50:0	Colourless	F_1	
2	n – H : EA	40:10	Light Orange	F_2	
3	n – H : EA	30:20	Light Green	F_3	
4	n – H : EA	20:30	Dark Green	F_4	
5	n – H : EA	10:40	Dark Blue	F_5	
6	n – H : EA	0:50	Yellow	F_6	EEPAa (S1)
7	EA : Met	40:10	Green	F_7	
8	EA : Met	30:20	Light Red	F_8	
9	EA : Met	20:30	Red	F_9	
10	EA : Met	10:40	Yellow	F_{10}	EEPAb (S2)
11	EA : Met	0:50	Green	F_{11}	
12	Met : nH	40:10	Dark Green	F_{12}	
13	Met : nH	30:20	Orange	F_{13}	EEPAc (S3)
14	Met : nH	20:30	Yellow	F_{14}	
15	Met : nH	10:40	Colourless	F_{15}	

Three fractions F_6 - EEPAa (S1), F_{10} – EEPAb (S2), F_{13} - EEPAc (S3) were selected for further phytochemical investigation.

Description of Isolated Fractions:

EEPAa (S1):

Fraction name: F_6

Colour: Yellowish White
Melting point: 125°C

EEPAb (S2):
Fraction name: F_{10}
Colour: Yellow
Melting point: 129 °C

EEPAc (S3):
Fraction name: F_{13}
Colour: Orange
Melting point: 130°C

Phytochemical analysis of isolated column fractions of *Piper attenuatum* B. Ham was carried out using the different chemical test. The Isolated fractions by Column chromatography on phytochemical investigation show the presence of alkaloids, tannins, glycosides, terpenoids, phenols and carbohydrates. All the three fractions are again subjected to TLC to find out the separation of a single compound and confirmation from the fraction using Ethylacetate and methanol (10:1) as solvent system.

TABLE 4: PHYTOCHEMICAL ANALYSIS OF ISOLATED COLUMN FRACTIONS OF *PIPER ATTENUATUM* B. HAM

Phytochemical Test	Fractions		
	EEPAa (S1)	EEPAb (S2)	EEPAc (S3)
Tannins test	+	+	+
Saponin test	-	-	-
Alkaloid test	+	+	+
Glycosides test	+	+	+
Cardiac glycosides test	+	+	+
Terpenoids test	+	+	+
Triterpenoids	+	+	+
Phenols	+	+	+
Carbohydrates	+	+	+

TABLE 5: TLC OF FRACTIONS OF *PIPER ATTENUATUM* B. HAM

S. no.	Fraction Used	Solvent Used	Ratio	Rf value range		
				366nm	254nm	Visible light
1	EEPAa (S1)	Ethylacetate & methanol	(10:1)	0.05 – 0.89	0.06 – 0.65	0.04 – 0.95
2	EEPAb (S2)	Ethylacetate & methanol	(10:1)	0.03 – 0.89	0.04 – 0.66	0.03 – 0.93
3	EEPAc (S3)	Ethylacetate & methanol	(10:1)	0.03 – 0.91	0.05 – 0.67	0.04 - 0.94

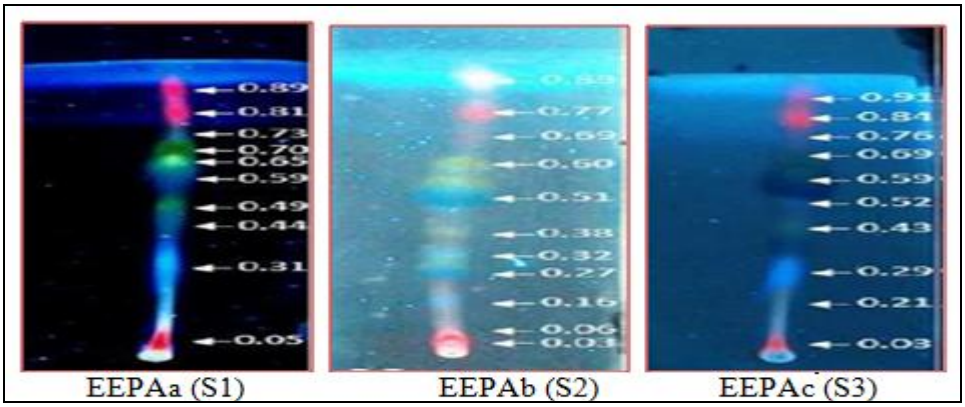


FIG. 3: TLC OF VARIOUS FRACTIONS OF ETHANOLIC EXTRACT OF *PIPER ATTENUATUM* B. HAM AT 366NM

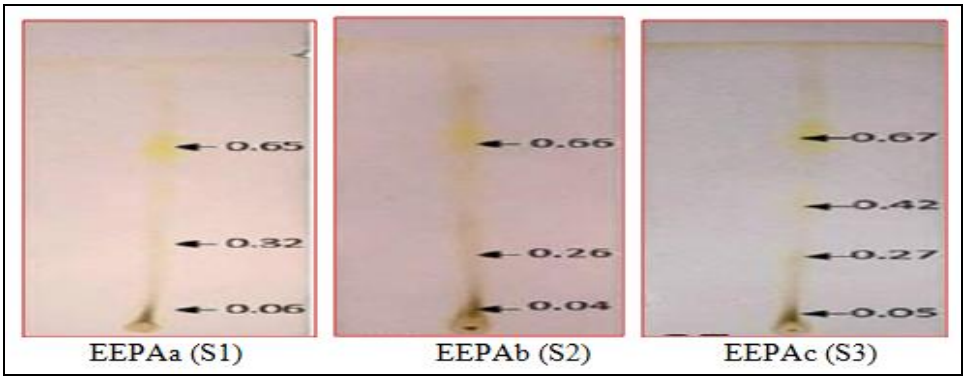


FIG. 4: TLC OF VARIOUS FRACTIONS OF ETHANOLIC EXTRACT OF *PIPER ATTENUATUM* B. HAM AT 254NM

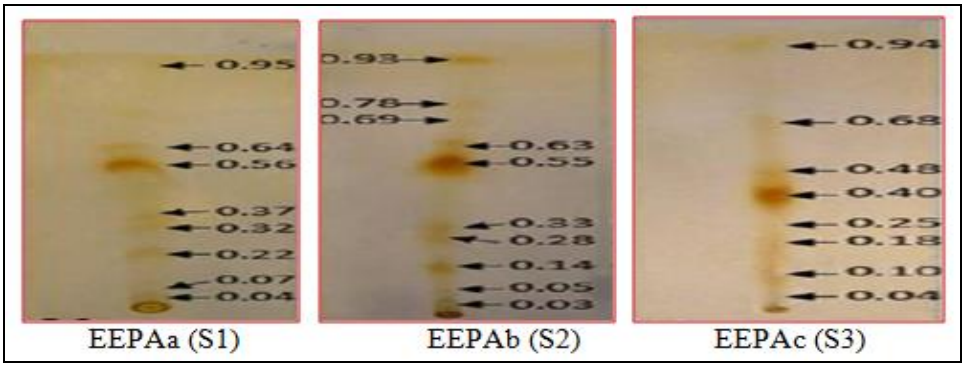


FIG. 5: TLC OF VARIOUS FRACTIONS OF ETHANOLIC EXTRACT OF *PIPER ATTENUATUM* B. HAM AT VISIBLE LIGHT

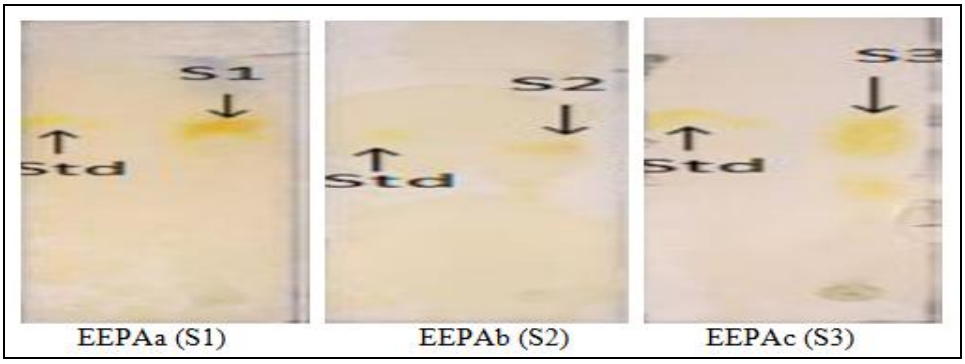


FIG. 6: TLC OF STANDARD PIPERINE COMPOUND & ISOLATED FRACTIONS FROM ETHANOLIC LEAF EXTRACT OF *PIPER ATTENUATUM* B. HAM, STD. – PIPERINE

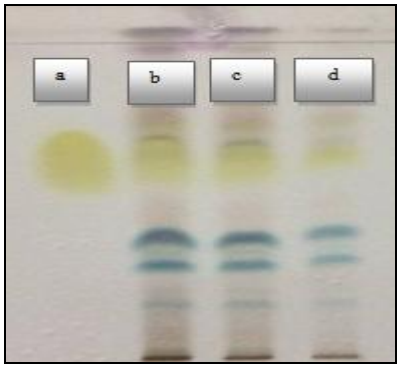


FIG. 7: A, B, C, D SPOT SHOWS A - STANDARD PIPERINE AND B, C, D ARE ETHANOLIC EXTRACT EEPAA (S1), EEPAB (S2) & EEPAC (S3)

Characterization of Bioactive Compounds from Ethanolic Leaf Extract of *Piper attenuatum* B. Ham by HPTLC: Three crystals EEPAa (S1), EEPAb (S2) and EEPAc (S3) obtained from column chromatography are subjected to HPTLC.

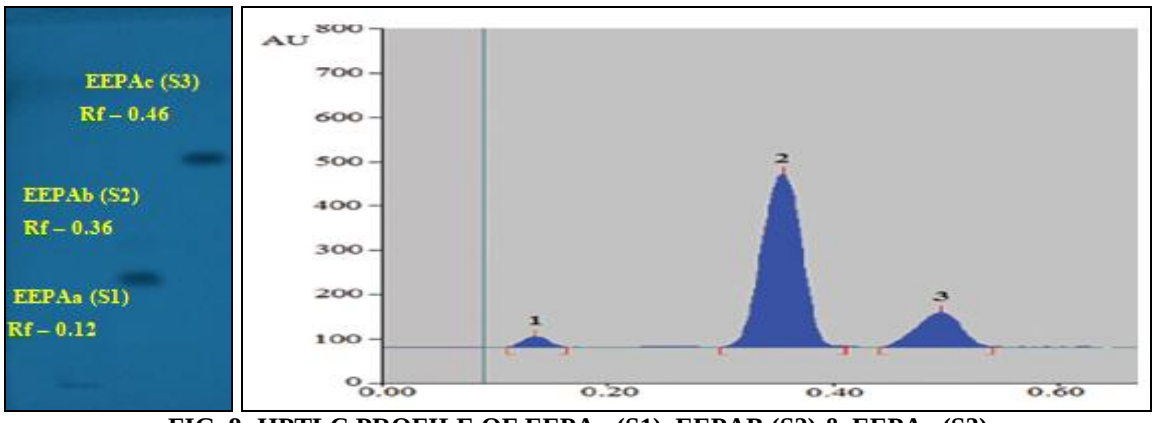


FIG. 8: HPTLC PROFILE OF EEPAa (S1), EEPAB (S2) & EEPAc (S3)

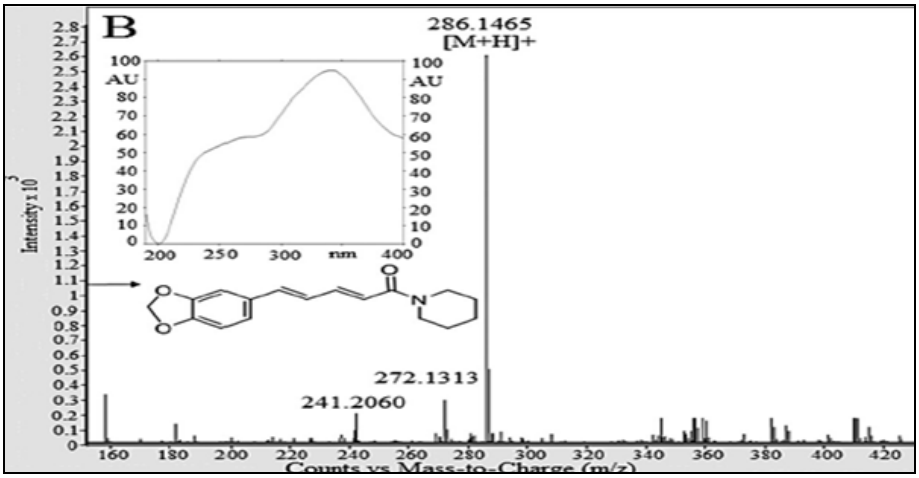


FIG. 9A: STRUCTURE ELUCIDATION OF EEPAb (S2) BY MASS SPECTROSCOPY

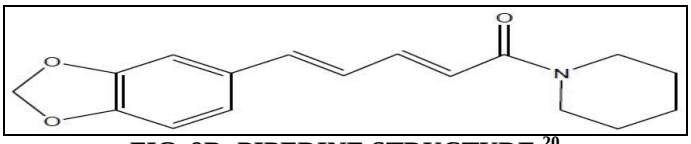


FIG. 9B: PIPERINE STRUCTURE [20]

In-vitro Anti-Inflammatory Activity of Ethanolic Leaf Extract of *Piper attenuatum* B. Ham

TABLE 6: *IN-VITRO* ANTI-INFLAMMATORY ACTIVITY OF ETHANOLIC LEAF EXTRACT OF *PIPER ATTENUATUM* B. HAM

Sample	Conc. (µg/ml)	Mean % Inhibition ± SD
Standard Acetyl Salicylic acid	500	88.06 ± 2.07***
	400	80.25 ± 1.34***
	300	77.15 ± 1.45***
	200	69.58 ± 0.83***
	100	50.56 ± 1.36**
Ethanolic extract of *Piper attenuatum* B. Ham	500	75.15 ± 2.91 ***
	400	63.43 ± 4.20***
	300	53.09 ± 2.60**
	200	36.78 ± 5.16**
	100	23.17 ± 3.29*

All values are Mean ± SEM, n = 5. One way Analysis of Variance (ANOVA) followed by Dennett's test was performed as the test of significance. The minimum value of $p < 0.05$ was considered significant. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ as compared with control group.

In-vivo Anti-Inflammatory Activity of Ethanolic Leaf Extract of *Piper Attenuatum* B. Ham

TABLE 7: *IN-VIVO* ANTI-INFLAMMATORY ACTIVITY OF ETHANOLIC LEAF EXTRACT OF *PIPER ATTENUATUM* B. HAM

	Paw Volume				
Groups	**0hr**	**30 min**	**60 min**	**90 min**	**120 min**
Control	4.08±0.059	4.24±0.093	4.44±0.037	4.01±0.056	4.07±0.067
Indomethacin (10mg/kg)	3.46±0.086	1.39±0.181***	1.00±0.099***	1.73±0.152***	1.88±0.110***
EEPA (200mg/kg)	4.01±0.042	2.12±0.067***	1.96±0.051***	2.33±0.107***	2.40±0.083***
EEPA (400mg/kg)	3.98±0.069	1.86±0.047***	1.58±0.042***	1.81±0.094***	1.89±0.070***

Data presented here as mean ± SD (n=6), the significance was tested using one way ANOVA, the result was very significant with P<0.001 each data was compared to control group.

TABLE 8: PERCENT OF INHIBITION OF INFLAMMATION, INDUCED BY CARRAGEENAN

	Paw volume reduction (%)				
Groups	**0 min**	**30 min**	**60 min**	**90 min**	**120 min**
Indomethacin (10mg/kg)	15.19	67.21	77.47	56.85	53.80
EEPA (200mg/kg)	1.71	50	55.85	41.89	41.03
EEPA (400mg/kg)	2.45	56.13	64.41	54.86	53.56

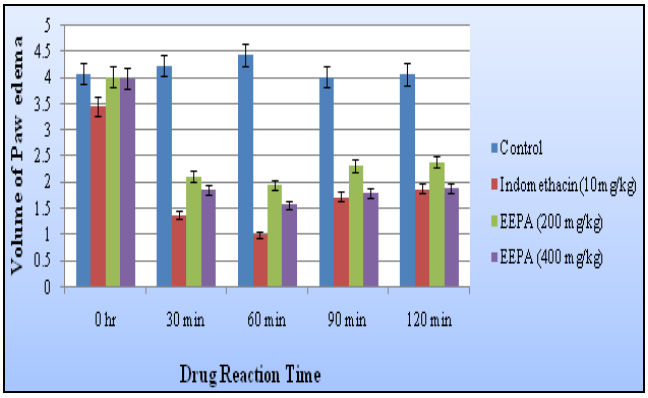


FIG. 10: *IN-VIVO* ANTI-INFLAMMATORY ACTIVITY OF ETHANOLIC LEAF EXTRACT OF *PIPER ATTENUATUM* B. HAM

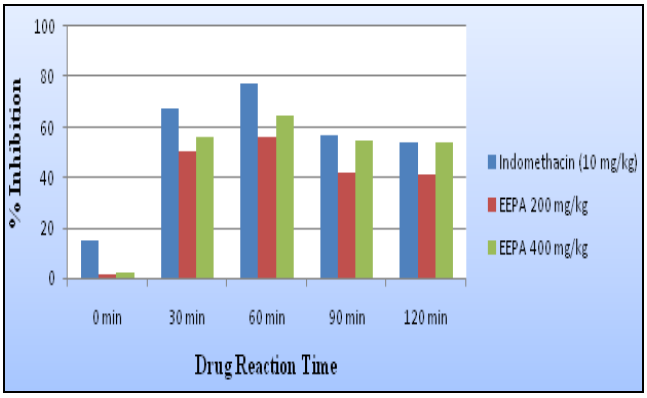


FIG. 11: PERCENT OF INHIBITION OF INFLAMMATION, INDUCED BY CARRAGEENAN

DISCUSSION: Inflammation is the immune system's response to harmful stimuli, such as pathogens, damaged cells, toxic compounds, or irradiation and acts by removing injurious stimuli and initiating the healing process. Inflammation is, therefore, a defense mechanism that is vital to health. However, uncontrolled acute inflammation may become chronic, contributing to various chronic inflammatory diseases.[30] Unhealthy lifestyles, including lack of physical activity, poor diet, stress, excessive tobacco and alcohol consumption, exposure to radiation, and infection with pathogenic microorganisms induce inflammation, leads to chronic diseases like Alzheimer's, arthritis, cancer, and cardiovascular disease (CVD), diabetes and Parkinson's [31]. Inflammation can be classified as either acute or chronic. Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes (especially granulocytes) from the blood into the injured tissues. A cascade of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue. Prolonged inflammation is known as chronic inflammation [32].

Inflammation represents a chain of organized, dynamic responses, including both cellular and vascular events with specific humoral secretions. A group of secreted mediators and other signaling molecules (*e.g.*, histamine, prostaglandins, leukotrienes, oxygen- and nitrogen-derived free radicals, and serotonin) are released by immune defense cells principally in the mechanism which can contribute in the event of inflammation [33].

In the present study, phytochemical evaluation is done using various tests that show various phytoconstituents in ethanolic leaf extract of plant *piper attenuatum* B. Ham given in **Table 1**. Various analytical methods like TLC, Coloumn chromatography, HPTLC and mass spectroscopy use for isolation, characterization and structure elucidation of bioactive compounds from ethanolic leaf extract. TLC of ethanolic leaf extract of *Piper attenuatum* B. Ham gives a maximum of 9 compounds which were shown in **Table 2** and **Fig. 2**. Compounds with *Rf* values of 0.92, 0.86, 0.53 and 0.13 were visualized in all TLC chromatograms. While isolation of ethanolic leaf extract of *Piper attenuatum* B. Ham done by column chromatography, we got 15 fractions given in **Table 3**; among these fractions, three fractions F6, F10 and F13 selected for further study. Phytochemical analysis of these three fractions shows the presence of various phytoconstituents given in **Table 4**. All these three fractions are again subjected to TLC to determine the separation of a single compound and confirmation from the fraction using Ethylacetate and methanol (10:1) as solvent system given in **Table 5** and **Fig. 3, 4, 5, 6**.

TLC of these three fractions against standard Piperine was done in **Fig. 7**. Three crystals, EEPAa (S1), EEPAb (S2) and EEPAc (S3) obtained from column chromatography are subjected to HPTLC for Characterization of Bioactive compounds, which show Rf value for EEPAa (S1), EEPAb (S2) and EEPAc (S3) were 0.12, 0.36 and 0.46 respectively shown in **Fig. 8**. Maximum peak compound EEPAb (S2) was selected for structure elucidation by using mass spectroscopy showing

the presence of Piperine alkaloid in EEPAb (S2) fraction of ethanolic leaf extract of *piper attenuatum* B. Ham **Fig. 9.** *In-vitro* and *in-vivo* anti-inflammatory activity was evaluated using standard acetylsalicylic acid and carrageenan-induced paw edema in rats. The method of anti-denaturation of egg albumin was chosen to evaluate the anti-inflammatory property of *piper attenuatum* B. Ham. In an anti-denaturation assay the denaturation of egg albumin is induced by heat treatment. The denatured protein expresses antigens associated with Type III hyper-sensitive reactions related to diseases such as serum sickness, glomerulonephritis *etc* [34]. Heat-denatured proteins are as effective as native proteins in provoking delayed hypersensitivity [35].

Moreover, it was already proved that conventional NSAID's do not act only by the inhibition of endogenous prostaglandins production by blocking COX enzyme but also by preventing of denaturation of proteins [36]. Thus anti-denaturation assay is a convenient method to check the *in-vitro* anti-inflammatory activity. *In-vitro* evaluation of anti-inflammatory activity (table 6) showed mean inhibition of protein denaturation of 75.15, 63.43, 53.09, 36.78 and 23.17% for doses of 500, 400, 300, 200 and 100 µg/mL, respectively, whereas, for standard acetylsalicylic acid it was found to be 88.06, 80.25, 77.15, 69.58 and 50.56% for same doses respectively.

The ethanolic extracts of *piper attenuatum* B. Ham showed good anti-inflammatory activity with a linear response. Maximum inhibition for ethanolic extract was observed at 75.15 ± 2.91% at 500 µg/mL and standard anti-inflammatory drug (Aspirin) showed the maximum inhibition, 88.06 ± 2.07% at the concentration of 500 µg/mL. *In-vivo* anti-inflammatory effects of the extract and standard drug are presented in **Table 7**. Carrageenan is a natural carbohydrate (polysaccharide) obtained from edible red seaweeds [37]. Carrageenan led to the time-dependent development of peripheral inflammation [38]. Carrageenan can induce acute inflammation beginning with the infiltration of phagocytes, the production of free radicals and the release of inflammatory mediators **Fig. 1.** Edema formation due to carrageenan in the rat is a biphasic event. The initial phase of edema is due to the release of histamine and serotonin, and the second phase of edema is due to the release of prostaglandins, protease, and lysosomal enzymes. Further, it has been demonstrated that the second phase is sensitive to the most clinically effective anti-inflammatory drugs. Free radicals, prostaglandin, and NO will be released when administrating with carrageenan for 1-6 h [39]. Piperine inhibits TNF-α, IL-6, IL-1β, and prostaglandin E2 production. Piperine was also found to inhibit IL-2, interferon-gamma, NO, and cyclooxygenase – 2 [40]. In control animals, the subplantar carrageenan injection produced local edema that increased progressively to reach a maximal intensity 60 minutes after injection **Fig. 10**.

The oral administration of both doses of the ethanolic leaf extract of *piper attenuatum* B. Ham significantly ($p < 0.001$) inhibited the inflammatory response induced by carrageenan in rats in a dose-related manner. The most prominent inhibition of 41.03% at 200 mg/kg and 53.56% at 400 mg/kg were observed at the 120 minutes of the study. The result was found to be highly statistically significant at 120 minutes after administration of the sample drugs ($p < 0.001$) **Table 7.**

CONCLUSION: Based on the present in the investigation, it can be concluded that phytochemical investigation showed that ethanolic leaf extract of *piper attenuatum* B. Ham has alkaloids, and mass spectroscopy confirm the presence of Piperine in fraction EEPAb (S2). *In-vivo* and *in-vitro* anti-inflammatory activity of ethanolic leaf extract of *piper attenuatum* B. Ham is due to Piperine alkaloid.

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