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IN VITRO SCREENING OF ICACINACEOUS PLANTS INDIGENOUS TO KERALA

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Abstract

This project work was focused on "*in vitro* screening of Icacinaceous plants indigenous to Kerala". From phytochemical analysis of ethyl acetate extract of leaves of *Sarcostigma kleinii* it was found that carbohydrates, alkaloids, flavonoids,tannins, phenols, glycosides are present and saponins, proteins & amino acids are absent. From phytochemical analysis of ethyl acetate extract of leaves of *Pyrenacantha volubilis* it was found that carbohydrates, proteins and amino acids, flavonoids,alkaloids, glycosides, tannins and phenolic compounds are present. *Sarcostigma kleinii* leaves are used to treat helminthiasis, ulcers, leprosy and skin conditions traditionally. *Pyrenacantha volubilis*leaves are mainly used to treat cancer. The present study deals with the anti-inflammatory activity and antibacterial activity of ethyl acetate extracts of leaves of *Sarcostigma kleinii* and *Pyrenacantha volubilis* of Icacinaceae family. The results suggest that ethyl acetate extract of leaves of these two Icacinaceous plants may serve as anti - inflammatory and antibacterial agents which may be due to the chemical constituents like alkaloids, phenols, tannins or flavonoids present in these plants.

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Introduction

Medicinal plants have been identified and used throughout human history. Plants have the ability to synthesize a wide variety of chemical components that are used to perform important biological functions at least, 12,000 such components have been isolated so far, a number estimated to be less than 10% of the total. Chemical components in the plant mediate their effects on the human body through processes identical to those already well understood for the chemical components in conventional drugs, thus herbal medicine does not differ greatly from conventional drugs in terms of how they work [1]. Natural products were used by human kind as a source of drugs and higher plants provided most of therapeutic agents. Today natural products still represent over 50% of all the drugs in clinical use, with higher plant derived natural products. World Health Organization estimates that 80% of the peoplein developing countries of the world rely on traditional medicine for their primary health care and about 85% of traditional medicine involves the use of plant extracts [2].

In Vitro Screening

In vitro screening systems and high throughput screening are critical aspects of the modern drug discovery process. Prior to the advent of the technologies, screening of compounds for biological activity was a major barrier in the identification of novel drugs. By the turn of the 21st

century, however, *in vitro* screening methods had become capable of generating data on hundreds to thousands of compounds per day, greatly enhancing the acquisition of biological data. Detection of the presence of radiolabeled material, as we are monitoring changes in fluorescence or absorbance can be used in conjunction with microtiter plates, advanced robotics and sophisticated software to determine the biological activity of a candidate compound(3). Recent developments and new experimental techniques allow easy access even to complex specific functions of these cells and render it possible to draw conclusions about molecular mechanisms. These *in vitro* approaches, therefore may contribute essentially to the reduction of animal use in such studies. Furthermore, they may give full insight into not only the therapeutic potential but also to the possible dangers of phytopharmaceuticals. Present study deals with anti-inflammatory and antibacterial activity studies. (4)

Anti Inflammatory Activity

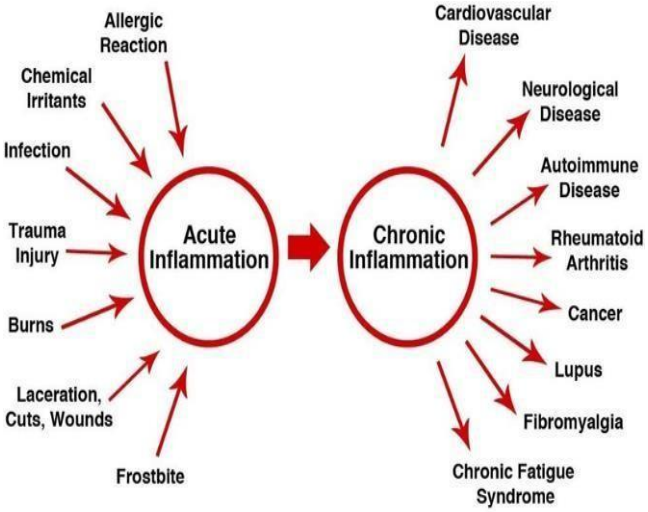

Figure 1 - Acute vs chronic inflammation [6]

Inflammation is defined as the local response of living tissue to injury to any agent. It is a body defense reaction for the purpose of eliminating or limiting the spread of injurious agent as well as to remove the consequent necrosed cells and tissues. Thus it is aimed at eliminating foreign substances invading the body, or abnormally generated self-compounds produced during tissue injury. Inflammation is not a single process and it is not simply binary in nature ("on" or "off"), but it can be modulated by many factors in the cell's environment [5].

The agents causing inflammation may be: -

- Physical agents like heat, cold, radiation, mechanical trauma.
- Chemical agents like organic and inorganic chemicals.
- Infecting agents like bacteria, viruses, and their toxins.
- Immunological agents like cell-mediated and antigen- antibody reactions

Anti Microbial Activity

Antimicrobial susceptibility testing can be used for drug discovery, epidemiology and prediction of therapeutic outcome. Antimicrobial testing methods are used for the *in vitro* investigation of extracts & pure drugs. Several bioassays such as disc diffusion, well diffusion, broth and agar dilution are well known and commonly used, but others such as flow cytofluorometric & bioluminescent methods are not widely used because they require specified equipment and further evaluation for reproducibility & standardization, even if they can provide rapid results of the antimicrobial agent's effect & a better understanding of their impact on the viability and cell damage inflicted to the tested microorganism [7].

Icacinaceous Plant

Icacinaceae is a family of flowering plants consisting of trees, shrubs and lianas primarily of the tropical areas. The family was traditionally circumscribed quite broadly, with around 55 genera totaling over 400 species. In 2001, through, this circumscription was found to be polyphyletic, and the family was split into four families in 3 different orders:-

- *Pennanticeae* (Apiales)
- *Stemonuraceae oliates* (Aquifoliates)
- *Cardiopteridaceae* (also Aquifoliates)

Other genera have moved to *Metteniusaceae* (*Metteniusales*). *Icacinaceae* belongs to the order Icacinales .

- Large tubers rarely present
- Leaves are alternate, simple, exstipulate
- Inflorescence axillary, extraaxillary, terminalor cauliflorus
- Flowers are actinomorphic, rarely zygomorphic, bisexual, rarely unisexual
- Fruits are drupe, ovoid, oblong, globose or rarely flattened Seeds, embryo commonly minute endosperm copious.

Sarcostigma Kleinii of Icacinaceae Family

Sarcostigma kleinii is a woody climber seen extensively in the western ghats. This plant is used widely in ayurvedha. *Sarcostigma kleinii* is a much -branched vigorous climbing shrub with stems upto 26metres long and upto 8 cm thick. Usually evergreen, the plant is sometimes leafless when flowering. The plant is harvested from the wild for local use as food,medicine and source for oil. Oil is extracted from theseeds and is popular for treatment of rheumatism in india. *Sarcostigma kleinii* also known in Malayalam as Erumathali, Odal, vattodal. It is a woody climber with leaves-oblong-lanceolate, apex-accuminate, base- rounded or obtuse, petiole 5-12cm long. Flowers- 3 to 6 together and yellow. Fruits-drupe, ovoid, orange yellow, glabrous. General habitat-evergreen and semi-evergreen forests, also in sacred grooves. Global distribution in Indo - Malaysia, state – kerala all districts [8, 9].

Materials and Methods

Table 1 : Instruments & chemicals used

SL.NO	NAME OF CHEMICAL	REQUIREMENTS
1	Alpha naphthol	Conical flask
2	Benedicts reagent	Standard flask
3	Chloroform	beaker
4	Copper sulphate	Funnel
5	Fehlings reagent A	Water bath
6	Fehlings reagent B	Filter paper
7	Ferric chloride	Glass rod
8	Ferrous sulphate	Petri dish
9	Glacial acetic acid	Cotton
10	Hydrochloric acid	Distilled water
11	Lead acetate	Test tubes
12	Ninhydrin	
13	Ntric acid	
14	Picric acid	
15	Potassium bromide	
16	Potassium iodide	
17	Pyridine	

Collection and Identification of Plants

Fresh leaves of *Sarcostigma kleinii Wight & Arn* was collected from near K.R.Narayanan National Institute Of Visual Sciences And Arts, Thekkumthala, Kottayam, Kerala on 16 th december 2021 and leaves of *Pyrenacantha volubilis Wight* was collected from Sree Irumkulangara Durga Bhagavathi Temple, Manacaud, Thiruvananthapuram, Kerala on 31st December 2021. The plants were identified and authenticated by Dr. Rogimon P Thomas, Assistant Professor, Department of Botany,C.M.S College Kottayam, Kerala, India.

Preparation of Plant Extract

Fresh leaves were washed and cleaned thoroughly under running water. The excess water was drained and the leaves were shade dried. Dried leaves were powdered to obtain a coarse powder. Dried leaf powder is placed inside a thimble, which is loaded into the main chamber of the Soxhlet extractor. The extraction of weighted leaf powder with respective volume of the solvent was carried out with its boiling point. Selected solvents were used for the extraction depending on their increasing polarity. The Soxhlet extractor is positioned into a flask containing the extracting solvent. The Soxhlet is then fitted with a condenser. The solvent is heated to reflux .The solvent vapour travels up a distillation tube and condensed into the extractor housing the thimble holding the solid. The condenser ensures that solvent vapour condenses and drips back down into the chamber housing the plant material. The chamber containing the material slowly fills with warm solvent. Some of the desired compound then dissolves in the warm solvent. When the Soxhlet chamber is almost full, the chamber is automatically emptied by a siphon side arm, with the solvent running back down to the extracting flask. This cycle may be allowed to repeat many times, over hours or days, until the solvent gets colourless in extracting chamber. During each cycle, a portion of the non-volatile compound dissolves in the solvent. After many cycles the desired compound is concentrated in the distillation flask. After extraction the solvent is removed typically by means of a rotary evaporator [10].

Phytochemical Analysis [11, 12]

Table 2: Phytochemical screening of EAESk &EAEPv

Experiment	Observation	Inference
1) Detection of Alkaloids The extracts were individually dissolved in dilute HCL and filtered. a) **Dragendroff's Test :** To 0.5 ml of filtrate added 1 ml of Dragendroffs reagent b) **Mayer's Test :** To 1 ml of filtrate, added a few drops of mayer's reagent c) **Hager's Test :** To 1 ml of filtrate, added a few drops of Hager's reagent d) **Wagner's test :** To 0.5 ml of filtrate add 1 ml of Wagner's reagent	An orange red Formation of white or pale precipitate Produce yellow precipitate Formation of yellow or brown precipitate	Indicates the presence of alkaloids Shows the presence of alkaloids Indicates the presence of alkaloids Confirm the presence of alkaloids

2) Detection of Carbohydrate **The extracts were individually dissolved in distilled water and filtered** **a) Molisch'sTest :Few drop of Molisch's reagent was added to 2ml of extract and shaken Con.H_2SO4 was added from the side of the test tube.** **b) Fehling's Test : Filtrates were hydrolysed with dil.HCL, neutralised with alkali and heated Fehling's A & B solutions and heated again for 5-10 min.** **3) Detection of Proteins and Aminoacids** **a) Xanthoprotein Test : The extracts were treated with drops of conc. Nitric acid** **b) Biuret Test : To 3 ml of the test extract, 4% NaOH and few drops of 1% CuSO4 solution was added.**	**Formation of violet ring at the junction of the solution** **Formation of red or brick red precipitate** **Formation of yellow colour** **Formation of violet or purple colour**	**Indicate the presence of carbohydrates** **Indicate the presence of carbohydrates** **Indicate the presence of proteins** **Indicate the presence of proteins**
4) Detection of Glycosides **Extracts were hydrolysed with dil.HCL and then**		
subjected to test for glycosides a) **Extract were treated with Iml water and aqueous NaOH** b) **Legal Test : To the extract,1 ml pyridine and 1 ml sodium nitroprusside** **was added**	**Formation of yellow colour Pink to blood red colour**	**Presence of glycosides** **Presence of glycosides**
5) Detection of Saponins **Foam Test : The drug extract of drug powder on vigorous shaking with water for 10 minutes**	**Foam formation**	**Presence of saponins**
6) Detection of Flavonoids a) **Alkaline reagent Test: Extract were treated with few drops of NaOH solution.** b) **Lead acetate Test: Extracts were treated with lead acetate solution.**	**Formation of yellow colour and disapperance of yellow colour on addition of dil. H_2SO4** **Formation of yellow colour**	**Indicates the presence of flavonoids** **Indicates the presence of flavonoids**
7) Detection of Tannins **Ferric chloride Test: To 2 ml of extracts, a few drops of 5% aqueous ferric chloride solution was added.**	**Formation of bluish blackcolour, which disappears onaddition of a few ml of dil. H_2SO4 and formation of yellowish brown colour**	**Indicates the presence oftannins**
8) Detection of Phenols **a) Ferric chloride test: To 1ml extract add 2 ml of distilled water followed by addition of 10% solution of aqueous ferric chloride drop wise** **Lead acetate test: 1 mlextract was diluted to 5 mlwith distilled water and tothis add few drops of 1%** **aqueous solution of leadacetate**	**Formation of blue or green precipitate** **Formation of yellow precipitate**	**Indicates the presence of phenols** **Indicates the presence of phenols**

Antibacterial Activity

Antibacterial activity was determined using agar disc diffusion method. The cultures were swabbed on nutrient agar plates and sterile discs (5mm) immersed in different concentrations (250 µg/ml, 500 µg/ml and 1000 µg/ml) of the samples (EAEPv, EAESk) , positive control (streptomycin – 1000 µg /ml) and ethyl acetate were placed over the agar surface using sterile forceps. The plates were then incubated at 37 0C for 24-48 hrs. After incubation, the zone of inhibition around discs was measured [13, 14].

Anti-Inflammatory Activity

The reaction mixture (0.5 ml) consisted of 0.4 ml BSA (3% aqueous solution) and varying concentrations of test sample. The samples were incubated at 37 ºC for 20 min and 2.5 ml phosphate buffered saline (pH 6.3) was added to each tube and and then heated at 80 ºC for 10 min. The absorbance was measured using spectrophotometer at 660 nm [15-17]. The percentage inhibition of protein denaturation was calculated as follows:

$$\frac{Abs\ control - Abs\ sample\ X100}{Abs\ sample}$$

Results and Discussion

Table 3 - Preliminary phytochemical screening of EAEPv

Phytochemical Constituents	Inference	
	EAESk	EAEPv
Alkaloids	Positive	Positive
Carbohydrates	Positive	Positive
Proteins & Aminoacids	Negative	Positive
Glycosides	Positive	Positive
Saponins	Negative	Positive
Flavonoids	Positive	Positive
Tannins	Positive	Positive
Phenols	Positive	Positive

The phytochemical analysis of EAESk and EAEPv was carried as the above described procedures. It has been found that tests for carbohydrates, flavonoids, alkaloids, glycosides, tannins and phenolic compounds give positive results and test for proteins & aminoacids as well as saponins give negative results in Sarcostigma kleinii and that tests for carbohydrates, proteins and aminoacids, flavonoids, alkaloids, glycosides, saponins, tannins and phenolic compounds give positive results in Pyrenacantha volubilis

Antibacterial Activity

Table 4 - Results of antibacterial activity upon treatment of EAEPv

		Zone of Inhibition (cm)	
Sample	Concentration (µg/ml)	*E.coli*	*B.subtilis*
EAEPv	250	0.6	0.7
	500	0.9	0.9
	1000	1.0	1.0
Solvent Control (Ethyl acetate)	-	0	0
Positive Control (Streptomycin)	1000	2.7	2.7

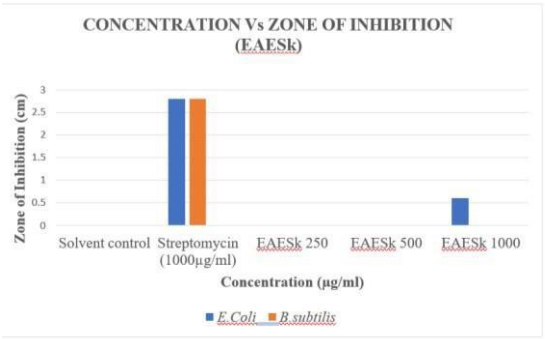

Figure 2 : Conc v/s Zone of inhibition EAESk

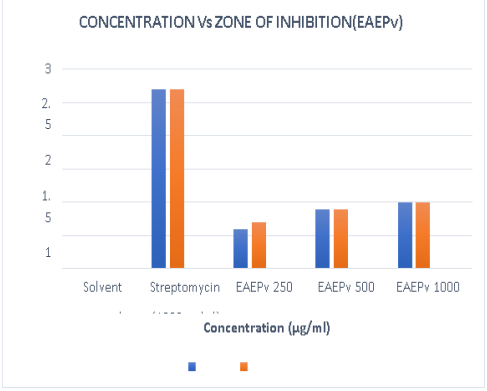

Figure 3: Conc v/s Zone of inhibitionPv

Table 5 - Results of antibacterial activity upon treatment of EAESk

		Zone of Inhibition (cm)	
Sample	**Concentration (µg/ml)**	***E.coli***	***B.subtilis***
EAESk	250	0	0
	500	0	0
	1000	0.6	0
Solvent Control (Ethyl cetate)	-	0	0
Positive Control (Streptomycin)	1000	2.8	2.8

Anti-Inflammatory Activity

Table 6 - % inhibition of Diclofenac

Standard	Concentration(µg)	OD at 660 nm	% of Inhibition
Control	-	0.807	-0-
Diclofenac	6.25	0.655	12.08
	12.5	0.631	15.30
	25	0.545	36.69
	50	0.403	45.90
	100	0.277	62.81

Table 7 - % inhibition of EAEPv

Sample I	Concentration (µg)	OD at 660 nm	% of Inhibition
Control	-	0.807	-0-
EAEPv	6.25	0.723	10.41
	12.5	0.716	11.28
	25	0.644	20.20
	50	0.545	32.47
	100	0.488	39.53

Table 8 - % inhibition of EAESk

Sample II	Concentration (µg)	OD at 660 nm	% of Inhibition
Control	-	0.807	-0-
EAESk	6.25	0.777	3.72
	12.5	0.747	7.43
	25	0.729	9.67
	50	0.632	21.69
	100	0.602	25.40

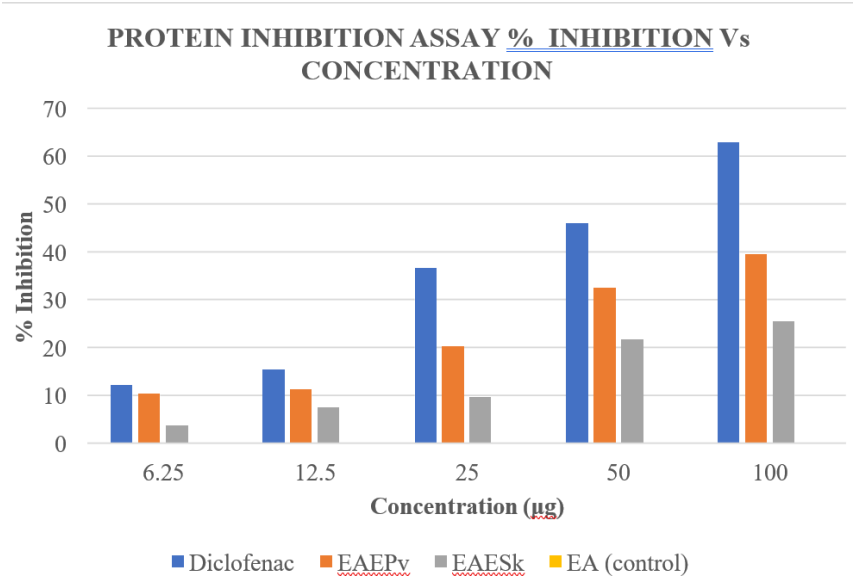

Figure 4: Protein inhibiton of EAESk & EAEPv

Summary and Conclusion

Present study was conducted for in vitro screening of Icacinaceous plants indigenous to kerala. Anti - inflammatory and antibacterial study of leaves of Sarcostigma kleinii & Pyrenacantha volubilis belonging to Icacinaceae family was performed. The phytochemical analysis of EAESk showed the presence of carbohydrates, flavonoids, alkaloids, glycosides, tannins and phenolic compounds. The phytochemical analysis of EAEPv showed the presence of carbohydrates, proteins and amino acids, flavonoids, alkaloids, glycosides, saponin, tannins and phenolic compounds. Activity of EAEPv & EAESk samples when tested microbiologically by the disc diffusion method, clear zones of inhibition were obtained. The zone of inhibition for *Pyrenacantha volubilis* was found to be 1cm for *E. coli* and *B. subtilis* at 1000 µg/ml. The zone of inhibition for *Sarcostigma kleinii* was found to be 0.6 cm for *E.coli* at 1000 µg/ml. *Pyrenacantha volubilis* showed better antibacterial activity than *Sarcostigma kleinii*. Anti-inflammatory activity study was carried out by protein denaturation inhibition assay using bovine serum albumin. Diclofenac was used as the standard and EAEPv& EAESk were the test samples. At 100 µg concentration Standard showed 62.81 % inhibition of protein, EAEPv showed 39.53 % and EAESk showed 25.40 % inhibition of protein.

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Conflict of interest

No Conflict of interest

Ethical approval and Inform Consent

Not Required

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