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Original Research Article

Anti-inflammatory activity of methanolic extract of *Ficus hispida* dried fruit

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ABSTRACT

Background: Natural products are a valuable resource of novel bioactive metabolites and these products exist in which the anti-inflammatory activity. The present investigation studies the *in vivo* and *in vitro* anti-inflammatory activity of methanolic extract of *Ficus hispida* in rat's model.
Methods: Plant material was extracted with methanol in a Soxhlet extraction apparatus. Indomethacin was used as a standard drug here, which is a known potent inhibitor of PG synthesis. The carrageenin and histamine induced paw oedema were selected to represent models of acute inflammations. The test compounds and standard drugs were administered orally. After 60 minutes paw oedema was induced by giving 0.1 ml of 1% Carrageenan and 0.1 % histamine by sub-plantar administration. Paw volume-Plethysmometer by mercury displacement method, before and after 1 hr to 4 hours of carrageenan and histamine administration. Performed MTT-based cytotoxicity assay of the *Ficus hispida* on the RAW264.7 cell line to determine the IC_{50} and calculate the pro-inflammatory cytokines viz, IL-6, IL-1β and TNF-α and compared to the LPS control.
Results: The result obtained from the *in-vivo* study shows that the *Ficus hispida* has significant anti- inflammatory activity in a dose dependent manner. This effect is similar to that produced by NSAIDS such as Indomethacin. The concentrations of IL-6, IL-1β and TNF-α, secreted by the cells after challenging with bacterial LPS (2 µg/ml) and subsequent treatment with 50 µg *Ficus hispida* has been found to reduce the production of all the three pro-inflammatory cytokines viz, IL-6, IL-1β and TNF-α as compared to the LPS control. The activity, in fact, is comparable to the standard NSAID Indomethacin.
Conclusions: All these findings and phytoconstituents present in the extract could be the possible chemicals involved in the prevention of inflammations.

Keywords: *Ficus hispida,* Anti-inflammatory activity, Pro-inflammatory methanol, Histamine, Carrageenan

INTRODUCTION

Inflammation is a non-specific, localized immune reaction of the organism, which tries to localize the pathogenic agents. Many consider the syndrome a self-defense mechanism. It consists in vascular, metabolic, cellular changes, triggered by the entering of pathogenic agents in healthy tissues of the body.[1] Oedema formation, leukocyte infiltration and granuloma formation represents such components of inflammation.[2] Oedema formation in the paw is the result of a synergism between various inflammatory mediators that increase vascular permeability/or the mediators that increase blood flow.[3] NSAIDs, steroidal drugs and immune-suppressant drugs which have been usually used in the relief of inflammatory diseases worldwide for a long time are often associated with severe adverse side effects, such as gastrointestinal bleeding and peptic ulcer Valiollah et al. For this reason, in recent time, more interest is shown in alternative and natural drugs for treatment of various diseases; but there is a lack of scientific evidence Shukla et al. The use of plant preparations and extracts of their anti-inflammatory properties has a long-standing history among Indian

physicians. In this regard, one such plant which has number of traditional uses is *Ficus hispida.*

Ficus hispida is a part of the *Moraceae* family. It is often a famous and extensively disbursed throughout subcontinent and India.[4] The crops were consists of phenanthroindolizidine alkaloids, triterpenoids, flavonoids, oxyterpene, n-alkanes, coumarins, tannins, and saponins, oleanolic acid, bergapten, β-sitosterol, β-amyrin, hispidin. The bark contains 10-ketotetracosyl arachidate, lupeol acetate, β-amyrin and triacontanol acetate. The fruit contains linalool, linalool oxide, terpeneol, and 2,6-dimethyl-1,7-octadiene-3,6-diol. The plant additionally includes ficushispimines A and B, ficushispidine, hispiloscine, N-triacontanyl acetate, ficusin A.[5-7] Newly isolated 2 enormous phenanthroindolizidine alkaloids, 6-O-methyltylophorinidine and 2-demethoxytylophorine, and a novel biphenyl-hexahydroindolizine hispidine.

Ficus hispida Linn is an important pharmacological activity, The extracts hold been stated to be bitter, astringent, and anti-dysenteric and recreation against piles, jaundice, psoriasis, anemia, then hemorrhage.[8,9] The fruit acts as like a coolant and tonic, the juice is instituted as a moderate purgative. Its leaves are an anti-diarrheal, anti-inflammatory, anti-tussive, anti-pyretic, astringent, haemostatic and anti-ulcer activity, anti-diabetic, anti-bacterial, hepatoprotective properties.[10-13] Beside antioxidant activity, phenolic compounds are antiallergic, antimicrobial activity, cardio-protective, anti-thrombotic and vasodilator effect.[14,15] A mixture of honey and its juice is a good antihemorrhagic.[16]

METHODS

Collection of plant material

The *Ficus hispida (FH)* fruits were brought from Guwahati, Assam India. The plant specimen has been identified and authenticated by Dr. P. P. Baruah, department of botany, Guwahati university and reg. no. Herb./Bot./GU./2020/49.

Extraction of Ficus hispida fruits by using Soxhlet's extractor and sample preparation

The dried fruits were powdered and passed through the sieve and used for the preparation of methanolic extract. 250 gm of dried powder of *Ficus hispida* fruits was subjected to successive extraction in a Soxhlet extractor with methanol, and dried extract used for further experimental test.[17-21]

Experimental animals

Wistar rat's male (8-10 weeks old) weighing 150-200 gm and *Albino mice* female (8-10 weeks old) weighing 20-25 gm were used for the experiment. All the experiments conducted on the animals were in accordance with the standards set for the use of the laboratory animal use and the experimental protocols were duly approved by the IAEC of Karnataka college of pharmacy, Bangalore (Reg. number: 1564/PO/Re/S/11/CPCSEA).

Acute oral toxicity studies for dose fixation

The acute oral toxicity study was performed according to the OECD guidelines no. 425. A dose of 1/10th and 1/20th was considered to be high dose and low dose prepared by dissolving in miliQ water. The doses were prepared as per the OECD guideline no. 425.

Detailed study plan

The following procedure was done, where n=6 animals in each group.

Table 1: Carrageenan induced rat paw edema model.[22,23]

Groups		No. of rats
Carrageenan induced rat hind paw edema model	Group I: Control group-0.1 ml of 1% carrageenan by sub-plantar administration.	6
	Group II: Standard group (Indomethacin 10 mg/kg/P.O.)	6
	Group III: Test group-FH Low dose	6
	Group IV: Test group-FH High dose	6

Table 2: Histamine induced rat paw oedema model.

Groups		No. of rats
Histamine induced rat hind paw edema model	Group I: Control group-histamine, 100 µl, 0.1%	6
	Group II: Standard group (Indomethacin 10 mg/kg/P.O.)	6
	Group III: Test group-FH low dose	6
	Group IV: Test group-FH high dose	6

Histamine-induced inflammation has been widely used to explore the anti-inflammatory effects of some medicinal plants.[24]

The following procedure was done, where n=6 animals in each group.

The test compounds and standard drugs were administered orally.

After 60 min paw oedema was induced by giving 0.1 ml of 1% carrageenan and 100 µl, 0.1% histamine by sub-plantar administration. Paw volume-Plethysmometer by mercury

displacement method, before and after 1, 2, 3 and 4 h of administration.

Evaluation

The anti-inflammatory effect of fruits of methanolic extract FH was calculated by the following equation: Anti-inflammatory activity (%)=Do-Dt/Do×100, where DO was the average inflammation (hind paw oedema) of the control group of rats at a given time; and Dt was the average inflammation of the drug treated (i.e. extracts or reference indomethacin) rats at the same time.

To study the *in vitro* anti-inflammatory activity by estimating followings; cell line-mouse macrophages RAW264.7. RAW264.7 cell line was procured from national centre of cell science, Pune.

Performing MTT assay-to find out the in vitro dose IC_{50} on the cell line[25]

RAW264.7 cell line was procured from national centre of cell science, Pune, cultured in DMEM medium, 10% fetal bovine serum and antibiotics incubated at 37°C in 5% CO_2.

A range of concentration of FH used per well as follows; 0.7, 1.5, 3.1, 6.25, 12.5, 25, 50, 100, 200, 400, 600, 800, 1000 µg/ml and incubated at 37^0C for 24 h and then processed for determining cell viability.

At the end of drug exposure period, the growth medium was aspirated from each well and 50 µL of MTT solution (5 mg/ml) was added to each well. The plate was incubated for 4 hours at 37^0C in dark to facilitate the formation of formazan crystals. After incubation 200 µL of acidified DMSO was added to each well to dissolve the formazan crystals to give a purple color. Add 25 µL of glycine buffer and measure absorbance at 570 nm in a plate reader. The IC_{50} of the test samples were calculated. The percentage of death of the cells was determined by 100-(Abs. of sample/abs. of control×100).

In vitro anti-inflammatory assay on RAW 264.7 cells and measuring the levels of inflammatory cytokines of IL-1beta, IL-6 and TNF-alpha by ELISA assay [26-30]

RAW264.7 cells were cultured in a 12-well plate at a seeding density of 5×10^5 cells per well and cultured at 37^0C in a humidified atmosphere of 5% CO_2 in air in DMEM medium.

After incubation, the culture media were aspirated from each well without disturbing the cell monolayer and centrifuged at 10,000 rpm at 4^0C for 5 mins to sediment particulate matter, if any. The supernatants were used for the estimation of the cytokines viz, IL-1β, IL-6 and TNF-α was done using antigen capture ELISA.

The ELISA well plates have been coated with 100 µl of IL-6, IL-1β and TNF-α primary antibodies (2.5 µg/ml) in carbonate buffer (Na_2HPO_4 and NaH_2PO_4, pH 9.6). Primary antibodies of IL-6, IL-1β, and TNF-α were used. Plates have been incubated overnight at 4^0C to facilitate proper adsorption of antibodies on to substrate. After 12-14 h of incubation, plates have been washed thrice with washing buffer i.e., NaCl and tween 20 in phosphate buffer, pH-7.4 and blocked with 250 µl of blocking buffer i.e., BSA in phosphate buffer, pH 7.4/ well.

After followed by incubation at 37^0C for 1 h, added standard cytokines for construction of calibration curve. Remaining wells had been coated with 100 µl of diluted cell lysate. Concentration range used for standards was-25, 12.5, 6.25, 3.125, 1.562, 0.781, 0.39 and 0.195 ng/ml.

The standard cytokines were used, incubated for 1h and washed thrice. Anti-cytokine antibodies such as; anti IL-6, IL-1β and then anti TNF-α monoclonal antibodies were diluted 1:1000 and added 100 µl per well to the strips containing the respective antigens and incubated at 37^0C for 1 hour. After incubation, 100 µl of HRP-conjugate was added. Plate incubated at 37^0C for 1 hour. Then 100µl of freshly prepared substrate i.e., TMB in DMSO containing H_2O_2 was added to all wells. After incubation in dark 37^0C for 15 mins, color has changed, the reaction has been terminated by adding 50 µl of 2.5 N H_2SO_4 per well and the A 450 nm was measured by using ELISA reader.

A standard calibration graph was plotted (abs vs conc.) and the concentrations of unknown samples have been determined from the graph.

Table 3: Each group was added of RAW264.7 cells in triplicate (n=3) manner and has been treated with test chemical.

S. no.	Groups
Group 1	Normal control-cells in growth medium, DMEM
Group 2	Lipopolysaccharide (LPS) control-2 µg/ml
Group 3	Treated group-LPS+ *Ficus hispida*, 50 µg/ml
Group 4	Standard: LPS + indomethacin, 2.5 µg/ml

Statistical analysis

The results are expressed as mean±S.E.M. from n=6 rats in each group. Data were analysed using statistical software Graph Pad prism version 5. The significance of difference among the groups was assessed using one-way analysis of variance (ANOVA) followed by Tukey's test compared between normal control (untreated) vs all groups $p<0.05$ were considered significant.

RESULTS

Yield of extraction

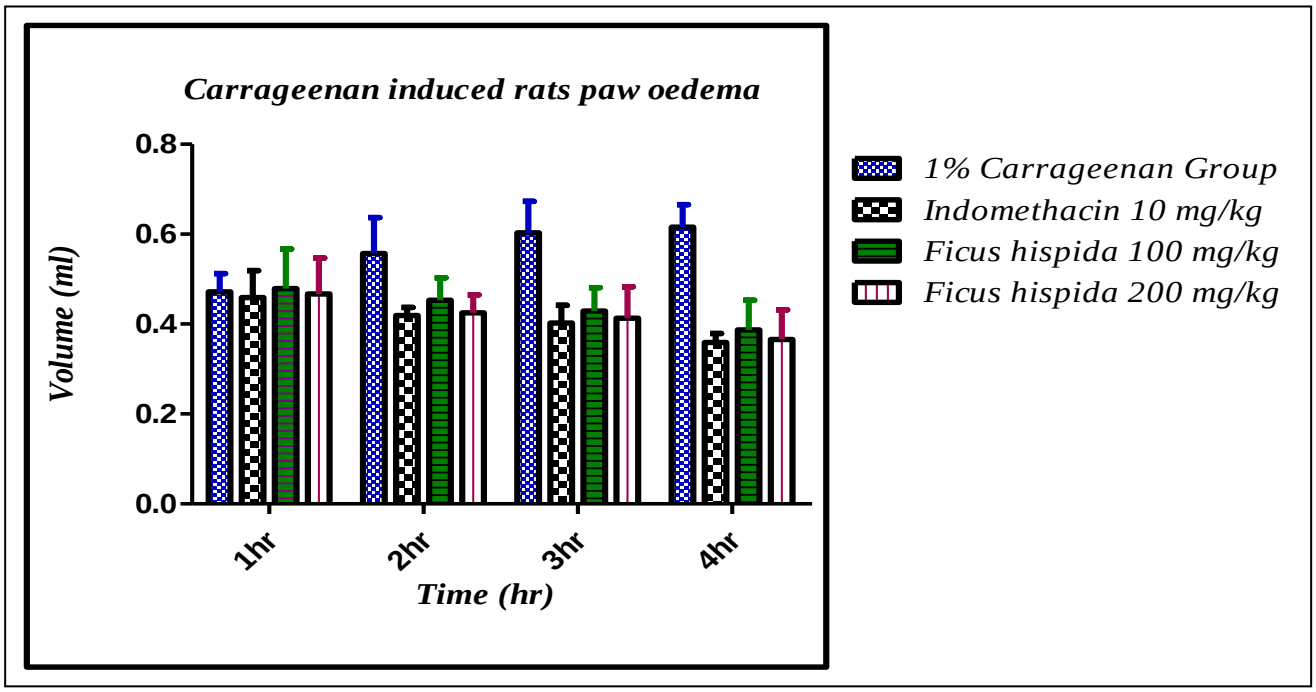
Yield of methanolic extract of *Ficus hispida* fruits was 46.08%.

Acute toxicity study

The 2000 mg/kg body weight was tolerated dose and no signs of toxicity have been found. Hence, 1/10th and 1/20th of the same dose was selected; 100 mg/kg and 200 mg/kg respectively and the further study were carried out.

Evaluation of in-vivo anti-inflammatory activity

Control group received an equivalent volume of vehicle only, standard group received indomethacin at the dose of 10 mg/kg and test drug *Ficus hispida* extract received low dose 100 mg/kg and high 200 mg/kg P.O. respectively.



Values are expressed as mean±SEM; n=6 $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$ when compared with carrageenan ($p<0.05$), $^{###}p<0.001$, $^{##}p<0.01$, $^{#}p<0.05$ when compared with Indomethacin.

Figure 1: Anti-inflammatory of methanolic extraction of fruits of *Ficus hispida* in carrageenan induced rat's model.

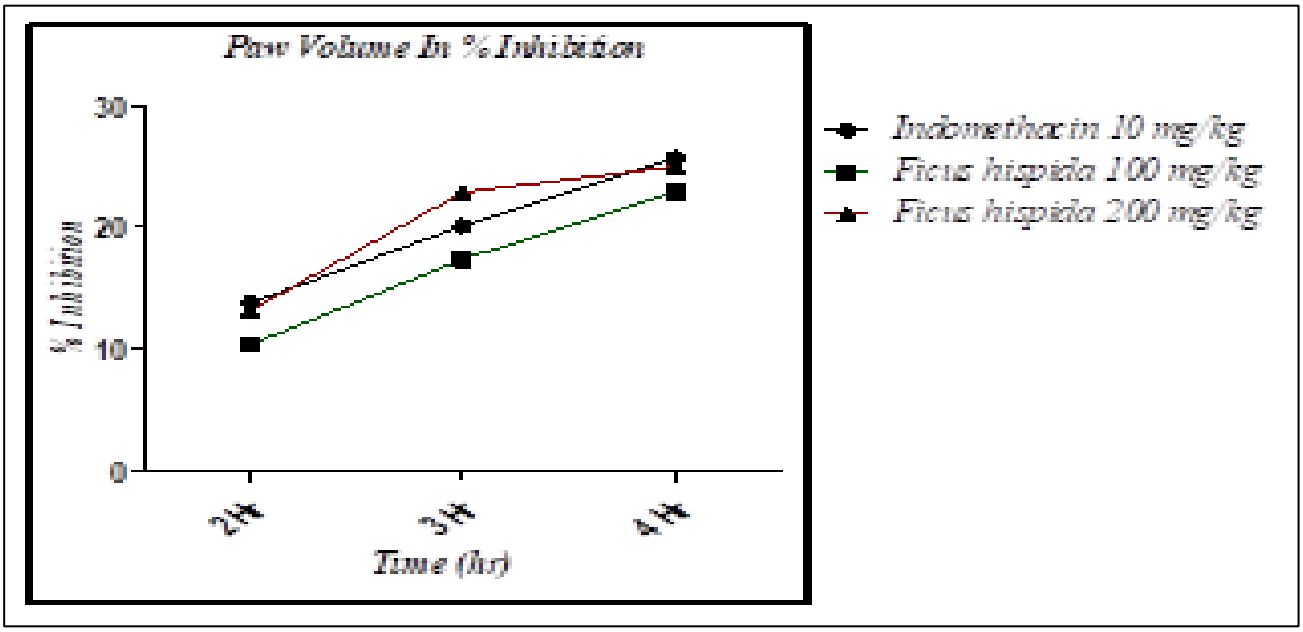
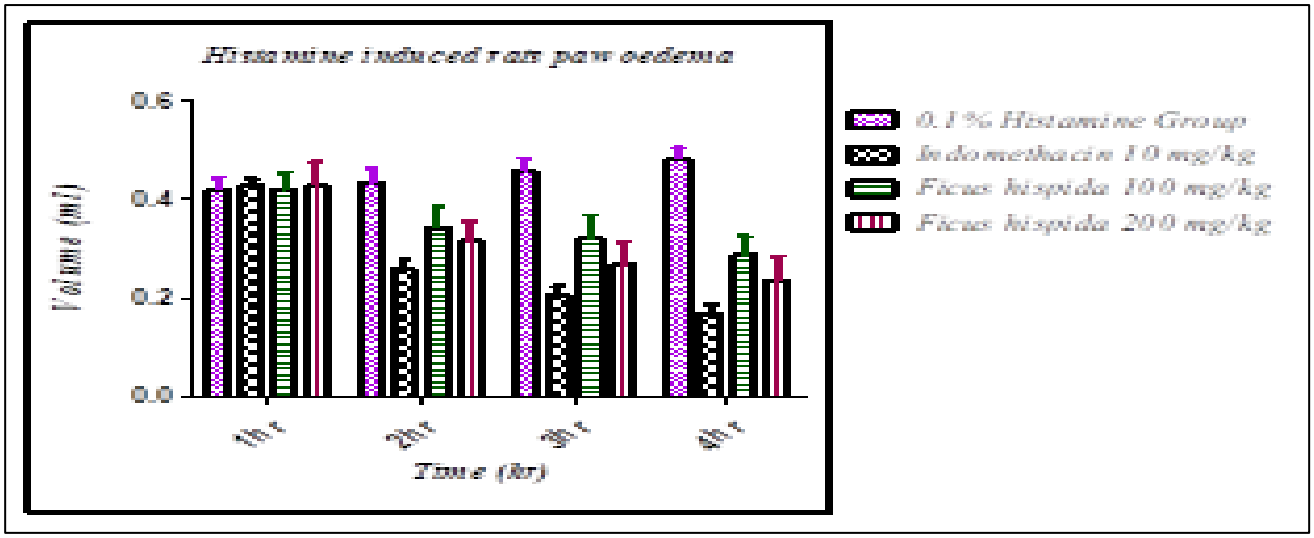


Figure 2: Paw volume in percent inhibition at different interval of time in carrageenan induced rat's model.



Values are expressed as Mean ± SEM; n=6, $^{***}p<0.001$ when compared with Histamine ($p<0.05$). $^{###}p<0.001$, $^{##}p<0.01$, $^{#}p<0.05$ when compared with Indomethacin.

Figure 3: Anti-inflammatory of methanolic extraction of fruits of *Ficus hispida* in histamine induced rat's model.

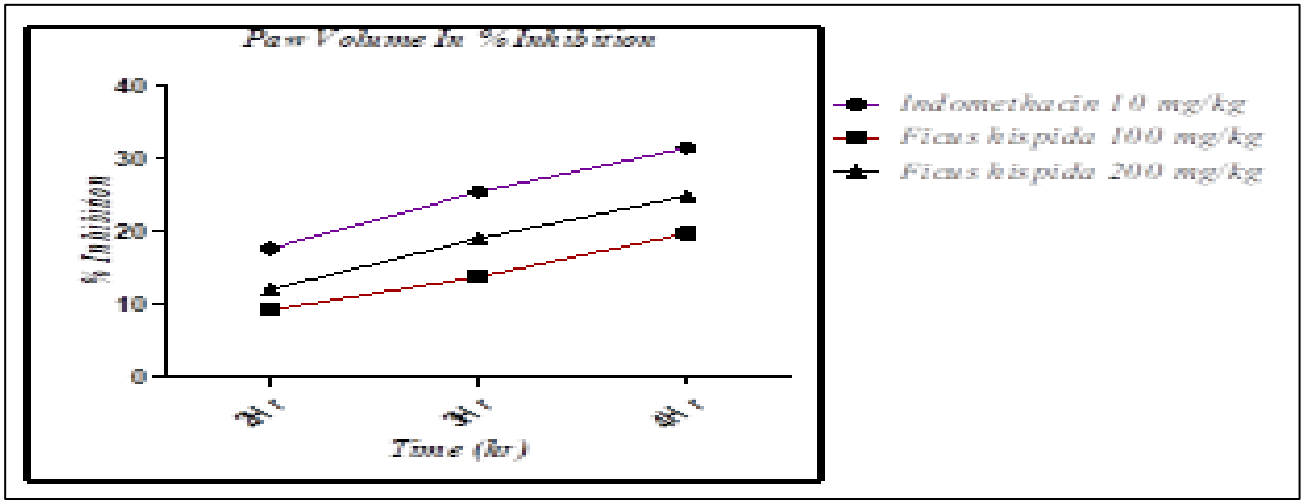


Figure 4: Paw volume in percentage inhibition at different interval of time in histamine induced rat's model.

Figure 5: 1 hour after inflammatory agents' injection.

Figure 6: 4 hours after test drug injection.

In this study, the methanolic extract of fruits of *Ficus hispida* exerted considerable inhibitory effect on carrageenan induced paw oedema in rats starting from the first hour after administration.

This effect was dose dependent and maximum inhibition induced by the extract was recorded after 4 hours with high dose of *Ficus hispida.*

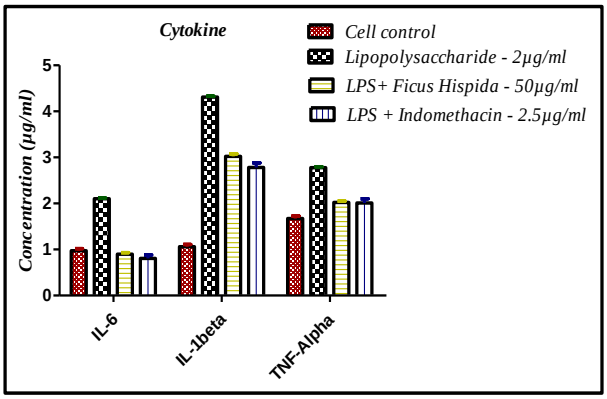
Evaluation of in-vitro anti-inflammatory activity

Table 4: MTT assay-the IC_{50} values of *Ficus hispida* and indomethacin on RAW 264.7

Samples	IC_{50} (µg/ml)
Ficus Hispida	125
Indomethacin	50

Control group received an equivalent volume of vehicle only, standard group received indomethacin at the dose of 2.5 µg/ml and test drug *Ficus hispida* extract received 50 µg/ml respectively.

In vitro anti-inflammatory assay on RAW 264.7 cells and measuring the levels of inflammatory cytokines of IL-1 beta, IL-6 and TNF-alpha by ELISA assay.



Values are expressed as mean±SEM; n=3 $^{***}p<0.001$ when compared with LPS, $^{###}p<0.001$, $^{##}p<0.01$ when compared with Indomethacin.

Figure 7: Cytokine ELISA.

Ficus hispida at a final concentration of 50 µg/ml has been found to reduce the production of all the three pro-inflammatory cytokines viz, IL-6, IL-1β and TNF-α as compared to the LPS control.

DISCUSSION

In an effort to search for novel anti-inflammatory agents from Natural plants, more than 300 plant extracts were tested for their ability to reduce the inflammation production. The methanolic extract of *Ficus hispida* dried fruits effective inhibition of inflammation release; this consequently led to contain oleanolic acid, bergapten, β-sitosterol, β-amyrin, hispidin, 10-ketotetracosyl arachidate, lupeol acetate, β-amyrin and triacontanol acetate, linalool, linalool oxide, terpeneol, and 2, 6-dimethyl-1, 7-octadiene-3, 6-diol, additionally includes ficushispimines A and B, ficushispidine, hispiloscine, N-triacontanyl acetate, ficusin A, newly isolated two enormous phenanthroindolizidine alkaloids, 6-O-methyltylophorinidine and 2-demethoxytylophorine, and a novel biphenyl hexahydro-indolizine hispidine being responsible for the observed activity.[5-7]

To the best of our knowledge this is the first time their isolation from the species *Ficus hispida* is reported. Moreover, this is the first time the compounds were shown to reduce pro-inflammatory production. In order to evaluate the compound's activity, we further examined the anti-inflammatory effects of this compound on other inflammatory models, cytokines and mediators. In the present study, carrageenin-induced paw oedema in rats and histamine-induced paw oedema were selected to represent models of acute inflammations. Control group received an equivalent volume of vehicle only, standard group received Indomethacin at the dose of 10 mg/kg and test drug *Ficus hispida* extract received low dose 100 mg/kg and high dose 200 mg/kg P.O. respectively. And study revealed that there were significantly changes in test group when we were compared with their respective control. However, despite the fact that the inflammatory response can be triggered by different routes, even though we had got 20 to 30% inhibition after 4 hours in *Ficus hispida* 100 and 200 mg/kg respectively in carrageenan induced rat paw oedema model in this study, the methanolic extract of fruits of *Ficus hispida* exerted considerable inhibitory effect on carrageenan induced paw oedema in rats starting from the first hour after administration. Similarly inhibitory effects of *Ficus hispida* administered orally on histamine induced paw oedema and the percentage inhibition was 20% in *Ficus hispida* 100 mg/kg and 25% inhibition in *Ficus hispida* 200 mg/kg. This effect was dose dependent and maximum inhibition induced by the extract was recorded after 4 hours with high dose of *Ficus hispida*.

Performing MTT-based cytotoxicity assay of the 'test compounds' on the cell line was to determine the IC_{50} for fixing the dose for *in-vitro* anti-inflammatory. The IC_{50} of *Ficus hispida* on raw 264.7 cell line has been found to be 125 µg/ml. The IC_{50} of indomethacin on RAW264.7 cell line has been found to be 50 µg/ml. The concentrations of IL-6, IL-1β and TNF-α, secreted by the cells after challenging with bacterial LPS (2 µg/ml) and subsequent treatment with 50 µg *Ficus hispida* have been found to be 0.897, 3.024 and 2.023 µg/ml respectively. The concentrations of IL-6, IL-1β and TNF-α, for cell control (without LPS and drug) have been found to be 0.974, 1.059 and 1.672 µg/ml respectively. The concentrations of IL-6, IL-1β and TNF-α, for LPS control (cells challenged with LPS; without drug) have been found to be 2.101, 4.312 and 2.776 µg/ml respectively. The concentrations of IL-6, IL-1β and TNF-α secreted by cells treated with Indomethacin (2.5 µg) have been found to be 0.805, 2.783 and 2.012

µg/ml respectively. *Ficus hispida* at a final concentration of 50 µg/ml has been found to reduce the production of all the three pro-inflammatory cytokines viz, IL-6, IL-1β and TNF-α as compared to the LPS control.

CONCLUSION

This chapter describes about anti-inflammatory activity of *Ficus hispida* dried fruits extracts in carrageenan and histamine induced paw oedema in rats. The crude extract from the *Ficus hispida* plant produced significant anti-inflammatory activity. Since methanolic extracts have produced lower activity than disease control which almost equal active with standard drug. The results were expressed as maximal paw oedema (maximal peak during the 4 hours) and as total paw oedema and presented as mean±SEM, n=6. The *Ficus hispida* extract was further subjected to *in vitro* anti-inflammatory activity. *Ficus hispida* at a final concentration of 50 µg/ml has been found to reduce the production of all the three pro-inflammatory cytokines viz, IL-6, IL-1β and TNF-α as compared to the LPS control. The activity, in fact, is comparable to the standard NSAID Indomethacin. These all findings and phytoconstituents present in the extract could be the possible chemicals involved in the prevention of inflammations.

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