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ATTENUATION OF INFLAMMATION AND MEDIATORS' MODULATION BY *PIPER PEDICELLATUM* C. DC. LEAF CHLOROFORM EXTRACT IN GRANULOMA-INDUCED RATS

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ABSTRACT: Objective: The study aims to evaluate the granuloma and associated inflammatory mediators inhibition potential of the chloroform extract of *Piper pedicellatum* C. DC. Leaves, as plant leaves, are being used traditionally in the mitigation of various inflammatory conditions; however, there is an insufficiency of scientific evidence about the relevant bioactivity of the plant. **Method:** The cotton pellet induced-granuloma model in rats is used in the study, providing insights into subsequent phases of inflammation. *Piper pedicellatum* C. DC. Leaf chloroform extract at 200 mg/kg and 400 mg/kg and indomethacin (reference standard, 10 mg/kg) were used during treatment. **Results:** The chloroform extract at 200 mg/kg and 400 mg/kg dose inhibited exudate formation by 25.23% and 38.43%, respectively, and inhibition of granuloma tissue was found to be 27.62% and 40.33%, respectively. Further significant decrease in serum inflammatory markers CRP and PGE2 and reduced cytokine TNF-α and IL-6 were observed in chloroform extract and indomethacin-treated rats. In contrast, a substantial increase in anti-inflammatory cytokine serum IL-10 levels was noted in treated rats. **Conclusion:** The findings suggested the plant extract possesses anti-inflammatory properties which support the ethnomedicinal uses of the plant and a pharmacological stand for further investigations to develop novel and safe therapeutic modalities.

INTRODUCTION: Inflammation involves several mediators and may be described as the immune system's first defensive reaction. Protection against microbial invasions, antigen entry, and any harm or damage to cells and tissues is the primary objective[1].The early symptoms are redness, pain, heat, swelling, and impairment of function. The inflammatory processes are crucial for sustaining the body's homeostasis.

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They are characterized by a complex network of numerous mediators, a wide variety of cells, and the involvement of many pathways. Effective and well-regulated inflammatory response prompts the elimination of harmful stimuli and the restoration of physiology back to normal, the processes being precisely governed by complex molecular cascades [1, 2].

However, when inflammatory mediators are released for an extended duration and detrimental signal-transduction pathways are activated, the inflammatory process continues, and a chronic pro-inflammatory state develops[3]. Steroidal and nonsteroidal drugs comprise most of the currently available anti-inflammatory drugs. However, chronic use of these medications has been linked to

intolerable side effects, and also the available therapies are often not productive [2, 4, 5]. Therefore, new anti-inflammatory drugs with selective activity and lower toxicity are necessary from the clinical therapeutics standpoint. With the advent of the increasing number of synthetic medicines, each with its own set of side effects, it is about time that consideration is given to the remedies that can be found in native medicinal plants. This has increased the global quest to identify and harvest plants having significant therapeutic benefits while causing minor adverse effects [6].

The *Piper pedicellatum* C. DC. is a shrub belonging to the family of Piperaceae, thrives mostly in foothills regions and is found in Arunachal Pradesh, Assam, North Bengal & Sikkim states of India, and also in Bhutan, China, and Bangladesh. The plant, mainly its leaves, are used to treat various inflammatory diseases such as body aches, digestive system disorders, pyrexia, and abscesses [7, 8]. The presence of several groups of terpenes and phytosterols are reported in the plant, as well as antioxidant activities along with phenolic, flavonoid, and flavonol content in the different plant extracts [9, 10, 11]. Although, scientific study regarding the pharmacological action and data is unavailable to support the traditional claim. This detailed study was designed to investigate the anti-inflammatory potential and underlying mechanisms of the chloroform extract of the plant leaves.

MATERIALS AND METHODS:

Collection and Extraction of Plant Material: The *Piper pedicellatum* C. DC. Leaves were collected from the Papum Pare district of Arunachal Pradesh, India, in November 2016. The plant leaves were identified and authenticated by the taxonomist at the Department of Forestry, North Eastern Regional Institute of Science & Technology, Nirjuli, Arunachal Pradesh, India. A herbarium specimen (no. DU-Ph. Sc. 05) is preserved for future reference.

Extract Preparation: The collected *Piper pedicellatum* C. DC. Leaves were cleaned, air-dried, and ground to a coarse powder. Then successive solvent extraction was carried out with petroleum ether and chloroform, respectively, and further concentrated in a rotary evaporator. The extracts were stored at 4^{o}C until further use. In the study, the anti-inflammatory potential of a chloroform extract (PPLCE) was assessed in granuloma-induced rats.

Experimental Animals: In the present study, Adult Swiss albino mice (20-30gm) and Wistar rats (140-180gm) of either sex were used. The polypropylene cages were used to maintain the selected animals at a regulated temperature, humidity, and 12-hour light/dark cycle. The animals were acclimatized and provided with the rodent diet with access to water. The animals were kept fasted for 18 hr before experiments with access to water. All the experimental procedures conducted in the study were approved by Institutional Animal Ethical Committee (vide approval No. IAEC/DU/189) under the Department of Pharmaceutical Sciences, Dibrugarh University, Dibrugarh, Assam, India.

Acute Toxicity Study: The Organization for Economic Cooperation and Development (OECD) guideline 425 [12] was followed to conduct the oral acute toxicity study of PPLCE. Female Swiss Albino mice (n=5) were used in the study. A limit dose of PPLCE, 2000 mg/kg body weight, was orally administered to the animals. The animals were closely monitored for 24 hrs after the treatment for any toxic signs, changes in behaviour, and death and further observed for 14 days for any delayed toxicity signs and mortality. Until 14 days, no signs of toxicity or mortality were observed; thus, the LD_{50} and of PPLCE was considered more than 2000 mg/kg body weight.

Induction of Cotton Pellet-induced Granuloma: Five groups (n=6) of Wistar rats were used in the study. The rats were anaesthetized and aseptically implanted with two sterilized cotton pellets (10±1.0 mg each) bilaterally in the axilla region of the rats (except group I). Drugs/vehicle were administered once daily for the next seven days [13, 14] as follows:

Group I: (Normal control): receives vehicle *p.o.*

Group II: (Disease control): receives vehicle *p.o.*

Group III: receives standard drug indomethacin 10 mg/kg *p.o.*

Group IV: receives PPLCE 200 mg/kg *p.o.*

Group V: receives PPLCE 400 mg/kg *p.o.*

On day eight of implantation, rats were sacrificed by euthanasia, blood samples were collected, and serum was separated and used to estimate inflammatory markers and cytokines. Implanted pellets were dissected out, and extraneous tissues were removed. Wet weights (for exudate tissue) of the pellets were recorded and then dried at 60^{o}C to constant weight for the dry weight (for granuloma tissue). The percentage inhibition of exudates/granuloma tissue was calculated as:

$$(W_{tc}\text{-}W_t/\ W_{tc}) \times 100.$$

Where W_{tc} is the mean pellet weight of the disease control group, and W_t is the mean pellet weight of the treated group.

Estimations of serum C-reactive protein (CRP), Prostaglandin E2 (PGE2), Tumor necrosis factor-α (TNF-α), Interleukin-6 (IL-6), and Interleukin-10 (IL-10) were done by using ELISA kits as per manufacturer's instruction.

The following commercially available kits were used, Rat CRP ELISA kit (Sigma Aldrich, St. Louis, USA), Rat PGE2 ELISA kit (Cayman Chemical, MI, USA), Rat TNF-α ELISA kit (BD Biosciences, CA, USA) and Rat IL-6 and IL-10 ELISA kit (R to D Systems, Minneapolis, USA).

Statistical Analysis: Mean & SEM was used to describe the data. Data were analyzed in Graph pad Prism Software using one-way ANOVA followed by Dunnet's multiple comparison tests. The *p*-value < 0.05 was considered significant.

RESULTS:

Exudate and Granuloma: The cotton pellet granuloma model is used extensively to access inflammation's exudative and proliferative (granuloma) phases. The results are shown in **Table 1**.

The PPLCE (200mg/kg and 400mg/kg) and standard drug indomethacin (10 mg/kg) used in the study exhibited significant inhibitory activity on exudate and granuloma formation when compared with the disease control group.

At the doses of 200mg/kg and 400mg/kg, PPLCE inhibited exudate tissue formation by 25.23% (p<0.01) and 38.43% (p< 0.01), respectively and inhibition of granuloma tissue was found to be 27.62% (p< 0.05) and 40.33% (p< 0.01) respectively. The standard drug indomethacin (10mg/kg) showed 43.45% (p<0.01) and 46.19% (p<0.01) reduction in the weight of exudate and granuloma tissues, respectively.

TABLE 1: EFFECT OF PPLCE ON EXUDATES AND GRANULOMA FORMATION IN RATS

Group (mg)	Wt. of exudates (mg)	% inhibition (exudate)	Wt. of granuloma tissue (mg)	% inhibition (granuloma)
Normal Control				
Disease Control	176.37±6.32		63.46±5.16	
Indomethacin (10 mg/kg)	99.74±4.51**	43.45	34.15±3.65**	46.19
PPLCE (200 mg/Kg)	131.87±8.14**	25.23	45.93±4.12*	27.62
PPLCE (400 mg/Kg)	108.59±6.83**	38.43	37.87±4.71**	40.33

Values are expressed as mean±SEM (n=6). The results were analyzed using one-way ANOVA followed by Dunnet's multiple comparison test. *p< 0.05, **p< 0.01 indicate statistical significance compared to the disease control group.

Inflammatory Markers (Mediators) CRP and PGE2: The effect of PPLCE on inflammatory markers CRP and PGE2 were investigated in serum. As shown in **Fig. 1** and **Fig. 2**, most inflammatory markers CRP and PGE2 were released from rats after induction with cotton pellet granuloma as an inflammatory response. Treatment with PPLCE significantly decreased the serum CRP and PGE2 levels in a dose-dependent manner. PPLCE at the dose of 200mg/kg and 400mg/kg decreased CRP levels to 25.13±1.24µg/mL and 18.45±0.75µg/mL, respectively, as compared to 31.72±1.14µg/mL in the disease control group. PPLCE 200mg/kg and 400mg/kg dose inhibited PGE2 levels by 2.57±0.13 pg/mL and 2.18±0.14 pg/mL, respectively, as compared to 3.12±0.13 pg/mL in the disease control group. Indomethacin treated group shows12.34±0.65 µg/mL of CRP and 1.31±0.14 pg/mL PGE2 levels.

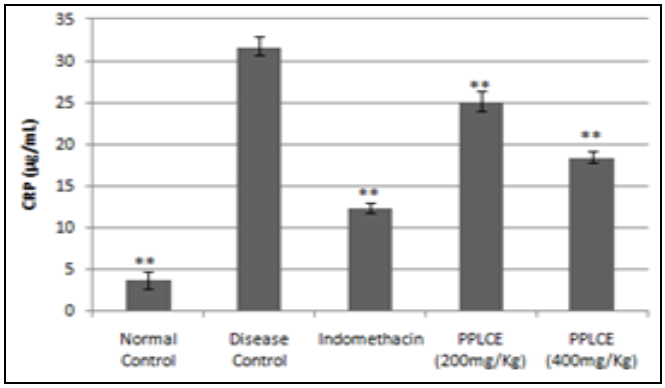

FIG. 1: EFFECT OF PPLCE ON CRP. Values are expressed as mean±SEM (n=6). ***p*<0.01 indicates statistical significance compared to the disease control group.

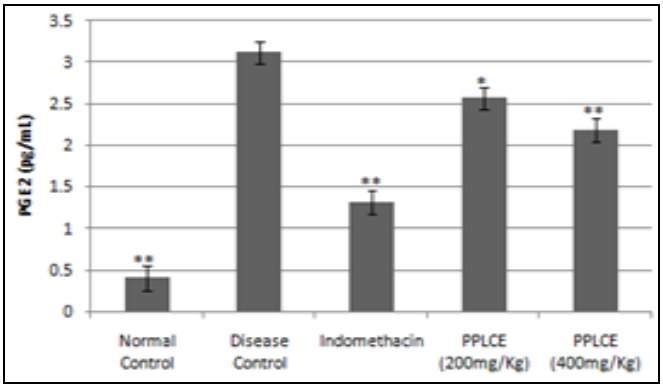

FIG. 2: EFFECT OF PPLCE ON PGE2. Values are expressed as mean±SEM (n=6). **p*<0.05,***p*<0.01 indicates statistical significance compared to the disease control group.

Pro-inflammatory Cytokines TNF-α and IL-6: After induction of inflammation, elevated levels of pro-inflammatory cytokines TNF-α and IL-6 were observed in rats. PPLCE treatment shows dose-dependent inhibition of serum TNF-α and IL-6 levels significantly against disease control rats, as shown in **Fig. 3** and **Fig. 4**. Serum TNF-α was lowered to 2.37±0.13pg/mL and 2.12±0.15pg/ ml by 200mg/kg and 400mg/kg PPLCE dose respectively. Also, serum IL-6 was decreased to 0.98±0.16pg/mL and 0.78±0.20pg/mL at 200mg/kg and 400mg/kg of PPLCE, respectively. Whereas indomethacin decreases serum TNF-α to 1.35±0.14pg/mL and IL-6 to 0.54±0.17pg/mL when compared to disease control group TNF-α (3.76±0.13pg/mL) and IL-6 (1.67±0.23pg/mL) levels.

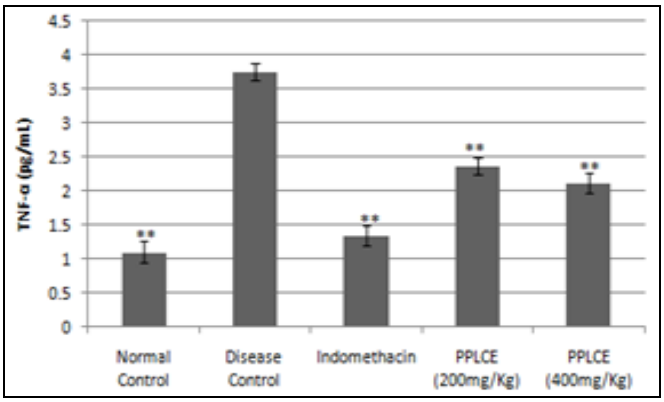

FIG. 3: EFFECT OF PPLCE ON TNF-α. Values are expressed as mean±SEM (n=6). ***p*<0.01 indicates statistical significance compared to the disease control group.

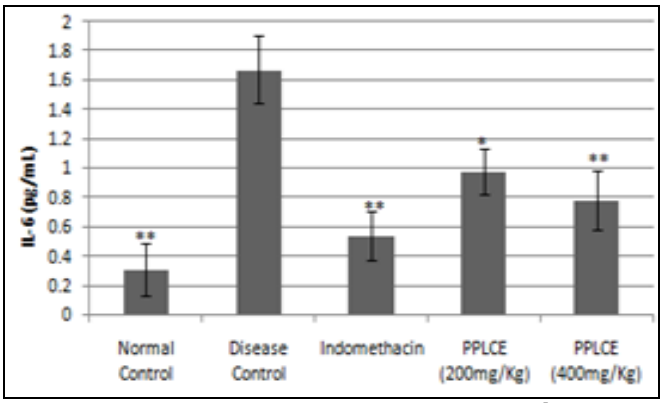

FIG. 4: EFFECT OF PPLCE ON IL-6. Values are expressed as mean±SEM (n=6). **p*< 0.05, ***p*<0.01 indicates statistical significance compared to the disease control group.

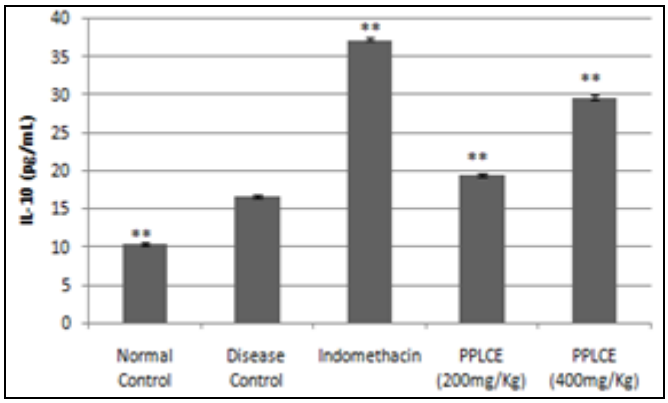

FIG. 5: EFFECT OF PPLCE ON IL-10. Values are expressed as mean±SEM (n=6). ***p*<0.01 indicates statistical significance compared to the disease control group.

Anti-inflammatory Cytokine IL-10: The rats treated with PPLCE and indomethacin displayed a marked escalation of serum IL-10 levels significantly, which is indicative of the healing phase as shown in **Fig. 5**. PPLCE at 200 mg/kg and 400 mg/kg dose-dependently augment serum IL-10 to 19.37±0.26 pg/mL and 29.67±0.24 pg/mL respectively, and indomethacin to 37.23±0.22 pg/mL in contrast to 16.64±0.25 pg/mL of the disease control group.

DISCUSSION: The cotton pellet-induced granuloma model mimics the primary features of chronic inflammation, such as monocyte infiltration, fibroblast proliferation, angiogenesis, and exudation, which manifested into three phases of inflammation and granuloma formation is the characteristic trait of this model [15, 16]. Perfusion of low protein fluid in the site of inflammation indicates the first transudative phase of the inflammation. The second exudative phase occurs after two to three days and is displayed by the exudation of protein-containing fluid. Finally, the third proliferative phase begins after about three days and is mainly characterized by the appearance of mucopolysaccharides and collagen and the multiplication of fibroblasts [17, 18]. In the study, the PPLCE treatment decreases the exudates and granuloma weights, which exhibits its ability to reduce both the exudative and proliferative phases of inflammation and reflects the potential to limit fibrosis and collagen synthesis.

The inflammatory pathways encompass a complicated network of interrelated positive and negative feedback systems involving various cytokines, inflammatory markers, and mediators that either increase or decrease inflammatory manifestations and symptoms [19]. CRP, a well-established clinical marker of inflammation, is an acute phase protein, the serum level raised during inflammation, tissue lesion, and infections. Inflammatory cytokines like IL-1 and IL-6 play a pivotal role in the modulation of the CRP gene [20, 21]. During inflammation, the degradation of cellular membrane phospholipids generates arachidonic acid, which is further converted to PGE2 by the actions of COXs and PG synthetases. PGE2 acts as a primary inflammatory lipid mediator, which causes vasodilation, migration of leukocytes, and fluid leakage, promoting oedema and soreness. Anti-inflammatory agents inhibit COX activities and lower PGE2 levels [22, 23]. TNF-α, IL-1, IL-6, and other pro-inflammatory cytokines are released more often throughout the inflammation process, which provokes the inflammatory conditions [24, 25]. TNF-α and IL-6, in particular, play critical roles in the inflammation progression by inducing the production of other cytokines, chemokines, and pro-inflammatory mediators such as PGE2 and CRP and fever [26, 27]. Inhibition of TNF-α prevents the release of IL-1, IL-6, and IL-8, which further suppresses COX-2 and PGE2 levels and limits inflammatory manifestations [28]. In contrast, IL-10 exhibits broad anti-inflammatory effects by down-regulating expressions of other pro-inflammatory cytokines and chemokines by targeting diverse cell types like neutrophils and monocytes/macrophages [29, 30].

Therefore, to limit inflammatory processes and their morbidities, the downregulation of pro-inflammatory cytokines and mediators and elevating anti-inflammatory cytokines like IL-10 are effective treatment targets. The results of the current study with the cotton pellet-induced granuloma model have shown that the anti-inflammatory activity of PPLCE is similar to that of NSAID Indomethacin due to modulation of several inflammatory mediators and cytokines like inhibition of CRP, PGE2 and TNF-α, IL-6 release while augmenting IL-10 levels in serum.

CONCLUSION: The study's findings showed that the PPLCE had potent anti-inflammatory properties in the experimental model investigated, and it also provides an understanding of the mechanism of its action. The ability of PPLCE to inhibit and control a variety of inflammatory mediators and cytokine pathways contributes to its anti-inflammatory activity. The study also offers a rationale from a scientific standpoint for the traditional use of plant leaves in inflammatory conditions. However, additional in-depth investigations are needed to further explore the plant's medicinal effects.

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CONFLICTS OF INTEREST: The authors declare no conflict of interest.

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