PHYTOCHEMICAL ANALYSIS OF *Alpinia officinarum* AND TO TEST ITS ANTI - OXIDANT, ANTI-MICROBIAL, ANTI-INFLAMMATORY AND ANTI - ARTHRITIC ACTIVITY

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ABSTRACT

Alpinia officinarum also known as sitharathai in Tamil belongs to the family Zingiberaceae. It is a traditional herb where the rhizome of the plant is mainly used to treat sore throat, cough and chest congestion. In this study, the phytochemicals analysis, the antimicrobial, anti-oxidant, anti- arthritics, anti-inflammatory were done using rhizome extract of Alpinia officinarum. The present study also proves that that it has many bioactive compounds like flavonoids, alkaloids, tannins, steroid and phenols justifies the use of rhizome extraction of the plant for the treatment of various diseases. Among all the properties that the extract possesses, the anti-oxidant activity was to be specifically high done by FRAP assay and DPPH assay. The antimicrobial activity was done against 3 gram-positive bacteria, 3 gram-negative bacteria and 2 fungi. Among which a fungi Aspergillus flavus showed highest zone of inhibition of 34 mm followed by bacteria Enterobacter faecium and Klebsiella pneumoniae with a zone of inhibition of 27 mm. The anti-arthritic activity was done by Bovine serum protein Denaturation method and Egg albumin Denaturation method and the result was 77.62% and 37.58% which was found to be considerably similar to that of the standard Diclofenac sodium i.e., 73.44% and 26.0%. The anti-inflammatory activity was done where the membrane stabilization and hemolytic activity was checked. It was found that as the concentration is increased from 500, 750 and 1000 µg/ml; the membrane stabilization is increased with decreasing hemolysis. Thus the study justifies the use of the plant as traditional medicine for treatment of various ailments.

Keywords*: Alpinia officinarum, Phytochemical Analysis, Rhizome extract, Anti-oxidant, Antimicrobial, Anti-arthritic and Anti-inflammatory.*

INTRODUCTION

Medicinal plants are a gift to us from the nature as they provide a number of health benefits to us. In India these medicinal plants are used for about centuries for their properties and are still used to this date. *Alpinia officinarum (figure-1)* also known as sitharathai in Tamil belongs to the family *Zingiberaceae*. It is a traditional herb where the rhizome of the plant is mainly used to treat sore throat, cough and chest congestion.

India has a variety of traditional medicinal systems like Ayurveda, Siddha, Unani and a huge class of ethnomedicine. As a report by World Health Organization (WHO), over 80% of the people of developing countries

are relaying on the traditional medicines that are extracted from the plants for their primary health needs. Use of these traditional medicines for the preparation of modern medicinal preparation is indispensable and thus 'Phytomedicines' are a link between traditional and modern medicine.

The chemicals that are produced by plants are called as phytochemicals. These are produced by the plants primary and secondary metabolism. These phytochemicals are important for the plants and protect them from disease and damage caused by environmental hazards. The phytochemicals are majorly classified as primary and secondary metabolites. The primary metabolites are responsible for the basic development of the plant which includes the sugars, amino acids, proteins, nucleic acids, chlorophyll, etc.

(Figure-1 Alpinia officinarum)

Secondary metabolites are those which are needed for the survival of the plant in harsh environment. They forms the smell, colour and taste of the plants and secondary metabolites such as flavonoids, tannins, saponins, alkaloids, steroids are found to have other commercial applications like they can be used as coloring agents, as drugs, as flavoring agents, insecticides, pesticides, anti-bacterial and snit-fungal products. Moreover they can also be used to protect human form many diseases like cancer, diabetes, cardiovascular diseases, arthritis and aging etc. and thus the phytochemicals are considered very important till this date.

Materials and methods

Sample preparation

The samples were purchased from the nearby herbal shop at Coimbatore, Tamil Nadu, and India. The samples were washed; shade dried and was grinded into a fine powder and was stored in an air tight container until use in room temperature.

Preparation of plant extract

The sample extraction process was simple; the extractions are made in 100 % alcohol like ethanol and methanol. About 5 gram of powdered sample was weighed and added to the conical flask to which ethanol or methanol was added the flasks were sealed airtight and were kept in an orbital shaker at 120 rpm for 24-36 hours. The contents were filtered using filter paper and were stored at 4^{o}C until use.

QUALITATIVE ANALYSIS OF PRIMARY METABOLITES

Test for Carbohydrates

1. Benedict's test: About 0.5ml of the filtrate was taken to which 0.5ml of Benedict's reagent was added. This mixture was heated for about 2 minutes in a boiling water bath. The appearance of red precipitate indicates the presence of sugars.

2. Molisch's test: To about 2ml of the sample, 2 drops of alcoholic solution of α-napthol was added and to the mixture after being shaken well. Few drops of conc.H_2SO_4 were added along the sides of the test tube. A violet ring indicates the presence of sugars.

Test for Proteins

1. Biuret test: 2ml of filtrate was taken to which 1 drop of 2% copper sulphate solution was added. 1ml of 95% ethanol was added. Them it was followed by excess addition of KOH. The appearance of pink colour indicates the presence of proteins.

2. 2ml of extract was mixed with 2ml of water and about 0.5% of conc.HNO_3 was added. The appearance of yellow colour indicates the presence of proteins.

Test for amino acids

1. To 1ml of the extract, few drops of ninhydrin reagent (10mg of ninhydrin in 200ml of acetone) was added. The appearance of purple colour indicates the presence of amino acids.

Miscellaneous compounds

Test for resins

1ml of extract was taken and to this few ml of acetic anhydride was added. To this 1ml of conc.H_2SO_4 was added. The appearance of orange to yellow colour indicates the presence of resins.

Test for fixed oils and fats

1. Spot test: Small quantity of the extract was taken and pressed between 2 filter papers. The appearance of spots indicates presence of oils.

2. Saponification test: To the extract, few drops of 0.5N alcoholic KOH and few drops of phenolphthalein was added. This mixture was heated for about 2 hours. The formation of soap or partial neutralization of alkali indicates the presence of fixed oils or fats.

Test for Gums and Mucilage

To 1ml of extract, distilled water, 2ml of absolute ethanol was added with constant stirring. White or cloudy precipitate indicates the presence of gums and mucilage.

Test for Carboxylic acids

To 1ml of extract a pinch of sodium bicarbonate was added. The production of effervescence indicates the presence of carboxylic acids.

QUALITATIVE ANALYSIS OF SECONDARY METABOLITES

Test for alkaloids

1. Mayer's test: To a few ml of filtrate, 2 drops of Mayer's reagent was added. A creamy white precipitate shows a positive result for alkaloids.

2. Wagner's test (iodine-potassium iodine reagent): To about an ml of extract few drops of Wagner's reagent were added. Reddish-brown precipitate indicates presence of alkaloids.

3. To 5ml of extract, 2ml of HCL was added. Then, 1ml of Dragendroff's reagent was added. An orange or red precipitate shows a positive result for alkaloids.

Test for Glycosides

1. Borntrager's test: To 2ml of filtrate, 3ml of chloroform was added and shaken. The chloroform layer was separated and 10% ammonia solution was added. The pink colour indicates the presence of glycosides.

2. 5ml of extract was hydrolyzed with 5ml of conc. HCL boiled for few hours in a boiling water bath. Small amount of alcoholic extract was dissolved in 2ml of water and aqueous 10% NaOH was added. The presence of yellow colour was a positive result for the glycosides.

3. 2ml of extract is mixed with about 0.4ml of glacial acetic acid containing traces of H_2SO_4 was added. The production of blue colour is positive for glycosides.

Test for Cardiac glycosides (Killer-Killani test)

5ml of solvent extract was mixed with 2ml of glacial acetic acid and a drop of ferric chloride solution was added followed by the addition of 1ml of conc.H_2SO_4. A brown ring in the interface indicates the presence of deoxy sugars of cardenoloides. A violet ring may appear beneath the brown ring while acetic acid layer a green ring may also form just gradually towards the layer.

Test for Phenol

1. Gelatin test: To 5ml of extract, 2ml of 1% solution of gelatin containing 10% of NaCl is added. Appearance of white precipitate indicates the presence of phenol.

2. Lead acetate test: To 5ml of extract, 3ml of 10% lead acetate solution was added and mixed gently. The production of bulky white precipitate is positive for phenols.

Test for Polyphenols

1. To the 3ml of the extract 10ml of ethanol was added and were warmed in a water bath for 15 minutes. To this few drops of ferric cyanide (freshly prepared) was added. The formation of blue – green colour indicates the presence of polyphenols.

2. To 1ml of extract few drops of 5% solution of lead acetate was added. The appearance of yellow precipitate indicates the positive result for polyphenols.

3. To the 5ml of ethanolic extract, 0.1% gelatin solution was added. The formation of precipitate was positive for polyphenols.

Test for tannins

To 5ml of extract, few drops of neutral 5% ferric chloride solution were added. The production of dark green colour indicates the presence of tannins.

Test for flavonoids

1. To the aqueous solution of the extract, 10% of ammonia solution was added and heated. The production of fluorescence yellow is positive of flavonoids.

2. 1ml of extract was taken and 10% of lead acetate was added. The yellow precipitate is positive for inference for the flavonoids.

3. The extract is treated with conc.H_2SO_4 resulting in the formation of orange colour indicates the positive result for flavonoids.

4. To 5ml of dilute ammonia, the plant extract is added and shaken well. The aqueous portion is separated and conc.H_2SO_4 was added. The yellow colour indicates the presence of flavonoids.

Test for Phytosterols

1. The extract is dissolved in 2ml of acetic anhydride and to which 1 or 2 drops of conc.H_2SO_4 was added along the sides. An array of colour change indicates the presence of Phytosterols.

2. The extract was refluxed with alcoholic KOH and saponification takes place. The solution was dilutes with ether and the layer was evaporated and the residue was tested for Phytosterols. It was dissolved in dilutes acetic acid and few drops of conc.H_2SO_4 are added. The presence of bluish green colour indicates the presence of polysterols.

Test for Phlobatannins

Aqueous extract was boiled with diluted HCL leading to the deposition of reddish precipitate indicates the presence of Phlobatannins.

Test for saponins

1. 0.5mg of extract was vigorously shaken with few ml of distilled water. The formation of frothing is positive for saponins.

2. The froth from the above reaction is taken ad few drops of olive oil was added and shaken vigorously and observed for the formation of emulsion.

Test for Steroids

To 2ml of the extract, 2ml of chloroform and 2ml of conc.H_2SO_4 was added. The appearance of red colour and yellowish green fluorescence indicates the presence of steroids.

Test for xanthoproteins

1ml of extract is taken and to this few drops of nitric acid and ammonia are added. Reddish brown precipitate indicates the presence of xanthoproteins.

Test for Terpenoids (Salkowski test)

3ml of extract was taken and 1ml of chloroform and 1.5ml of conc.H_2SO_4 was added along the sides of the tube. The reddish brown colour in the interface is considered as positive for presence of Terpenoids.

Test for Coumarins

To 2ml of the extract, 3ml of 10 aqueous solution of NaOH was added. The production of yellow colour indicates the presence of coumarins.

Test for quinones

To 1ml of extract, alcoholic KOH is added the presence of red to blue colour indicates the presence of quinones

Antimicrobial activity

Antimicrobial activity of the sample was done by well diffusion technique using 3 gram-positive bacteria, 3 gram-negative bacteria and 2 fungal pathogens. The gram-positive bacteria include *Enterobacter faecium, Streptococcus pyogenes, Staphylococcus aureus* and gram-negative bacteria which includes *Escherichia coli, Klebsiella pneumoniae, and Pseudomonas aeruginosa*. And two fungal pathogens include *Candida albicans and Aspergillus flavus* all the above said microorganisms are acquired from the Kovai Medical center and Hospital, Coimbatore. The bacterial culture was spread on the Muller-Hinton agar plates and the fungal culture on Sabouraud dextrose agar. The plates were then swabbed with respective pathogens. Wells were cut using well puncture at 6mm in diameter which was then filled with 50µl of extract in aseptic condition. The plates were then incubated at 37^{o}C for 24 hours for bacterial culture and 28^{o}C for 72 hours for fungal culture. The antimicrobial activity was calculated by measuring the zone of inhibition.

Anti-oxidant activity

1. FRAP Assay

To 100µl of the sample, 400µl of water was added. To this 1.25ml of phosphate buffer (pH-7.0) and 1.25ml of potassium ferrocyanide was added and heated in water bath for 15 minutes. Then 1.25ml of 10% trichloroacetic acid and 0.5ml of ferric chloride was added. Presence of green colour indicates the presence of anti-oxidant in the sample.

2. DPPH Assay

The test samples and references dissolved in the methanol were mixed with 0.1ml of alcoholic DPPH solution. The test samples and references were taken I different test tubes. The mixture was shaken vigorously at room temperature (25°C) I dark and left to stand for 20 minutes. Absorbance was measured at 517nm in UV-Vis spectrophotometer against the blank. The free radical scavenging activity is calculated by using a formula.

$$y = (A0 – A1/ A0) \times 100$$

Where,

A0 = DPPH control

A1 = sample extract

Anti-Arthritic activity

1. Bovine Serum Protein Denaturation Method:

Preparation of reagents

0.5% of Bovine serum Albumin (BSA): 500mg of BSA was dissolved in 100ml of water.

Phosphate Buffer Saline pH 6.3: 8g of sodium chloride (NaOH), 1.44g of disodium hydrogen phosphate (Na_2PO_4) and 0.24g of potassium dihydrogen phosphate (KH_2PO_4) was dissolved in 800ml of distilled water.

Test solution: 0.45ml of bovine serum albumin and 0.05ml of test solution of various concentrations was prepared.

Test control solution: 0.45ml of bovine serum albumin and 0.05ml of distilled water was prepared.

Product control solution: 0.45ml of distilled water and 0.05ml of test solution was prepared.

Standard solution: 0.45ml of bovine serum albumin and 0.05ml of Diclofenac sodium of various concentrations was prepared.

Method

To 0.05ml of various concentrations of test drugs (100, 200, 300, 400 and 500 µg/ml) standard drug Diclofenac sodium (100, 200, 300, 400 and 500 µg/ml) was taken and mixed with 0.45ml of BSA. The samples were incubated at 37°C for 20 minutes and the temperature was increased to keep the samples at 57°C for 3 minutes. Add 2.5ml of phosphate buffer to the above solution after cooling. The absorbance was measured using UV-Visible spectrophotometer at 225nm. The control represents 100% protein Denaturation. The results were compared to Diclofenac sodium. The percentage inhibition of protein Denaturation can be calculated.

Percentage Inhibition = 100 – [{(optical density of the test solution – optical density of control)/ optical density of test}/ x 100]

2. Egg Albumin Denaturation Method

The reaction mixture (5ml) Consisted of 0.2ml of egg albumin, 2.8ml of phosphate buffered saline (pH 6.4) and 2ml of varying concentrations of drug (100, 200, 300, 400 and 500µg/ml). Equal volume of double distilled water was used as control. The mixtures was then incubated at 37 ± 2° in a BOD incubator for 15 minutes and the heated at 70°C for 5 minutes. The absorbance was measured at 660nm using the vehicle as blank. Diclofenac sodium at concentrations of 100, 200, 300, 400 and 500µg/ml was used as reference drug. The percentage inhibition of protein Denaturation was calculated by using the following formula

% inhibition = 100 x [Vt / Vc- 1]

Where,

Vt = absorbance of the test sample, Vc = absorbance of control.

Anti-inflammatory activity:

Membrane stabilization:

Preparation of Red Blood cells suspension (RBCs)

Blood was collected from healthy human volunteer who has not taken any steroidal anti-inflammatory drugs for 2 weeks prior to the experiment and transferred to the centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10 minutes and were washed 3 times with equal volume of normal saline. The volume of blood was measured and reconstituted as 10% v/v suspension with normal saline and mixed with equal volume of Alsever solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.42% NaCl) and centrifuged at 3000rpm. The packed cells were washed with isosaline and a 10% suspension was made. Extracts at various concentrations (500, 750, and 1000 µg/ml) was prepared using distilled water and to each concentration 1ml of phosphate buffer, 2ml of hypo saline and 0.5ml of HRBC suspension was added. It was then incubated at 37°C for 30 minutes and centrifuged at 300rpm for 20 minutes. The hemoglobin content of the supernatant solution was estimated spectrometrically at 560nm.

% inhibition of hemolysis = 100 x (OD1-OD2/OD1)

Where,

OD1= Optical density of hypotonic-buffered saline solution alone

OD2= Optical density of test sample in hypotonic solution

RESULT AND DISCUSSION

In this study, the phytochemicals present in the alcoholic extracts of *Alpinia officinarum* was analyzed using standard screening methods. The results reveal that the plant contains various bioactive constituents like phenols, tannins, alkaloids, flavonoids, steroids, quinones are present while proteins, carbohydrates, glycosides, amino acids are absent in the sample. The phytochemicals characteristics are summarize in the table – 1.

Table – 1: Qualitative Analysis of Phytochemicals in Rhizome of *Alpinia officinarum*

S. No	Phytochemicals	Extract of Rhizome of *Alpinia officinarum*
1.	Carbohydrates	Absent
2.	Proteins	Absent
3.	Amino Acids	Absent
4.	Gums and mucilage	Present
5.	Resins	Absent
6.	Fixed oils	Present
7.	Carboxylic acids	Absent
8.	Quinones	Present
9.	Alkaloids	Present
10.	Glycosides	Present
11.	Cardiac Glycosides	Present
12.	Phenol	Present
13.	Tannins	Present
14.	Flavonoids	Present
15.	Phytosterols	Present
16.	Phlobatannins	Absent
17.	Saponins	Absent
!8.	Steroids	Present
19.	Xanthoproteins	Present
20.	Terpenoids	Absent
21.	Coumarins	Present

Antimicrobial activity

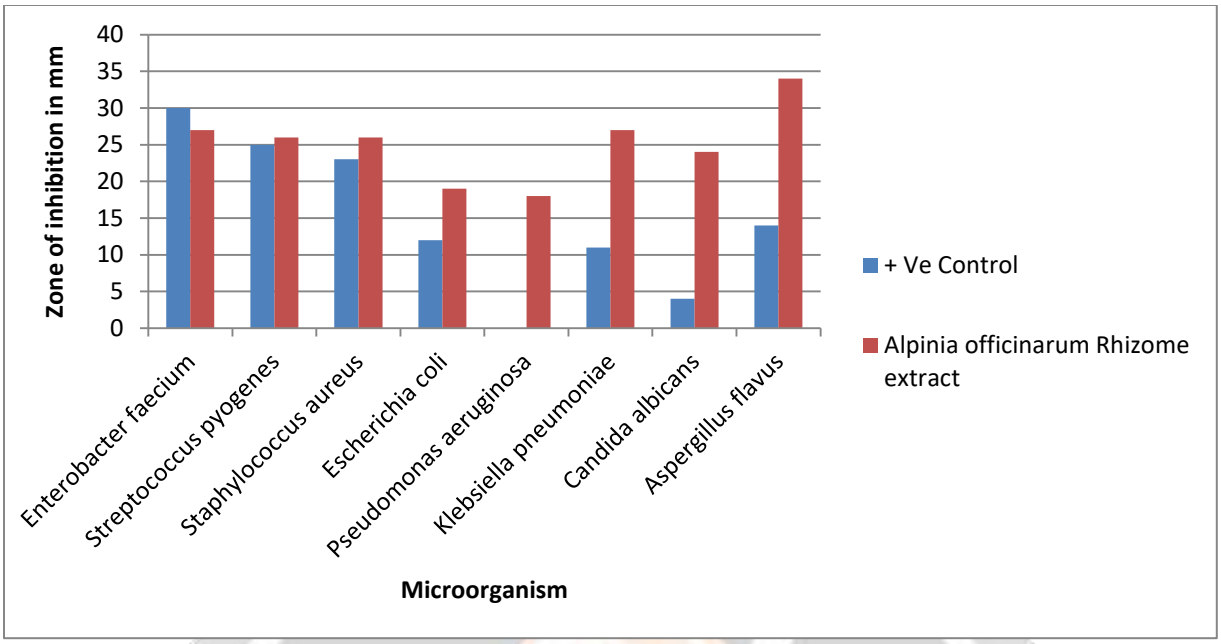
The present study shows the antimicrobial activity against three gram-positive bacteria *Enterobacter faecium, Streptococcus pyogenes, Staphylococcus aureus*, three gram-negative bacteria *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and two fungal cultures *Candida albicans and Aspergillus flavus*.

Tetracycline was used as the positive control. The extract of rhizome of *Alpinia officinarum* showed inhibition against all the microbial culture. The maximum zone of inhibition was against the fungi *Aspergillus flavus* 34mm and *Klebsiella pneumoniae* and *Enterobacter faecium* 27mm. The zone of inhibition of other cultures is shown in the table-2, figure -2 and figure -3.

Table-2: Antimicrobial activity of *Alpinia officinarum* Rhizome extract

S. No	Microbial culture	Zone of Inhibition (mm)	
		+ Ve Control	Sample
1.	*Enterobacter faecium*	30 mm	27mm
2.	*Streptococcus pyogenes*	25 mm	26mm
3.	*Staphylococcus aureus*	23 mm	26mm
4.	*Escherichia coli*	12 mm	19mm
5.	*Pseudomonas aeruginosa*	-	18mm
6.	*Klebsiella pneumoniae*	11 mm	27mm
7.	*Candida albicans*	4 mm	24mm
8.	*Aspergillus flavus*	14 mm	34mm

Figure-2: Antimicrobial activity of *Alpinia officinarum* Rhizome extract



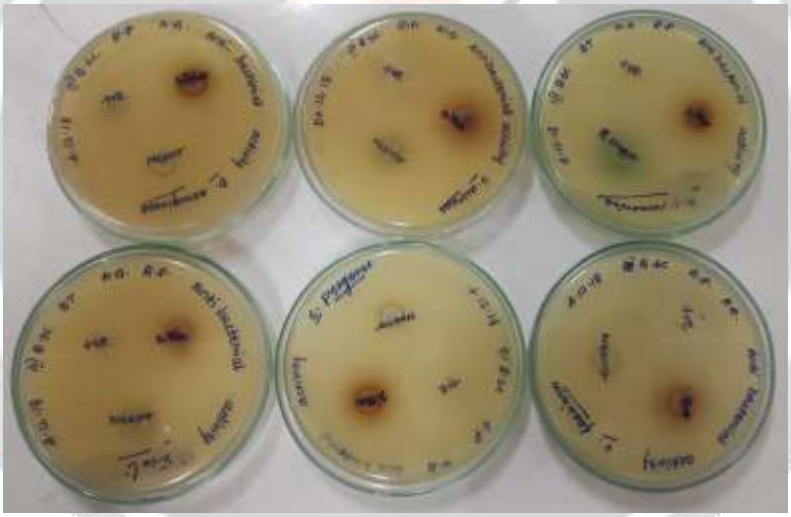


Figure 3- Antibacterial activity of *Alpinia officinarum*

Anti-oxidant activity

Antioxidants are the substances which protect the cells from caused by free radicals. They are produced in our body during break down of food or when exposed to tobacco smoke or radiations. These free radicals are one the important cause for heart disease, cancer and other diseases. The antioxidant potential of *Alpinia officinarum* rhizome extract was done by FRAP assay (figure-4) and DPPH assay. Since it a qualitative analysis, only the absorbance is measured. In FRAP assay, the production of green colour indicates the presence of antioxidants in the sample. In DPPH assay, the absorbance is measured at 517nm in UV-Visible spectrophotometer. The radical

scavenging activity of the extract was determined as a decrease in the absorbance of DPPH. It is said that, lower the absorbance value, higher the scavenging activity of free radicals.

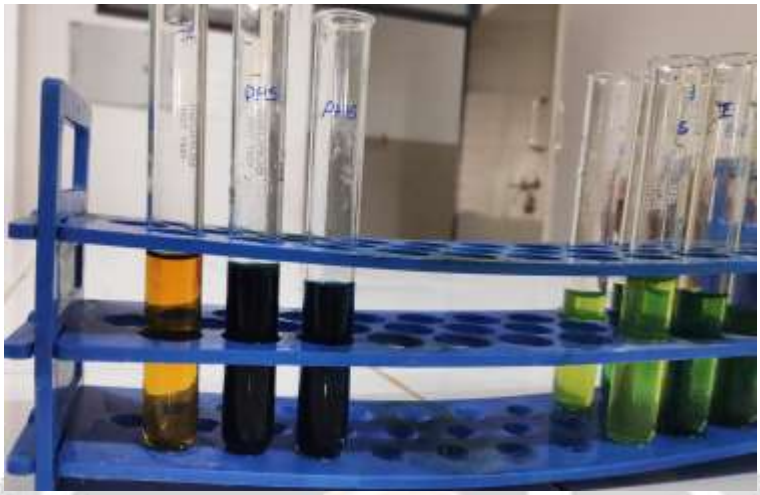


Figure-4: Antioxidant activity, FRAP assay

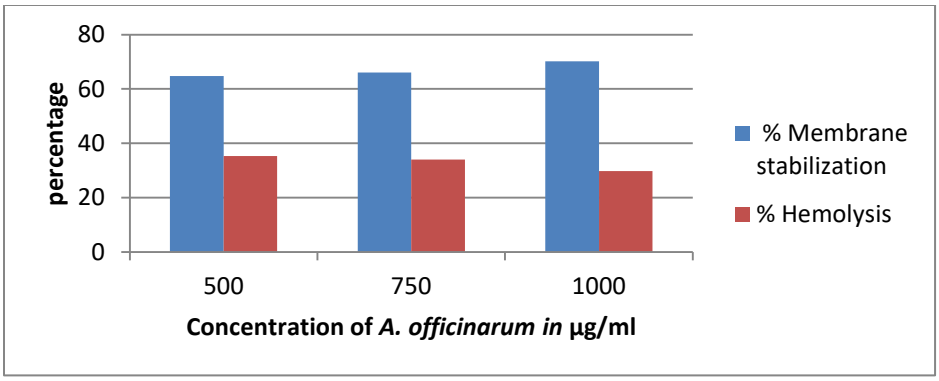
Anti-inflammatory activity

The anti-inflammatory activity of rhizome extract of *A. officinarum* was done by Bovine serum protein Denaturation method and egg albumin Denaturation method. The extract was taken at three different concentrations (500, 750 and 1000 µg/ml). The absorbance was read at 560nm at UV-Visible spectrophotometer. The extract of *A. officinarum* showed maximum inhibition rate of 35.29% at 500µg/ml and lowest inhibition rate of 29.83% at 1000µg/ml. As the concentration is increases, the membrane hemolysis is decreased and membrane stabilization is increased as shown in table 3 and figure -5. Hence the extract was effective in all the concentrations

Table-3: Anti-inflammatory activity of rhizome extract of *Alpinia officinarum*

Conc. µg/ml	%Membrane stabilization	% Hemolysis
	Alpinia officinarum	*Alpinia officinarum*
500	64.71	35.29
750	66.03	33.97
1000	70.17	29.83

Figure -5: Anti-inflammatory activity of rhizome extract of *Alpinia officinarum*



Anti-arthritic activity

The ant-arthritic activity by bovine serum protein Denaturation method at the concentration of 500µg/ml *Alpinia officinarum* extract showed 77.62% whereas in standard Diclofenac it was 73.44. In egg albumin Denaturation method at the concentration of 500µg/ml *A. officinarum* showed 37.58% where in standard diclofenac it was 26%. The anti-arthritic activity at different concentrations is shown in the table-4, figure -6 and table-5 and figure -7.

Table-4: Anti-arthritic activity

Bovine Serum Protein Denaturation method

Concentration µg/ml	% Anti – arthritic activity	
	Alpinia officinarum	Diclofenac sodium
100	57.88	59.31
200	60.73	59.85
300	67.01	62.12
400	75.18	68.36
500	77.62	73.44

Figure-6: Bovine Serum Protein Denaturation method

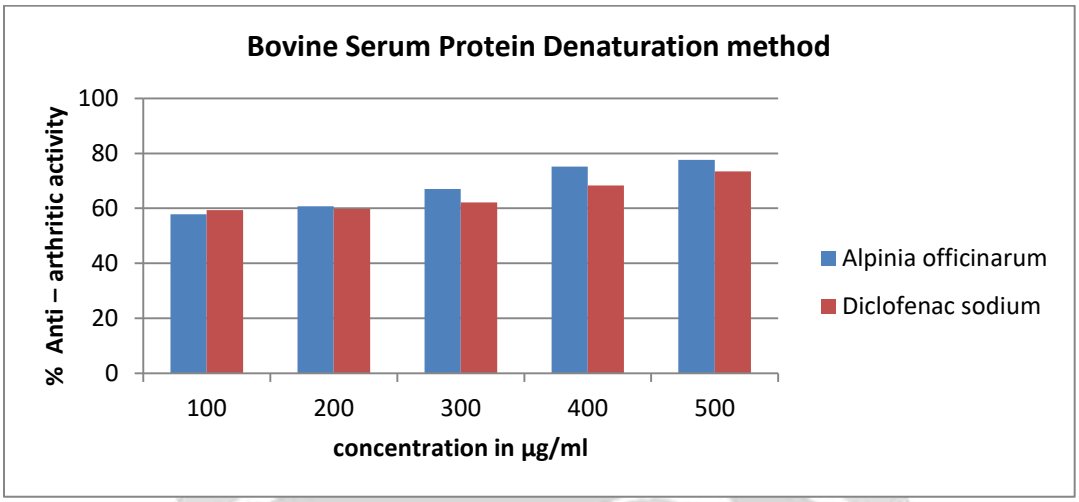
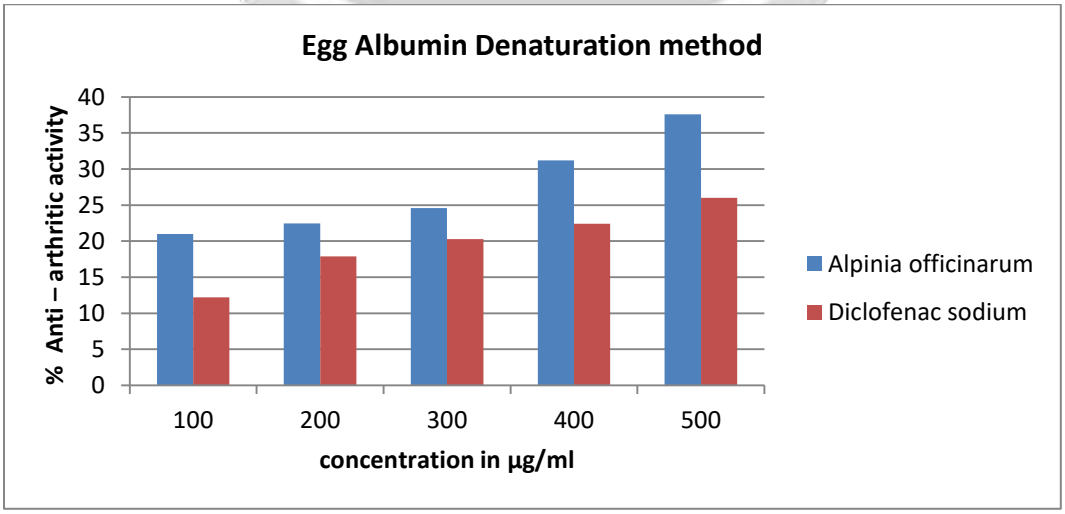


Table-5 Egg Albumin Denaturation method

Concentration µg/ml	% Anti – arthritic activity	
	Alpinia officinarum	Diclofenac sodium
100	21.0	12.2
200	22.45	17.9
300	24.58	20.3
400	31.20	22.4
500	37.58	26.0

Figure -7: Egg Albumin Denaturation method



Conclusion

Alpinia officinarum belongs to the family Zingiberaceae with high medicinal values and used as traditional medicine. The rhizome of this plant is mainly used for the treatment of various diseases. The rhizome was dried and powdered and dissolved in alcoholic solvent. The filtrate was then used for the study. The study reveals that the extract contains many bioactive compounds like alkaloids, flavonoids, phenols, tannins, steroids and also exhibit many activities such as anti-microbial, anti-inflammatory, anti-arthritic and anti-oxidant activities. Among which the anti-oxidant activity of the rhizome extract of *Alpinia officinarum* is considerably high compared to others. The extract also showed high rate of inhibition against microbial cultures. Thus this study justifies that the extract of Alpinia Officinarum can be used as an antimicrobial agent against bacteria and fungi and also can be used as an anti-inflammatory, anti – arthritic drug and as an anti- oxidant also.

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