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# Cassava starch film loaded with extract of *Piper betel* leaf for anti-inflammatory activity

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The aim of the present research work was to formulate a transdermal film of *Piper betel* L. leaf extract along with diclofenac sodium and evaluate its anti-inflammatory activity. The ethanolic extract of *P. betel* leaf was obtained by solvent extraction method using a Soxhlet apparatus. The *P. betel* leaf extract was analysed for inhibition of the albumin denaturation and it showed a significant inhibition of the albumin denaturation. The topical film was then prepared by solvent casting method using diclofenac sodium and *P. betel* leaf extract. The film was evaluated for drug content, thickness, weight uniformity, folding endurance, moisture content, moisture uptake, and barrier properties of the film. The formulated film was thin, flexible, and possessed satisfactory physicochemical properties. The anti-inflammatory potential of the film was evaluated by using carrageenan-induced rat paw oedema method on albino Wistar rats. The anti-inflammatory activity of film was found comparable with standard diclofenac sodium film and exhibited inhibition of oedema within 4 h. This signifies that the combination of *P. betel* leaf extract and diclofenac sodium has remarkable potential to serve as synergistic topical anti-inflammatory film preparation than simple topical film formulation of diclofenac sodium.

**Keywords:** Anti-inflammatory activity, Cassava starch, Diclofenac sodium, *Piper betel*, Transdermal film.

**IPC code; Int. cl. (2021.01)-** A61K 36/00, A61K 127/00, A61P 29/00

## Introduction

Drug delivery by the topical route is an emerging and convenient method. The topical route has variety of advantages compared to oral route such as avoidance of first pass metabolism, ease of administration, convenient, helps to achieve control and sustained release of drug in plasma and better patient compliance[1]. The topical application of the agent generally refers to delivery of the drug through the skin either for systemic or for local action. The transdermal delivery of drug in the form of film provides the advantage of avoiding dose dumping and in case, if any toxicity develops, it allows the patient to terminate the drug therapy by simply removing the film[1,2]. In response to various stimuli such as pathogens, damaged cells and irritants, body initiates its defence mechanism called as inflammation. There is an increased blood flow to the area of injury or infection due to the release of chemicals and results in pain, redness, and swelling in the affected area. In response to injury or infection, the immune system triggers this physical reaction. The inflammatory response is provoked by the production of mediators and chemotactic factors[3-5]. Non-steroidal anti-inflammatory drugs (NSAIDs) are generally preferred for the treatment of inflammation. The oral dosage form of the NSAID undergoes hepatic first-pass metabolism and only half of the administered dose reaches the systemic circulation[6-8]. On prolonged usages it causes ulcer, increases gastric irritation, nausea, and vomiting. NSAIDs have severe side effects such as delay in muscle regeneration, ulceration, haemorrhage, tissue damage, etc. and on prolonged use cause gastrointestinal bleeding, heart attack, stroke, high blood pressure, and kidney damage[8]. Due to these, disadvantages, there is a need to search for an alternative to overcome the problems associated with the use of NSAIDS[9-11]. Nowadays, interest is increasing day by day towards natural remedies with a basic approach towards nature. The dependency on the use of herbal or traditional medicine has increased from all over the world with approximately 80% of the world population inclined

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towards the use of herbal medicine to cater to their primary health care needs.

*P. betel* L. is cultivated in India, Sri Lanka, Malaysia, Indonesia, and East Africa. Its extracts are reported to cure urinary tract infections, cervicitis, vaginitis, gastrointestinal disorders, skin infections, etc[12,13]. Currently, there are abundant scientific findings reporting the beneficial effects of betel leaves including antioxidant, anti-carcinogenic, anti-inflammatory, antibacterial, antifungal, and anti-diabetic activities[12-17].

Cassava starch is a polysaccharide and is considered as one of the most promising natural biopolymers. The films developed from Cassava starch are easily biodegradable, tasteless, odourless, colourless, and non-toxic[18-21].

Considering the anti-inflammatory properties of *P. betel* leaf extracts, an attempt was made to develop a transdermal film of *P. betel* leaf extract and diclofenac sodium. As per the literature review, this is the first work reported to combine the extract of *P. Betel* and the synthetic drug diclofenac sodium with an intention that this combination would reduce the dose of the diclofenac sodium, which ultimately could help to reduce the side effects associated with the use of NSAIDS (diclofenac sodium) for a longer duration of time along with the benefits of topical route. The research work of combining *P. betel* leaf extract and diclofenac sodium is based on the hypothesis that, if the individual constituent exerts similar effect, then their combination will sometimes exhibit enhanced effect. The synergistic effect achieved could allow the use of lower doses of the active pharmaceutical ingredient and help to reduce adverse effects related to their prolonged use[22].

## Materials and Methods

Cassava starch was obtained from Shree Ram Bio Starch Polymers Private Limited, Vadodara, India. All other chemicals used in project work were of analytical grade.

### Plant collection and identification

*P. Betel* leaves were collected in the month of July 2018. The identification was done in the Department of Botany, SGPKM, Girad, Wardha, where a voucher specimen (ATNPIPERB-04) was deposited. The leaves, stems, and roots were separated and cut into small pieces. They were dried in an oven at 40 °C to constant weight. The drying time and percentage of water loss were determined in the process.

### Preparation of *Betel* leaf extract

The leaves of *P. betel* were washed with distilled water, dried in shade and crushed into a fine powder. The dried powder was extracted using ethanol (1:15) using a Soxhlet apparatus for 24 h at 60 °C. The extract was dried in an oven for 3 days at 40 °C until the entire solvent was evaporated until the residue was left. The dried extract was weighed, recorded and stored in a tightly closed container[15,21].

### Phytochemical screening of plant extract

To obtain ethanolic extract of *P. betel*, few drops of different reagents were added in an individual test tube such as 5% ferric chloride solution, lead acetate solution, bromine water, potassium dichromate, dilute iodine solution, dilute nitric to determine the phenolic group present in the plant extract[14].

### Inhibition of albumin denaturation

To investigate the anti-inflammatory activity of the *P. betel* leaf extract, a method suggested by Sakat *et al.* was followed with minor modifications[23]. The ethanolic extracts of *P. betel* leaf (sample) and diclofenac sodium (control) at different concentration ranging from 10-80 µg/mL were mixed with 1% aqueous solution of bovine albumin. To this mixture, a small amount of 1N hydrochloric acid solution was added to adjust the pH. The samples were then kept in the incubator for 20 min at 37 °C. Thereafter, the samples were heated to 57 °C for 20 min. After cooling, the turbidity produced was determined spectrophotometrically at 660 nm. The percent inhibition of protein denaturation was then calculated using the following equation:

$$\text{Percentage inhibition of protein denaturation (\%)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \text{ X } 100\%$$

### Fourier transform infrared spectroscopy

The FTIR spectra of *P. betel* leaf extract, diclofenac sodium, and the formulated medicated film of (*P. betel* leaf extract + diclofenac sodium) were recorded using FTIR spectrophotometer (Shimadzu) using the single reflection horizontal attenuated total reflectance (ATR) to determine drug excipient compatibility. The FTIR spectrum was recorded from 4000 to 390 $cm^{-1}$(Ref. 24,25) (Fig. 1a-d).

### Film preparation

Initially, Cassava starch 4 g was added into distilled water and was boiled at 90 °C to carry out the gelatinization of starch. While boiling, the solution

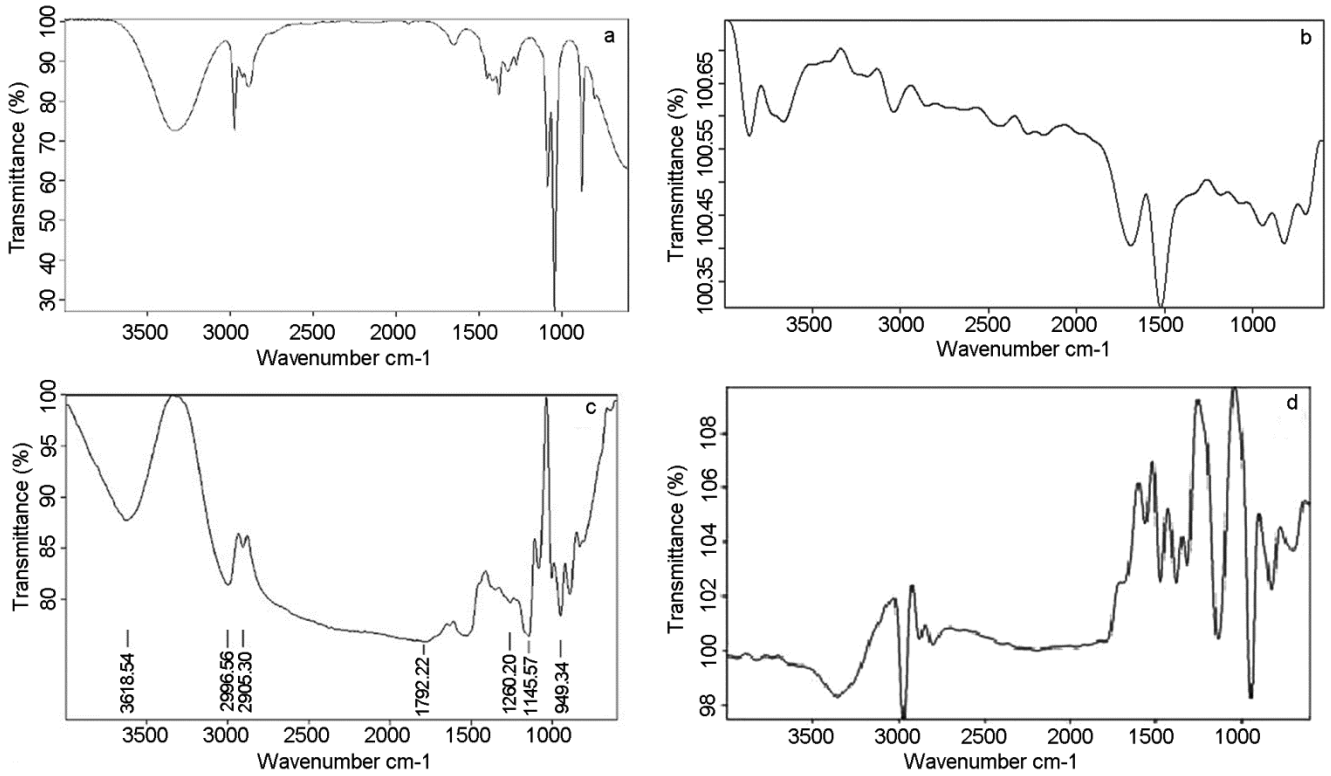


Fig. 1 — FTIR Spectrum, a) *Piper betel* leaf, b) Diclofenac sodium, c) Cassava starch, d) Test film (*Piper betel* leaf extract and Diclofenac sodium).

was stirred continuously until homogeneous. Thereafter, glycerine 2 mL was added into that solution. This mixture was cooled at 40-45 °C. Then, the ethanolic extract of *P. betel* leaf (40 mg) and diclofenac sodium (100 mg) was added into this mixture. The mixture was then transferred into the Petri plate and kept in the oven for drying for 24 h. After drying, the film formed in Petri plate was peeled off carefully[26].

**Physicochemical characterization of the film**

The formulated film was evaluated for the following physicochemical characterization methods

**Thickness**

Digital micrometre screw gauge was used to measure the thickness of the film. The thickness was measured at three different places of film and the mean value was calculated[21].

**Weight variation**

The weight variation was studied individually for each film[26].

**Folding endurance**

The numbers of times the film could be folded at the same place without breaking denotes folding endurance. The film was repeatedly folded at the same place until it broke.

**Drug content**

To measure the drug content, 2×2 $cm^2$ size of the film was kept in a phosphate buffer pH 5.5 and shaken continuously for 24 h. The solution was ultrasonicated for 15 min. After filtration, the drug (diclofenac sodium) content was estimated spectrophotometrically at $\lambda_{max}$ of 278 nm.

**Moisture uptake**

The film was weighed and kept in a desiccator at room temperature for 24 h. The film was then exposed to 84% relative humidity (a saturated solution of aluminium chloride) and was weighed again. The percentage of moisture uptake was calculated as the difference between final and initial weight with respect to initial weight as shown in following equation:n

$$\text{Moisture uptake}(\%) = \frac{\text{W final} - \text{W initial}}{\text{W initial}} \text{ X } 100$$

**Moisture content**

The films were weighed individually and dried in an oven at 105 °C for 24 h. After removal of the film

from the oven, the films were weighed again and their weights were recorded. The percentage of moisture content was calculated as the difference between initial and final weight with respect to final weight as shown in following equation:

$$\text{Moisture content (\%)} = \frac{\text{W initial} - \text{W final}}{\text{W final}} \text{ X } 100$$

**Barrier properties of the film**

Sterile nutrient broth solution about 10 mL was poured into two clean test tubes. Cassava starch film (2 $cm^2$) loaded with *P. betel* leaf and diclofenac sodium extract was applied to one test tube, while the other test tube was kept uncovered. Both the test tubes were kept aside for 24 h in the incubator and the growth of microorganisms was observed after 24 h[18].

***In vivo* studies**

Healthy male Albino Wistar rats weighing 200-250 g were used in the study. The rats were maintained in the animal house of JSPM's Rajarshi Shahu College of Pharmacy and Research, with controlled temperature, 25±1 °C for 10-12 h light-dark cycle at 23 °C and provided with water and food. The experimental protocol was approved by IAEC (Institutional Animal Ethics Committee), JSPM's Rajarshi Shahu College of Pharmacy and Research, Tathawade, Pune protocol number RSCPR/IAEC/2019/05. Laboratory animal handling and the research protocol was performed in accordance with CPCSEA guideline[27-29].

**Carrageenan–induced rat paw oedema**

The modified method of Winter *et al.* 1962; Otterness and Oore, 1988 was used in the present study to develop carrageenan–induced rat paw oedema[30-32]. Twenty four male Wistar rats of 180-200 g were used and divided into four groups, each group consisting of six rats ($n$=6). Group I served as normal control group and received no treatment. Groups II served as the positive control received carrageenan injection (0.1 mL, 1% w/v in normal saline). Group III received transdermal film (containing 40 mg of *P. betel* leaf extract and 100 mg of diclofenac sodium) and served as treatment group. Group IV received diclofenac sodium patch (containing 200 mg of diclofenac sodium) and served as a standard group. Oedema was induced by injecting freshly prepared carrageenan (0.1 mL, 1% w/v in normal saline) into the sub-planter tissue of each right hind paw in all groups animal except normal control group. The paw circumference was measured before and at 1, 2, 3, and 4 h after carrageenan injection using screw gauze method. Both standard and test patch were applied on sub plantar tissue of right hind paw and the result of both were observed and compared with the positive control group. The percentage value of oedema inhibition was calculated by the following formula[23]:

$$\text{Percentage inhibition (\%)} = 1 - \frac{y-x}{b-a} \text{X}100$$

where, x=Initial paw thickness of test group animals, y=Paw thickness of test group animal after treatment, a=Initial paw thickness of control group animal, b=Paw thickness of control group animal after treatment.

**Haematological parameters**

After 4 h to carrageenan induction and after application of formulation film and standard film, blood samples were collected by retro-orbital route in heparin tube and submitted to a pathological laboratory in Pune. Haematological parameter namely white blood cells (W.B.C.) count was analyzed[29].

**Statistical analysis**

The Graph pad prism version 5 (Graph Pad Software Inc., La Jolla, CA) was used for statistical analyses which were presented as mean±SEM. The data were statistically analysed by two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test, where $P$ <0.01 was considered as statistically significant.

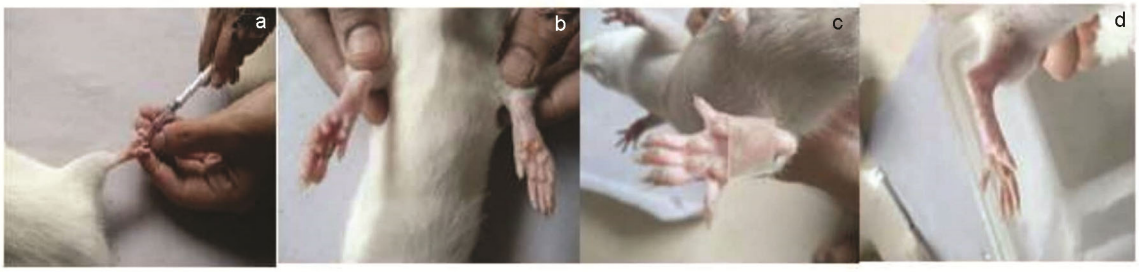


Fig. 2 — a) Injection of 1% carrageenan solution, b) Observation after injecting carrageenan solution, c) Application of test film, d) Removal of test film after 6 h.

## Results and Discussion

### Phytochemical analysis

In the present study, the preliminary phytochemical analysis of the ethanolic extract of *P. betel* leaf was carried out to detect the presence of the active constituent phenol. On addition of 5% ferric chloride solution into the extract, the colour of the solution changed from green to deep blue, lead acetate solution changed the colour of the extract from green and formed white precipitate. Bromine water caused the decolouration of the test solution; potassium dichromate changed the colour of the extract from green to red; on addition of nitric acid into the extract, the colour changed from green to reddish yellow, thus indicating the presence of phenol in the extract.

### Inhibition of albumin denaturation

The ethanolic extract of *P. betel* at a dose of 40 µg/mL exhibited an anti-inflammatory activity as presented in Table 1. Protein denaturation is an indicator of inflammatory disease. The agents that can prevent protein denaturation can act as an anti-inflammatory agent. The anti-inflammatory effect of *P. betel* leaf extract was highest at a concentration of 40 µg/mL.

### Thickness and weight uniformity, drug content

All the films were found to be uniform in thickness, homogeneous with smooth surface without any sign of cracking. The average thickness of all formulated films were observed to be in the range of 0.1 mm, the weight of the film was in the range of 2.145-2.366 mg and the drug content was found in between 91.24-98.64%.

### Folding endurance, tensile strength

The films exhibited good flexibility with sufficient mechanical properties. The result indicated that the film would not break and maintain their integrity when applied on the skin. Without cracks, the folding endurance value of the film was 130 when folded at the same place, thus indicating good folding endurance.

Table 1 — Percent Inhibition of albumin denaturation

| Concentration (µg/mL) | Inhibition of albumin denaturation (%) |
|---|---|
| 10 | 14.2±0.3 |
| 20 | 30±0.5 |
| 30 | 30.43±0.2 |
| 40 | 50±0.6 |
| 50 | 38±0.1 |
| 60 | 35±0.6 |
| 70 | 40±0.8 |
| 80 | 38.46±0.1 |

### Moisture uptake

All the film showed an increase in the moisture uptake with the time, with increase in the concentration of *P. betel* leaf extract, the percentage of moisture uptake also increased[21]. This could be because of the presence of the phenolic group in the extract.

### Moisture content

The moisture content of the prepared formulation was low, which helps in maintaining its stability throughout long-term storage at dry condition, thereby preventing the film from drying and becoming brittle.

### Barrier property of the film

No turbidity was observed in the test tube sealed with the film which indicated that the film acted as a barrier with antimicrobial properties preventing the entry of micro-organism into the nutrient broth, whereas the test tube which was uncovered exhibited turbidity due to the growth of micro-organism.

### Anti-inflammatory activity

Carrageenan-induced rat paw oedema method is the most widely used and most accepted model for inflammation. Carrageenan induces inflammation by releasing different inflammatory mediators such as histamine, serotonin, and bradykinin. In the present study, carrageenan was used to induce inflammation in all the groups except normal control Group I. Carrageenan was injected in the sub-plantar region of paw circumference of rats in Group II, Group III, and Group IV[28-30]. Each group was observed 4 h after the injection of carrageenan. Also, the effect of carrageenan on the rat paw oedema was observed after the application of the standard marketed film of diclofenac sodium and the test film (*P. Betel* extract and diclofenac sodium). The result for the anti-inflammatory activity was predicted in terms of per cent inhibition of paw circumference. In the normal control group, the paw circumference was 0.374±0.04 mm. In Group II which received carrageenan injection showed a significant increase in paw circumference as compared to control Group I (no carrageenan was injected). Group III also exhibited an increase in paw circumference before application of test film. After treatment with the test film, the paw circumference was reduced (Fig. 2 a-d). In the group III, after the injection of carrageenan, the

readings recorded for the decrease in the paw circumference at the time interval of 1, 2, 3, 4 h were 0.698±0.08, 0.547±0.02, 0.511±0.02, 0.0483±0.01 respectively and % inhibition of paw circumference at 1, 2, 3, 4 h were 38.12, 51.50, 54.69, 57.18% respectively for the groups considered for the study. In comparison to Group II ( no treatment), the rats in the Group III, which received the test film showed less significant ($P$ <0.05) decrease in paw circumference at 2nd and 3rd h interval, while it showed significant decrease in paw circumference at 4th h and was found to be statistically significant ($P$ <0.001). Group IV showed an increase in paw circumference before application of the standard film, which then found to decrease after the application of the standard film containing (diclofenac sodium). The paw circumference reading after the application of the film at 1, 2, 3, and 4 h were 1.118±0.04, 0.874±0.006, 0.553±0.018, 0.518±0.025, 0.479±0.033 respectively and % inhibition of paw circumference at 1, 2, 3, 4 h were 21.82, 50, 53.66, 57.15% respectively (Fig. 3). After the injection of Corrageenan, the increase in the rat paw circumference as shown in Fig. 3 was significantly reduced in Group III and Group IV. The per cent inhibition of the paw circumference of rats in Group III and Group IV showed a significant decrease in paw circumference at 3rd and 4th h interval as shown in Table 2. This indicates that the combination of diclofenac sodium (100 mg) with the *P. betel* leaf extract showed comparable anti-inflammatory activity when compared with the standard diclofenac sodium film containing 200 mg of diclofenac sodium. The results indicated that combination of *P. betel* leaf extract with diclofenac sodium (100 mg) had positive impact on the anti-inflammatory activity of diclofenac sodium and exhibited same anti-inflammatory activity as observed with diclofenac sodium 200 mg. Thus, the combination of *P. betel* leaf extract with diclofenac sodium resulted in reducing the dose of diclofenac sodium to almost 50%. The hypothesis by the authors suggesting the combination of *P. betel* leaf extract and diclofenac sodium can be supported by research work by various researchers. Nitric oxide (NO) is an important signalling molecule which is produced at the inflammation site by the action of NO synthase present in leucocytes. NO is a pro-inflammatory mediator known to induce inflammation[31]. *P. betel* leaf contains polyphenols which are reported to be the potent inhibitors of nitric oxide synthase and nitric oxide production[32,33,34]. *P. betel* leaf extract also possess free radical and peroxynitrite (ONOO) (-) scavenging activity[35]. The findings by Alam B *et al.* suggested that *P. betel* may be used as a supplementary herbal remedy for the treatment of pain and inflammatory disease[36]. The authors further proposed that the analgesic and anti-inflammatory effect exerted by *P. betel* extract may prove to be valuable when combined with analgesic and anti-inflammatory drugs[36]. Earlier studies have reported that the *P. betel* leaf extract exhibited inhibition of cyclooxygenase synthesis[37,38]. Cyclooxygenase is known to initiate the formation of prostaglandins and the increased level of prostaglandins in the tissue initiates pain, oedema, and

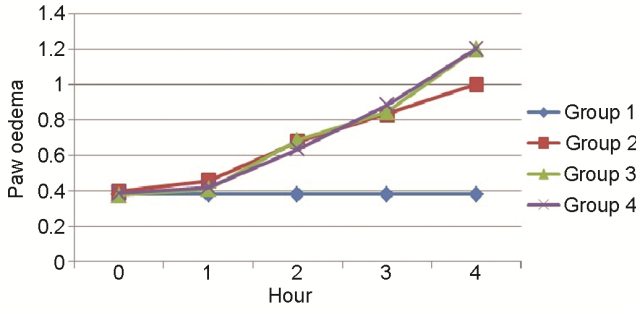


Fig. 3 — Carrageenan induced paw oedema.

Table 2 — Percent inhibition of paw circumference in different groups of rats

| Groups | Before application of film Paw circumference | Paw size in mm at time in h. and % inhibition 1h | 2h | 3h | 4h |
|---|---|---|---|---|---|
| Group I | 0.373±0.04 | 0.373±0.04 | 3.73±0.004 | 3.73±0.04 | 3.73±.004 |
| % inhibition of paw circumference | 0% | 0% | 0% | 0% | 0% |
| Group II | 1.123±0.05*** | 0.850±0.009*** | 0.668±0.009*** | 0.659±0.009*** | 0.632±0.008*** |
| % inhibition of paw circumference | 0% | 24% | 40.51% | 42.11% | 43.72% |
| Group III | 1.128±0.04 | 0.698±0.08** | 0.547±0.02* | 0.511±0.02* | 0.483±0.01** |
| % inhibition of paw circumference | 0% | 38.12% | 51.50% | 54.69% | 57.18% |
| Group IV | 1.118±0.04 | 0.874±0.006 | 0.553±0.018 | 0.518±0.025* | 0.479±0.033** |
| % inhibition of paw circumference | 0% | 21.82% | 50% | 53.66% | 57.15% |

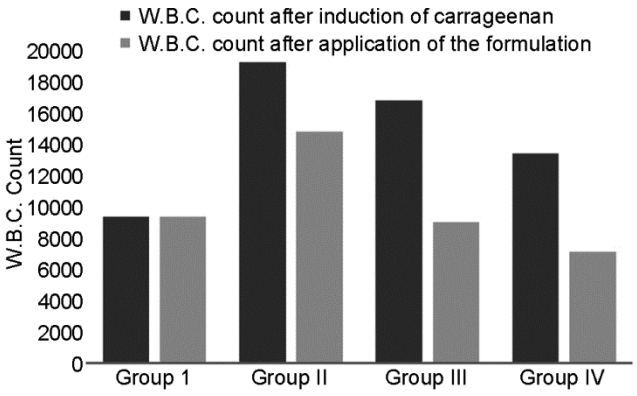


Fig. 4 — Histogram showing W.B.C. count.

fever. Inhibitory effect of the *P. betel* leaf extract on cyclooxygenase is comparable to that produced by NSAIDs. These NSAIDs also acts by similar mechanism by inhibiting cyclooxygenase enzyme. Hence, the combination of *P. betel* leaf extract and diclofenac sodium would prove to be beneficial as an anti-inflammatory agent. The result of this research work supports the hypothesis of combining *P. betel* leaf extract with NSAIDs to improve the anti-inflammatory activity and reduce the dose of diclofenac sodium by exerting synergistic effect.

This approach of combining *P. betel* leaf extract with diclofenac sodium would reduce the load on the synthetic drug thus providing an alternative to reduce the side effects associated with the use of synthetic agents.

**Haematological parameter**

The result for the haematological parameter is given in Fig. 4. The W.B.C. count in the Control group I was found to be 9200 cu/mm$^3$. In Group II, the WBC count was increased to 19000 cu/mm$^3$ after injection of carrageenan. In Group III, after the injection of carrageenan, rise in W.B.C. count was observed to 166000 cu/mm$^3$ which was found to decrease after the application of test film (*P. betel* leaf extract and diclofenac sodium film) to 8900 cu/mm$^3$. In Group IV standard film i.e., diclofenac sodium patch treated group showed an increase in W.B.C. count after injection of carrageenan solution to 13200 cu/mm$^3$ which was reduced to 7000 cu/mm$^3$ after application of the standard film. The total WBC count for an adult ranges from 5,000 to 10,000/mm in a normal condition[39]. WBC > 10,000/cu/mm$^3$ represents inflammation (possibly due to allergies) and tissue damage[40]. Carrageenan, a chemical obtained from red algae, stimulates the release of inflammatory and pro-inflammatory mediators. Cytokines are a series of pro-inflammatory mediators activated by macrophages and are capable to stimulate WBC by enhancing the attachment and migration of WBC's on the endothelial cells, thereby increasing their levels in the blood as observed in Group II indicating the development of inflammation[41]. In Group III rats treated with the test film a decline in the WBC count was observed due to anti-inflammatory effect of *P. betel* leaf extract. It can suppress the secretion of pro-inflammatory cytokines responsible to stimulate WBC[42,43]. The decline in WBC count in Group III was almost comparable to that observed in Group IV. The reason for this might be the combination of *P. betel* leaf extract and diclofenac sodium which must have resulted in decline in the WBC count which was evident even in the dose of 100 mg of diclofenac sodium and almost comparable to that observed in group IV, wherein the dose of diclofenac sodium was 200 mg. The results of haematological parameter also support the hypothesis of combining *P. betel* leaf extract with NSAIDs to improve the anti-inflammatory activity and reduce the dose of diclofenac sodium.

## Conclusion

The study indicates combination of *P. betel* leaf extract with diclofenac sodium would prove to be beneficial in improving the anti-inflammatory activity of diclofenac sodium. The study suggested that the combination of diclofenac sodium (100 mg) with *P. betel* leaf extract exhibited the same anti-inflammatory activity as that of diclofenac sodium (200 mg). This in turn would prove to be beneficial to reduce the untoward effects associated with the use of higher dose of NSAIDs, thereby improving patient compliance.

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## Conflict of interest

Authors declared 'no conflict of interest'.

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