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EVALUATION OF ANTI-INFLAMMATORY ACTIVITY OF THE EXTRACT OF MADHUCA INDICA USING IN VITRO AND IN VIVO MODELS OF INFLAMMATION

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ABSTRACT

Madhuca indica flower and seeds have been found to contain several phytoconsitutents. In the present investigation, the bark of *Madhuca indica* was extracted using various solvents and the anti-inflammatory potential of the extract was investigated. The extraction abilities of different solvents for recovering extractable components from leaves followed the order: ethyl acetate>ethanol>water>n-hexane. The phenolic content was found to be highest in the ethyl acetate extract (59.57±3.566 %w/w) followed by ethanol (41.5±1.804 %w/w) and aqueous (20.87±1.054 %w/w) extracts. The ethyl acetate extract of the plant (EAEx) was subjected to *in vitro* and *in vivo* determination of anti-inflammatory potential. Inhibition of albumin denaturation was used as the *in vitro* model while carrageenan induce rat paw edema was used as the in vivo model for anti-inflammatory activity. The ethyl acetate extract of *Madhuca indica* exhibited the inhibition of albumin denaturation at all doses from (100-500 µg/ml) in a dose dependent manner. The 500 µg/mL concentration of the extract had shown the greatest inhibition capacity (39.6±3.869%) whereas the lowest inhibition capacity was exhibited by concentration 100 µg/mL (7.5±2.291%). EAEx at dose level of 200 mg/Kg exhibited maximum reduction in edema after 4h with 23.98% reduction whereas the change decreased at the 4th hour to 12.95%. EAEx dose of 400 mg/Kg was indeed able to exhibit better reduction in edema as compared to the control with 37.28% reduction after 3h. The reduction decreased to 36.44% after the 4th hour. The effect of EAEx was therefore found to be short acting.

KEYWORDS: *Madhuca indica*, albumin denaturation, carrageenan, edema, inflammation.

INTRODUCTION

Inflammation is a part of the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells or irritants. It is characterized by redness, swollen joints, joint pain, its stiffness and loss of joint function. Inflammation is currently treated by NSAIDs.[1] While over-the-counter painkillers can be really helpful in managing the pain associated with inflammatory conditions, and in some cases may be the best option, they can also cause gastrointestinal upset and definitely aren't the best long-term strategy for coping with chronic pain. The goal is, of course, to try and help relieve pain by developing a new herbal remedy for the inflammatory action. Among the various Complementary and alternative medicine, "herbal medicine" is the most popular and fastest growing approach used to treat various ailments worldwide. The side effects produced by the synthetic agents include dizziness, weakness and increasing the gatric ulceration, elevates liver enzyme (diclofenac), nausea, vomiting, pruritis, headache, insomnia, Steven-Johnson syndrome, peptic ulcer and nephrotoxicity caused by NSAIDs (aniline derivative), etc.

The key to reduce chronic inflammation in our body starts with our diet and being liberal in the use of high-quality herbs and spices is one simple way to boost the quality of our food. They are an inexpensive "secret weapon" that just about everyone can take advantage of. Spicing up our meals is not enough, however, if processed foods comprise the bulk part of our life. Thus there is a need to replace synthetic agents by safe and effective plant based herbal remedies as anti-inflammatory agents. Many plants extracts have been used as anti-inflammatory agent in folklore claim and in traditional medicines.

Madhuca indica is a medium sized to large growing deciduous shady tree belonging to the Sapotaceae family and grows about 16-20 meters tall.[2] The leaves of Mahua tree contain saponin, an alkaloid, and glycoside. Saprogenic and other basic acid are found in the seeds. Various Phytochemical studies on Mahua include characterization of Sapogenin, triterpenoids, steroids, saponin, flavonoids and glycosides, madhucic acid (penta cyclic triterpenoids), madhushazone, four new oleanane type triterpene glycosides and madhucosides A and B. The fresh flowers contains 2- acetyl-l- 1-pyrroline, the aroma molecule. They also contain

polysaccharide which on hydrolysis gives D-galactose, D-glucose, L-arabininose, L- rhamose, D- xylose and D-glucuronic acid.[3]

Madhuca indica has been reported to possess several pharmacological actions.[4-13] Most of these actions are linked to the flower of the plant. A few reports have also highlighted the presence of flavonoids and steroids in the bark and leaves of the plant. Hence it was decided to investigate the anti-inflammatory potential of the extract of bark of *Madhuca indica*.

EXPERIMENTAL

Collection and identification of the plant material

The bark of *Madhuca indica* were collected from the local surrounding of Damoh, Madhya Pradesh in the month of April and authenticated.

Preparation of the plant material

The collected plant bark after authentication was washed with distilled water and was dried under shade. The completely dried bark was converted to fine powder form using a low speed blender. The powdered bark were stored in air tight container until further studies.

Extraction of the bark[14]

The bark powder prepared using the above procedure was used for extraction process. Hot continuous extraction was performed for extracting out the phytochemicals from the bark powder. Briefly, 180 g of the bark powder was evenly packed in the extractor of the soxhlet apparatus and extracted successively with various solvents of increasing polarity. The solvents used for extraction included hexane, ethylacetate, ethanol and water. The extraction process was carried out for about 6-8 h for each solvent. The extracts were filtered while hot through Whatman filter paper and concentrated by distillation to reduce the volume to one tenth. The concentrated extracts were then transferred to evaporating dish and the remaining solvents were evaporated on thermostatically heated water bath. The oleo-resinous extracts were collected and placed in desiccators to remove the excessive moisture**.** The dried extracts were stored in desiccators until used for further investigational procedures.

Preliminary phytochemical testing[15]

All the extracts were subjected to qualitative phytochemical testing procedures for identifying the presence or absence of usual plant secondary metabolites. The test was performed for alkaloids, triterpenes/steroids, glycosides, tannins, flavonoids, saponins, and phenolic acids. The color, intensity of color or the precipitate formation was used as observational responses to the reactions occurring in these tests.

Total Phenolic Content[16]

One gram of the extract was added to 15 ml of methanol (50% v/v) and extracted for three times by maceration for 2h, then filtered and made up the volume with methanol (50% v/v) in volumetric flask upto 100 ml. One ml aliquot of the sample was taken in a test tube and diluted with 10 ml of distilled water. Then 1.5 ml Folin Ciocalteu's reagent was added and allowed to incubate at room temperature for 5 min. Four ml of 20% (w/v) Na_2CO_3 was added, adjusted with distilled water up to the mark of 25 ml, agitated and left to stand for 30 min at room temperature. Absorbance of the sample was measured at 765 nm against blank, i.e., distilled water.

Standard solutions of gallic acid (10-100 ppm) were similarly treated to plot the analytical curve. The control solution contained 200 µL of methanol and suitable reagents, and it was prepared and incubated under the same conditions as the rest of the samples.

The total phenolic content (%w/w) was calculated using the formula [34]:

Total phenolic content (%w/w) = $GAE*V*D*10^{-6}*100/W$

Where GAE – Gallic acid equivalent (µg/mL), calculated from calibration curve equation and absorbance of sample; V –Total volume of sample (mL); D – Dilution Factor; W- Sample weight

In vitro anti-inflammatory activity

Inhibition of albumin denaturation[17]

The extracts were dissolved in DMSO and appropriately diluted to prepare solutions of 100, 200, 300, 400 and 500 µg/mL concentration. A solution of 1% BSA in deionized water was prepared for the test. Ibuprofen solution of concentration 1 µg/mL was used as the positive control.

The test containers were filled with 200 µL of BSA, 1400 µL of PBS and 1000 µL of the test solution (extract). Ibuprofen solution was used in the positive control and distilled water was used in the negative control vessels in place of extract.

The reaction mixtures were incubated at 37°C for 15 min and then heated at 70°C for 5 min. The mixtures were then allowed to cool to room temperature and the absorbance of constituent of each vessel were analyzed in UV-Visible spectrophotometer at 660 nm. The inhibition of percent denaturation of albumin was determined using the following formula:

$\% \text{ Denaturation inhibition} = (1-D/C) \times 100\%$

Where D is the absorbance reading of the test sample, and C is the absorbance reading without test sample (negative control).

Anti-inflammatory action using carrageenan induced rat paw edema method

The carageenan induced rat paw edema method was used for evaluating the anti-inflammatory activity of the ethylacetate extract of *Madhuca indica* bark (EAEx).[18]

Paw oedema was induced by subcutaneous injection of 0.1mL (1% solution) of Carrageenan into the plantar surface of the right hind paw of the rat. The test sample was administered in different groups of animals, 30 min prior to carrageenan injection. Ibuprofen (10 mg/kg i.p.) was used as a standard anti-inflammatory drug which was administered 30 min prior to carrageenan injection. Animals were divided into 4 groups (n = 6) as follows
Group -- I - Control - treated with vehicle (normal saline)
Group -- II - Standard drug – Ibuprofen
Group – III– EAEx was administered in dose of 100 mg/kg.
Group – IV– EAEx was administered in dose of 200 mg/kg.

Paw diameters were measured immediately before the administration of the Carrageenan and thereafter at 1, 2, 3 and 4 h using vernier caliper. The results obtained were compared with control group. The percentage inhibition of paw inflammation exhibited by each group was calculated by using following formula:
% inhibition = C-T/ C x 100

C= Paw volume (mm) in vehicle treated group (control);
T= Paw volume (mm) in drug treated group

RESULTS AND DISCUSSION

The bark of *Madhuca indica* was scrapped from the tree and dried (Figure 1). The authenticated sample was powdered coarsely (Figure 2) and used for extraction.

Figure 1 Bark of *Madhuca indica.*

Figure 2 Powdered bark of *Madhuca indica.*

Extraction Yields

The extraction abilities of different solvents for recovering extractable components from leaves followed the order: ethyl acetate>ethanol>water>n-hexane.

Phytochemical Screening

The findings suggest the presence of alkaloids in the ethylacetate and ethanol extract, saponin glycosides and proteins in aqueous extract, flavonoids in ethylacetate, ethanol and aqueous extracts, terpenoids in the n-hexane and ethylacetate extracts of the bark of *Madhuca indica*.

Total Phenolic content

The ethyl acetate, ethanol and aqueous extracts of *Madhuca indica* contained phenolic components and hence were evaluated for quantifying the total phenolic content concentrations in extracts. Standard curve of gallic acid was calculated and plotted in distilled water for determining absorption data. From this Beer's law range and regression coefficient is determined. The linear equation of gallic acid was found to be y = 0.0046x + 0.0024. The results of the total phenolic content of the extracts examined, using Folin-Ciocalteu method, are depicted in Table 1. The total phenolic content in extracts, expressed as percent w/w. The phenolic content was found to be highest in the ethyl acetate extract followed by ethanol and aqueous extracts.

Table 1: Total phenolic content.

Extract	Total phenolic content (%w/w)
Ethyl acetate	59.57±3.566
Ethanol	41.5±1.804
Aqueous	20.87±1.054

Data expressed as Total phenolic content (%w/w), Values are mean ± Standard deviation of triplicate determinations.

In vitro anti-inflammatory activity

Protein denaturation has been significantly correlated with the occurrence of the inflammatory response and may lead to various inflammatory diseases including arthritis.[19-21] Tissue injury during life might be due to denaturation of the protein constituents of cells or of intercellular substance. Hence, the ability of a substance to inhibit the denaturation of protein signifies obvious potential for anti-inflammatory activity.[22, 23]

The ethyl acetate extract of *Madhuca indica* exhibited the inhibition of albumin denaturation at all doses in a

dose dependent manner (Table 2, Figure 3). The 500 µg/mL concentration of the extract had shown the greatest inhibition capacity (39.6±3.869%) whereas the lowest inhibition capacity was exhibited by concentration 100 µg/mL (7.5±2.291%). The inhibition protein denaturation by 100 µg/mL solution of standard drug Ibuprofen was found to be 87.33 ± 3.561%.

Table 2: Albumin denaturation inhibition activity.

Treatment	100 µg/mL	200 µg/mL	300 µg/mL	400 µg/mL	500 µg/mL
Ibuprofen	87.33±3.561	-	-	-	-
EAEx	7.5±2.291	16.1±2.156	27.3±2.167	33.5±2.167	39.6±3.869

Results are expressed as mean ± SD, n = 5

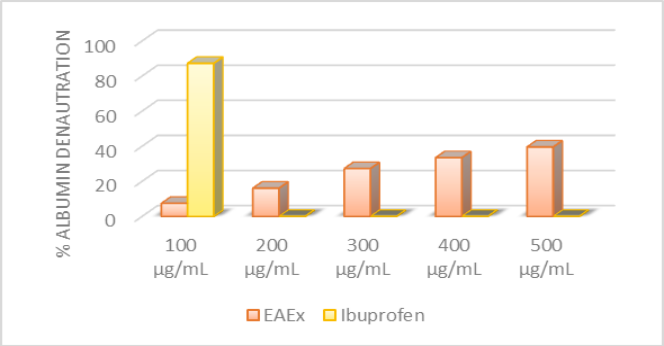

Figure 3 % albumin denaturation by EAEx.

***In vivo* anti-inflammatory activity**

The percent change in rat paw volume with respect to the control group was calculated and the results are presented in Table 3. The change in thickness of paw was measured with the aid of vernier caliper and the % change in thickness was determined. It can be seen from the results that after 4h of administration, Ibuprofen was able to reduce 75.90% of edema caused by carageenan injection. On the other hand, the two doses of EAEx were able to reduce the volume very vividly. EAEx at dose level of 200 mg/Kg exhibited maximum reduction in edema after 4h with 23.98% reduction whereas the change decreased at the 4th hour to 12.95%. EAEx dose of 400 mg/Kg was indeed able to exhibit better reduction in edema as compared to the control with 37.28% reduction after 3h. The reduction decreased to 36.44% after the 4th hour. The effect of EAEx was therefore found to be short acting.

Table 3: Effect of EAEx on rat paw thickness.

Group	Change in Paw thickness (mm) [% inhibition of edema]			
	1h	2h	3h	4h
Vehicle Control	1.35 ± 0.043	2.93 ± 0.072	3.46 ± 0.070	3.32 ± 0.064
Ibuprofen	0.43 ± 0.056 [68.14%]	1.02 ± 0.075 [65.18%]	0.98 ± 0.061 [71.67%]	0.80 ± 0.064 [75.90%]
EAEx (200 mg/Kg)	1.31 ± 0.039 [2.96%]	2.69 ± 0.04 [8.19%]	2.63 ± 0.041 [23.98%]	2.89 ± 0.043 [12.95%]
EAEx (400 mg/Kg)	1.01 ± 0.036 [25.18%]	2.15 ± 0.051 [26.62%]	2.17 ± 0.053 [37.28%]	2.11 ± 0.059 [36.44%]

Results are expressed as mean ± SD, n=5

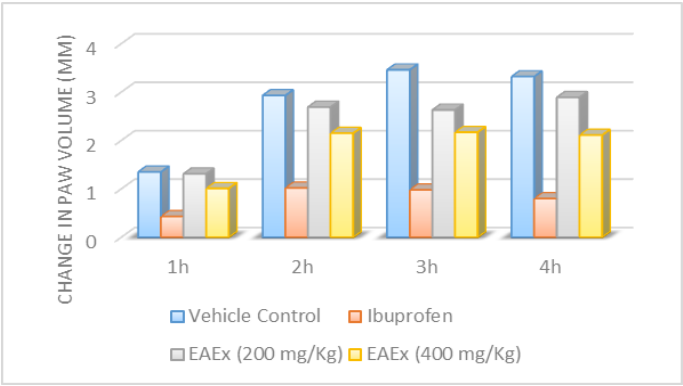

Figure 4 Statistical representation of effect of EAEx on rat paw edema.

As depicted from the results the extracts were not able to significantly decrease the edema in the rats though they exhibited anti-inflammatory activity. The percent change in paw thickness is presented in Figure 5.

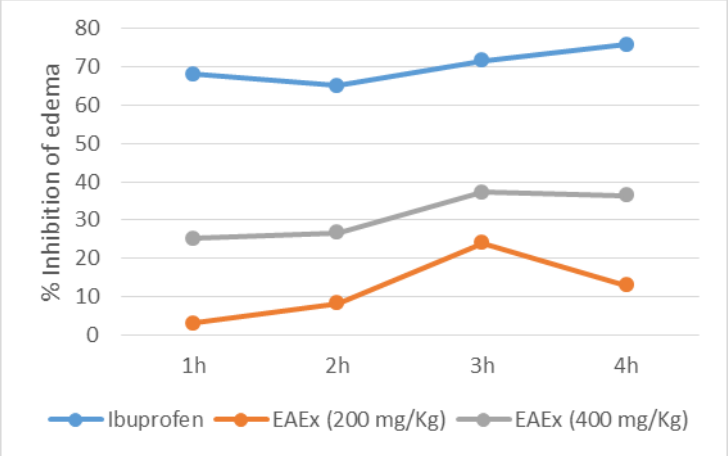

Figure 5 Percent change in paw thickness.

The above figure suggests that the highest inhibition by the extract was not very comparable to that by standard drug and also the effect of extract started to diminish after 3h of administration.

CONCLUSION

The objective of the present study was to assess the anti-inflammatory potential of extract of bark of *Madhuca indica* using the *in vitro* and *in vivo* models. The results obtained led to the conclusion that *Madhuca indica* bark are a rich source of potential phytochemicals and exhibits anti-inflammatory action.

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