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HUMAN RED BLOOD CELL MEMBRANE STABILITY TESTING FOR THE ESTIMATION OF ANTI-INFLAMMATORY ACTIVITY OF METHANOLIC EXTRACT OF MILLETTIA PACHYCARPA BENTH LEAVES

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ABSTRACT: The present study is carried out with the methanolic extract of leaves of *Millettia pachycarpa* benth. Traditional use of it inspired us to investigate the blood corpuscular protective power of this plant as it used as blood tonic in Chinese traditional medicine. By the phytochemical screening we have both flavonoids and steroids. There's also alkaloids are found which have extensive physiologic action embodying analgesic activity. This investigation is made following the most simple, reliable and less time consuming method. As the human red blood corpuscular membrane is similar to lysosomal membranes that influence inflammatory process. Result obtained using methanolic extract of *M. pachycarpa* have better acceptance as it shows good response in inhibiting hemolysis (34.30%) at highest concentration and these investigation surely stimulate further screening and isolation process.

INTRODUCTION: Inflammation is one common and major cause of sufferings now and every time past. Those drugs that are available are known as NSAID, i.e. non-steroidal anti-inflammatory drugs, act by inhibiting the function of prostaglandin. Prostaglandin is an autocoid that release extracellularly and initiate pain.

Anti-inflammatory agents either block this autocoid synthesis by inhibiting COX enzyme or protecting lysosomal membrane from break down.

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Plant is a source of wide variety of chemicals. Most of them need to be synthesized. One plant may consist of several compounds that have several effects on physiology. The main source of medicine from the beginning of mankind till modern time is plant. The synthesis of plant is both dangerous and harmful as they may be toxic. But in our daily life deliberately or undeliberately we take plants as food and they shows their regular biochemical action which is unnoticed to us, like tea shows stimulatory action for caffeine, betel nut cause aphrodisiac action for some alkaloidal content etc.

Millettia pachycarpa belongs to Fabacea family and have some local use. It is endemic to south-east Asian region including Bangladesh, Bhutan, China, India, Myanmar, Nepal, Taiwan, Thailand and Vietnam [1].

It locally used as fish poison as well as in medicine, in Chinese medicine it used as blood tonic and it functions to induce growth of blood. It also recommended for daily dietary menu for LDL reduction as it have phytosterol including β-sterol, also found in the treatment of pro-inflammatory medication of osteoarthraitis mediated cartilage degeneration [2-6].

METHODS AND MATERIALS:

Plant material preparation: The plant material was collected from wild and hilly part of Bangladesh (Bandarban, Chittagong) and was identified by Forest Research Institute of Bangladesh. The collected plant was dried for a few days in natural way and then at hot air oven (37 ± 2^0C) for 3 hours. It macerated to powder form and about 250gm powder was dissolved in 500ml methanol (95%) following cold extraction [7, 8]. It takes couple of days for proper dissolution then filtered through Buchner funnel and again dried at water bath (40^oC) for evaporation of methanol and extract preserved at $<4^o$C for next use.

Primary phytochemical screening: Phytochemicals of the selected plants were carried out by using aqueous and powdered form of the plant following Harborne (1973) Trease and Evans (1989).

1. **Test for Tannin:** About 0.5gm powdered sample was boiled in water (20ml), filtered it, and a few drops of 0.1% ferric chloride solution was added to see the brownish green or blue black coloration.

2. **Test for saponin:** About 2gm of dried sample was taken with 20ml of distilled water for boiling in water and filtered. 10ml filtrate was added with 5ml distilled water and vigorous shaking; 3-4ml olive oil was added for formation of emulsion.

3. **Test for Flavonoid:** 5ml of ammonium solution was added to a portion of aqueous extract following the addition of concentrated H_2SO_4. Yellow coloration confirms the presence of flavonoids. On standing for few moments it disappears. 1% aluminum solution, 2-3 drops, was added to a portion of aqueous extract filtrate. Yellow coloration concludes the presence of flavonoid [9].

4. **Test for steroid:** 5ml of aqueous extract was added to 2ml chloroform successively 3ml concentrated H_2SO_4 added, cautiously for reddish brown intermittent layer, which confirm positive result.

5. **Test for alkaloid:** Alkaloids are basic nitrogenous compounds with definite physiological and pharmacological activity. The alkaloid solution produces white-yellowish precipitation, when a few drops of Meyer's reagent were added [10]. Most alkaloids are precipitated from neutral or slightly acidic solution by Meyer's reagent [11]. The methanolic extract was evaporated to dryness and the residue was heated on boiling in water bath with 2% HCl. After cooling, the mixture was filtered and treated a few drops of Meyer's reagent. The samples were then holding to observe turbidity or yellowish white precipitation.

Study of anti-inflammatory activity by membrane stability assay: Anti-inflammatory activity of methanolic extract of *Millettia pachycarpa* was evaluated by using *in vitro* human red blood cell stability method. Blood sample was collected from a fresh volunteer, who doesn't have anti-inflammatory or contraceptive drugs at least since a week. The collected blood was mixed with sterilized Alsever solution. Alsever solution was prepared by 2% dextrose, 0.8% sodium citrate, 0.05% citric acid and 0.42% sodium chloride dissolved in distilled water.

Blood sample was centrifuged at 3000rpm and packed cell was washed with isosaline and a 10% (V/V) suspension of isosaline was made. Three different solution of *M. pachycarpa* were mixed with 1ml phosphate buffer, 2ml hyposaline and 0.5ml HRBC suspension. Aspirin was used as contrastable drug and instead of hyposaline 2ml water was used as control. The hemoglobin content in supernatant was calculated using Spectrophotometer at 560nm spectrum.

The result was estimated by following equations [12, 13];

$$Percentage\ of\ hemolysis = \frac{OD\ of\ test}{OD\ of\ control} \times 100$$

The percent of membrane protection was calculated by the following equation-

$$Percent\ of\ protection = 100 - \frac{OD\ of\ test}{OD\ of\ control} \times 100$$

RESULT AND DISCUSSION: The extractive preliminary phytochemical analysis that performed earlier results the presence of alkaloid, flavonoid, steroid, saponin etc. (**table 1**). It was qualitative analysis only, performed to find out and predict why the plant has anti-inflammatory effect. Alkaloid, a nitrogenous group of phytochemical that has wide diversity in classification and distribution, has good evidence of pain killing activity [14]. Recent observations from animal and human studies have demonstrated anti-inflammatory effects of phytosterols. For example, several animal and human studies report reductions in the levels of pro-inflammatory cytokines, including C-reactive protein, after consumption of dietary plant sterols [15].

Flavonoids have the hepatoprotective reputation as anti-oxidant phytoagent. So anyone in the present phytomaterials may cause the pain reduction that needs further concern.

TABLE 1: PRELIMINARY PHYTOCHEMICAL SCREENING OF METHANOLIC EXTRACT OF *M. PACHYCARPA* LEAVES

Phytochemicals	Status
Alkaloid	+
Steroid	+
Tannin	-
Saponin	+
Glycoside	+
Flavonoid	+

(+) = present, (-) = absent

TABLE 2: ANTI-INFLAMMATORY ACTIVITY OF METHANOLIC EXTRACT OF *M. PACHYCARPA* LEAVES BY USING HRBC MEMBRANE PROTECTION TESTING

No. of sample	Concentration		
	100µg/ml	200µg/ml	300µg/ml
Aspirin	30.17±1.08%	35.95±1.78%	38.84±0.67%
M. pachycarpa	29.34±0.97%	31.82±0.83%	34.30±1.22%

n=3, mean ± SEM, this percentage represents the percent of protection provided by both groups, the statistical analysis shows significant value as $p<0.005$

The main function of anti-inflammatory agent is to inhibit the function of cycloxygenase (COX) enzyme that is responsible for conversion of arachidonic acid to prostaglandin (PG). When the nociceptor or pain receptor activated it influence the release of that enzyme and which then function then extracellularly by converting arachidonic acid to prostaglandin. Non-steroidal anti-inflammatory drugs either stop or inhibit the conversion or protect the lysozomal membrane to inhibit inflammation.

The above table (**table 2**), shows the anti-inflammatory action of *M. pachycarpa* methanolic extract. This was only a preliminary testing where we have such kind of concentration dependent percent of membrane protection. As the HRBC membrane is likely to the membrane of lysosome therefore the stabilizing ability of HRBC will be implied as its ability to protect the lysosomal membrane as well.

In this test, aspirin, as standard, has 38.84% of protection at 300µg/ml where extract has 34.30% at the same concentration. It seems the extract has significant activity on anti-inflammatory functioning.

CONCLUSION: In the conclusion, it can be said that the experiment was helpful for further isolation of natural product as in pain reduction purposes. Most of drugs are not safe when they are synthetic but once when they are natural it becomes better than that.

No agent can be initiate or considered for clinical trial or for animal model induction, because it is quite harmful and offensive as well so if the agent have *in vitro* good result then these steps can be considered. Now from this study we have both phytochemical knowledge and membrane stabilizing data we may proceeds for future investigation on this behalf.

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