IN VITRO ANTI-ARTHRITIC ACTIVITY OF *PHYLLANTHUS ACIDUS* (L).

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Running title: Anti-Arthritic Activity of *phyllanthus acidus* (L.) by protein denaturation and HRBC method.

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Herbalism has a long tradition of use outside conventional medicine. It is becoming more main stream as improvement in analysis and quality control, along with advances in clinical research, show the value of herbal medicine in treating and preventing the disease. For example, one study found that 90% of the people with arthritic use alternative therapies such as herbal medicine. Arthritis is an auto immune disorder characterized by pain, swelling and stiffness. It is a form of joint disorders that involve inflammation in one or more joints. *Phyllanthus acidus* (L.) is a valuable medicinal plant which have been valuable in centuries in ayurvedic medicine. phytochemical analysis of *phyllanthus acidus* (L.) of fruit extracts revealed the presence of various biochemical compounds such as flavonoids, glycosides, alkaloids, starch, amino acid, lignin, volatile oils , fats, fixed oils, proteins, steroids and triterpenoids. Since glycosides and flavonoids have remarkable anti-inflammatory activity. Our present work aims at evaluating the invitro anti arthritic activity of *phyllanthus acidus* (L.) by protein denaturation and HRBC method. Denaturation protein is a well-documented cause of inflammation and rheumatoid arthritis. The data of our studies suggest that *phyllanthusacidus* (L.) of fruit extract showed that significant anti arthritic activity. Therefore our studies support the isolation and active constituents of *phyllanthus acidus* (L.) and supports in treating anti arthritic activity.

Keywords: Herbal medicines, Arthritis, In vitro, *phyllanthus acidus* (L.), Protein denaturation, HRBC.

INTRODUCTION:

Herbal medicines are also called as botanical medicine or phytomedicine refers to using a plants seeds, berries, roots, leaves, bark or flowers for medicinal purpose. Herbalism has a long tradition of use outside conventional medicine. It is becoming more main stream as improvement in analysis and quality control, along with advances in clinical research, show the value of herbal medicine in treating and preventing the disease. Herbal medicine is used to treat many conditions, such as allergies, asthma, eczema, premenstrual syndrome, rheumatoid arthritis, migraine, fibromyalgia, menopausal symptoms, irritable bowel syndrome and cancer, among others. It is best to take herbal supplements under the guidance of a trained provider. For example, one study found that 90% of the people with arthritic use alternative therapies such as herbal medicine. [(1)]

Invitro studies allows scientists to isolate specific cells, bacteria and virus and study them without the distractions of having to look at the whole organisms. Unfortunately, this means that sometimes results found in invitro studies do not translate well to real life. Humans are much more complicated than test tubes. However, compare to invivo studies, invitro studies are substantially faster.

The name "Phyllanthaceae" was first validly published by Ivan Ivanovich Martynov in 1820 in a Russian book entitled *Tekhno-botanico Slovar*. A proposal to conserve this name was published in 2007.Phyllanthaceae isa family of flowering plants in the eudicot order Malpighiales. It is most closely related to the family Picrodendraceae. The Phyllanthaceae are most numerous in the tropics, with many in the South Temperate Zone, and a few ranging as far north as the middle of the North Temperate Zone. Phyllanthaceae comprises about 2000 species. Depending on the author, theseare grouped into 54 to 60 genera. Some of the genera are poorly defined, and the number of genera in the family is likely to change as the classification is further refined. The genus *Phyllanthus*, one of the largest genera of flowering plants, with over 1200 species, has more than half of the species in the family.[(2)]

ARTHRITIS

Arthritis is an auto immune disorder characterized by pain, swelling and stiffness. It is a form of joint disorders that involve inflammation in one or more joints.

Types:

- Rheumatoid arthritis
- Osteo arthritis
- Lupus
- Gout

Signs and symptoms:

- Inability to use the hand or walk
- Stiffness, which may be worse in the morning
- Malaise and fatigue
- Weight loss
- Poor sleep
- Muscle ache and pains
- Tenderness and Difficulty in moving joints

DIAGNOSIS

Diagnosis is made by clinical examination from an appropriate health professional and maybe supported by other test such as radiology and blood test depending on the type of suspected arthritis. All arthritis potentiallyfeatures pain. Pain patterns may differ depending on the arthritis and the location. Rheumatoid arthritis is generally worse in the morning and shower.

- Blood test
- X-ray
- Computerised topography
- Magnetic resonance imaging
- Ultrasound

TREATMENT

There is no known cure for either rheumatoid or osteoarthritis. Treatment options vary depending on the type of arthritis and include physicaltherapy, lifestyle changes (including exercise and weight control), orthopaedicbracing and medications. Joint replacement surgery may be required in eroding forms of arthritis. Medications can help in reducing inflammation in the joints which decreases pain. Moreover, by decreasing inflammation, the joint damage may be slowed.

Physical therapy

In general, studies have shown that physical exercise of the affected jointcan have noticeable improvement in terms of long term pain relief. Furthermore, exercise of the arthritic joint is encouraged to maintain thehealth of the particular joint and the overall body of the person. Individuals with arthritis can benefit from both physical and occupational therapy. Exercise often focuses on improving muscle strength, endurance and flexibility.in some cases, exercise maybe designed to train balance.

Medications

There are several types of medications that are used for the treatment of arthritis. Depending on the type of arthritis, the medications that are given may be different.

- NSAIDs (ibuprofen, indomethacin, cox-2 inhibiters like celecoxib andbaldecoxib)
- Analgesics (morphine and acetaminophen)
- Glucocorticoids or prednisolone

MATERIALS AND METHODS

MATERIALS

- Bovine Albumin
- Phosphate Buffer Saline (PBS) pH6.3
- Human Blood

METHODS[3,4]

1. Plant Material Collection and Authentication

The fresh mature leaves of P. Acidus were collected from the area of Dharmapuri district, Tamilnadu, India.

2. Preparation of Extract

- **Alcohol extract**

  The collected leaves were shade dried without exposing them to direct sunlight. The dried leaves were ground to coarse powder with a mechanical grinder (Grinding mill) and powdered sample was kept in clean closed glass containers pending extraction. About 250 g of dried sample was subjected to extraction by 99% ethanol with a volume of 1000 ml for 15 days with stirring and agitation for allowing total extraction process. After the extraction process the P. acidus leaf extract was filtered with sterilized cotton filter followed by what man filter paper. The filtrate was collected in a beaker. The extract obtained after filtration was concentrated by using a rotary evaporator at 60°C.

- **Petroleum ether extract**

  The coarse powder extract with 2 to 3 litres of petroleum ether (60- 80°C) by continuous hot percolation using soxhlet apparatus. After completion of extraction, it was filtered and the solvent was removed by distillation under reduced pressure. The extract was stored in a desiccator, a yellowish green colour residue was formed.

- **Aqueous extract**

  The marc left after alcohol extraction was dried and macerated with 2-3litres of chloroform water (0.25%) in mouthed bottle for 3 days. After completion of extraction it was filtered and the solvent was removed by distillation under reduced pressure. The extract was then stored in desiccator. A black colour residue was obtained.

  All the above extract was used for identification of constituents by phytochemical test from the weight of the drug, the extract content was calculated.

Extractive value (%) = Weight of Extractive **x 100**

Weight of Drug

PHARMACOLOGICAL SCREENING

INVITRO ANTIARTHRITIC ACTIVITY

Anti-denaturation studies are performed by using bovine serum albumin (BSA). When BSA is heated it undergoes denaturation and express antigens associated with type -3 hypersensitivity reaction and that is related to disease such as serum sickness, rheumatoid arthritis, glomerular nephritis and system lupus erythematosus.

INHIBITION OF PROTEIN DENATURATION METHOD[5, 6]

Preparation of reagents

5% bovine serum albumin (BSA)

5g of BSA was dissolved in 100 ml of water 8g of sodium chloride(nail),0.2g of potassium chloride(kill),1.44g of disodium hydrogen phosphate(NA2HPO4) and 0.24 g of potassium dihydrogen phosphate (KH2PO4) where dissolved in 800 ml of distilled water. The PH was adjustedto 6.3 using 1N HCL and makes the volume of 1000 ml with distilled water.

Test solution

0.45ml of bovine serum albumin and 0.05ml of test solution of various concentrations were prepared.

Test control solution

0.45 ml of BSA and 0.05ml of distilled water were prepared.

Product control solution

0.45ml of distilled water and 0.05 ml of test solution.

Standard solution

0.45ml of serum albumin and 0.05ml of aspirin of various concentrations.

METHODS

- 0.5ml of test solution, test control solution, product control solution, standard solution were prepared.
- Various concentrations (100, 200, 400,800 µg/ml) of test drug. (Petroleum ether, aqueous and methanol extracts) and standard drug aspirin, (100,200,400,800µg/ml) were prepared.
- 1N HCL was used to adjust the pH to 6.3 for all above solutions. The samples were incubated at 37ºC for 20 min and the temperature wasincreased to keep the samples at 57ºC for 3 min.
- After cooling 2.5ml of phosphate buffer was added to the above solution. The absorbance was measured at 4.6nm.
- The control represents 100% protein denaturation. The percentageinhibition of protein denaturation can be calculated as **% Inhibition = 100 – {(optical density of test control - optical density of Product) / optical density of test solution} * 100**
- The control represents 100 % protein denaturation.

MEMBRANE STABILIZATION TEST[7]

Preparation of RBC suspension

Fresh whole human blood (10ml) was collected and transferred to the heparinised centrifuged tube. The tubes were centrifuged at 3000 rpm for 10 min and were washed 3 times with equal volume of normal saline. The volume of the blood was measured and reconstituted at 10%v/v suspension with normal saline.

Heat induced haemolysis

The reaction mixture (2ml) consisted of 1ml of test drug solution and 1ml of 10% HRBC suspension. Instead of drugs only saline was added to the control test tube. Aspirin was taken as a standard drug. All the centrifuged tubes containing mixture were incubated in the water bath at 56ºc for 30 min.at the end of the inhibition, the tubes were cooled under running tap water.

The reaction mixture was centrifuged at 2500 rpm for 5min and the absorbance of the supernatant was taken at 560nm. The experiments were performed in triplicates. Percent membrane stabilization activity was calculated by using the formula,

$$100 * (V_T / V_C - 1)$$

Where as

V_T = **absorbance of test sample** V_C = **absorbance of control**

RESULTS AND DISCUSSION:

PHTOCHEMICAL INVESTIGATION:

- The dried powder of the fruits was extracted by continuous hot percolation(soxhlet apparatus) with different solvents of increasing polarity and percentage of extracts were calculated.
- The various extracts were subjected to phytochemical staining, the extract answered **positively** for **alkaloids, phenols, steroids, terpenoids and flavonoids** were mentioned in Fig.1.
- It **negatively** answered for **anthroquinone, terpenoids, saponin and tannins.**
- Invitro anti arthritic activity was performed using most popular method such as inhibition of protein denaturation activity. Concentration ranges from 100 t0 800 **µg/ml** were tested to find out the percentageinhibition.in inhibition of protein denaturation assay it was found that the **ethanol extract showed better activity than other extracts** when compared to standard drug activity were mentioned in Table.1 and the comparison of inhibitions were mentioned in Fig.2.

- The various concentration of compound *phyllanthus acidus* (L.) ranging from 100 **µg/ml** to 800 **µg/ml** were tested for its protein denaturation and HRBC method. The results were clearly demonstrated that the compound *phyllanthus acidus* at different concentration have anti denaturation activity. Maxinum percentage of protein denaturation inhibition 63% was observed from ethanol extract followed by petroleum ether 52% at the maximum concentration of 800 **µg/ml.** Aspirin, a standard anti-inflammatory drug showed the maximum inhibition 93% at the concentration of 800 **µg/ml.** In HRBC method ethanol extract of maximum concentration 800 **µg/ml** shows 70%, petroleum ether extract 54% followed by aqueous shows 40% aspirin a standard drug showed the maximum inhibition of 90% at the concentration of 800**µg/ml**. A dose dependent increase in the percentage inhibition was observed forall the concentration tested increases in arthritic activity which was shown in Table.2 and fig.3.

CONCLUSION:

Our present work aims at evaluating the invitro anti arthritic activity of *phyllanthus acidus* (L.) by protein denaturation and HRBC method. Denaturation protein is a well-documented cause of inflammation and rheumatoid arthritis. The data of our studies suggest that *phyllanthusacidus* (L.) of fruit extract showed that significant anti arthritic activity. Therefore our studies support the isolation and activeconstituents(L.) supports in treating anti arthritic activity.

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TABLE.1: EFFECT OF DIFFERENT EXTRACTS ON INHIBITION OF PROTEIN DENATURATION.

Extract	**Concentration (µg/ml)**		
	100	**200**	**400**
Ethanol	26.6	45.9	51.2
Petroleum Ether	18.4	33.2	41.9
Aqueous	17.4	21.7	36.5
Aspirin	54.4	69.9	88.8

TABLE. 2: INVITRO ANTI ARTHRITIC ACTIVITY BY MEMRANE STABILIZATION METHOD.

Test sample	**Conc.(µg/ml)**	**Protection (%)**
Ethanol	**100**	**38.8**

	200	**44.2**
	400	**53.6**
	800	**70.4**
Petroleum ether	**100**	**28.8**
	200	**29.8**
	400	**42.2**
	800	**54.6**
Aqueous	**100**	**20.3**
	200	**21.6**
	400	**26.7**
	800	**40.4**
Aspirin(standard drug)	**800**	**90.2**

Phytochemicals	**Extract(*phyllanthus acidus*)**		
	FRUIT		
	Methanol	**Petroleum ether**	**Aqueous**
Alkaloids			
Mayer's test	+	-	+
Wagner's test	-	-	+
Flavonoids			
Lead acetate test	+	-	-
H_2SO_4 test	+	-	-
Steroids			
Libermann-burchard test	+	-	+
Terpenoids			
Salkowskitest	-	+	-
Anthraquinones	-	-	-
Phenols			
Ferric chloride test	-	+	-
Lead acetate test	+	-	-
Saponin	-	+	+
Tannin	-	-	-
Carbohydrates	+	+	-
Oils & resins	+	+	+

Fig. 1: Phytochemical test observations of different extracts of *Phyllanthus Acidus* (L).

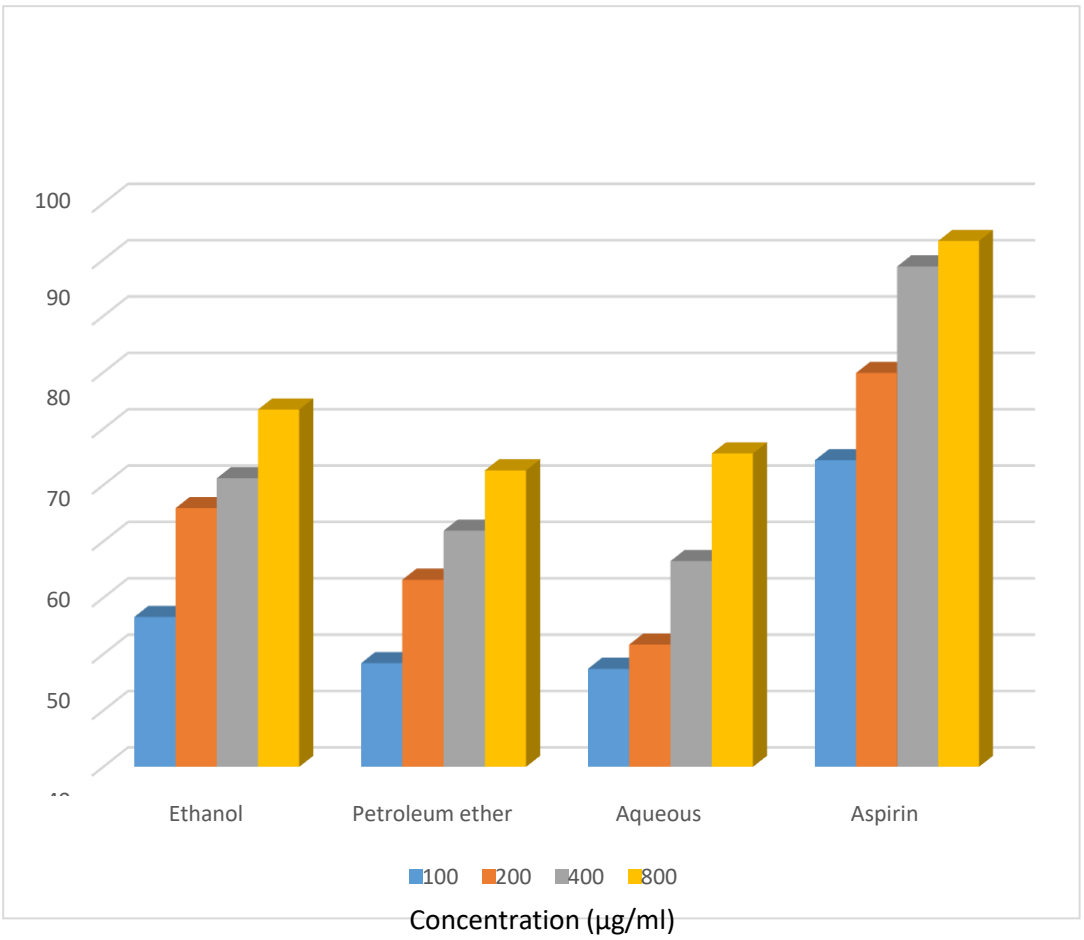

Fig. 2: Effect of different extracts on inhibition of protein denaturation.

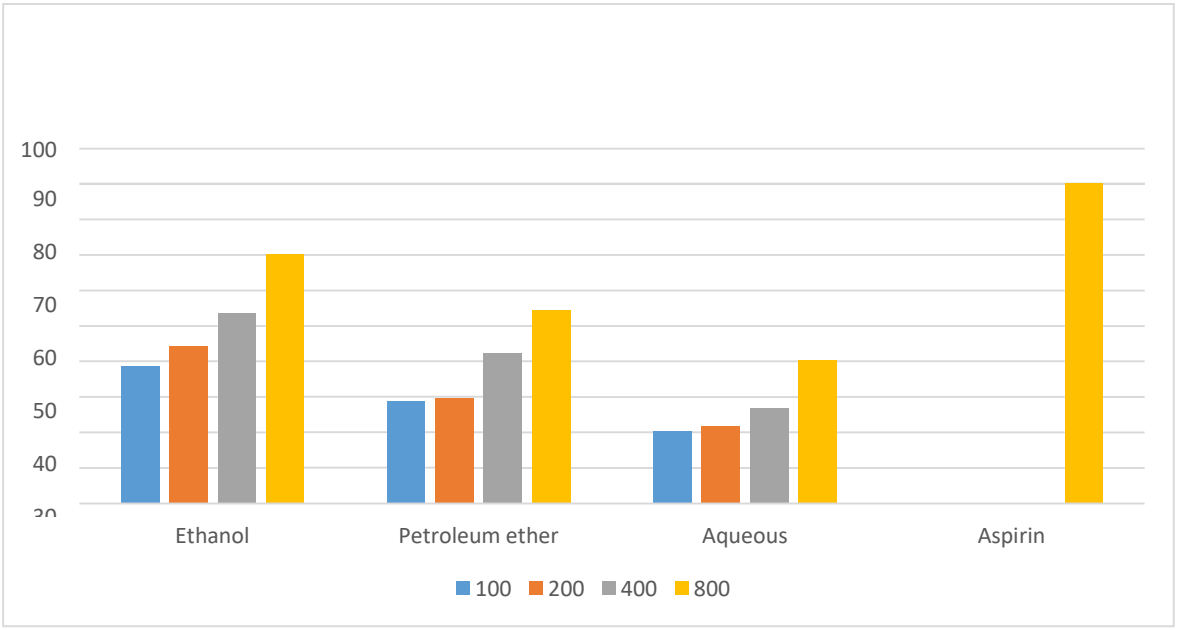

Fig. 3: Invitro anti arthritic activity by membrane stabilization method.

Tables and Figure titles:

TABLE.1: EFFECT OF DIFFERENT EXTRACTS ON INHIBITION OF PROTEIN DENATURATION.

TABLE.2: INVITRO ANTI ARTHRITIC ACTIVITY BY MEMRANE STABILIZATION METHOD.

Fig. 1: Phytochemical test observations of different extracts of *Phyllanthus Acidus* (L).

Fig. 2: Effect of different extracts on inhibition of protein denaturation.

Fig. 3: Invitro anti arthritic activity by membrane stabilization method.