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Identification & observation of anti-microbial activity in punica granatum leaf extract

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Abstract

This study finally indicated that leaf extracts were found to posse's good anti-microbial activity.Inhibition zone was observed by the leaf extracts. Hence, anti fungal activity was found by ethanol and aqueous extracts of leaf of *Punica granatum.*With this project on "Anti-microbial activity" Investigation on the leaf extract of *"Punica granatum"* We conclude that leaf extract of *Punica granatum* shows good Anti- microbial activity against Gram (+) ve and Gram (-) ve microorganisms. This may be because of Glycosides & Flavonoids.The powdered of leaf extract of Punica granatum has been subjected Soxhlet extraction by ethanol and Water as menstrum.By performing qualitative tests, water and Ethanol extract reveals the presence of alkaloids, carbohydrates, glycosides and phenolic compounds.Aqueous extract reveals the presence of alkaloids, glycosides and carbohydrates.The Anti-Microbial activity of extracts has been performed and its activity was determined.

Keywords: Punica granatum, Glycosides, Flavonoids.

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Introduction

Punica granatum, also known as *Punica Florida Salisb*, *Punica grandiflora hort. ex Steud*, *Punica nana L.*, and Punica spinosa Lam, is a native plant of northern Africa and the Caucasian. Mountains are widely distributed throughout the southern united state. The name pomegranate comes from the Latin Pomum meaning apple and granatus meaning full of seeds. The botanical name is derived from old French; Pomegrenate- Pomegranate apple it belongs to familyLythraceae [1,3].

INTRODUCTION TO PLANT

The Pomegranate, botanical name Punica granatum, is a fruit-bearing deciduous shrub or small tree that grows between 5 and 8 m (16 and 26 ft.) tall. It is also called Anar in India. It is a native plant of northern Africa and the Caucasian. Mountains are widely distributed throughout the southern united state. The name pomegranate comes from the Latin Pomum meaning apple and granatus meaning full of seeds. The botanical name is derived from old French; Pomegrenate- Pomegranate apple it belongs to family Lythraceae. Although previously placed in its own family Punicaceae, recent phylogenetic studies have shown that Punica belongs in the family Lythraceae, and it is classified in that family by the Angiosperm PhylogenyGroup [2,4].

DESCRIPTION

Punica granatum

The Punica granatum has glossy, leathery leaves that are narrow and lance shaped. A shrub or small tree growing 5 to 10 m (16 to 33 ft) high, the pomegranate has multiple spiny branches and is extremely long-lived, with some specimens in France surviving for 200 years. *P. granatum* leaves are opposite or sub opposite, glossy, narrow oblong, entire, 3–7 cm (1 ¼–2 ¾ in) long and 2 cm (¾ in) broad. The flowers are bright red and 3 cm (1.2 in) in diameter, with three to seven petals. Some fruitless varieties are grown for the flowersalone. Fruits are berries with a strong skin, like leather, intermediate in size between a lemon and a

grapefruit, 5–12 cm (2.0–4.7 in) in diameter. Fruits have many seeds with fleshy and edible coats (called sacrotesta).

The number of seeds in a pomegranate can vary from 200 to about1400. *P. granatum* var. *nana* is a dwarf variety of *P. granatum*popularly planted as an ornamental plant in gardens and larger containers, and used as a bonsai specimentree [5,6].

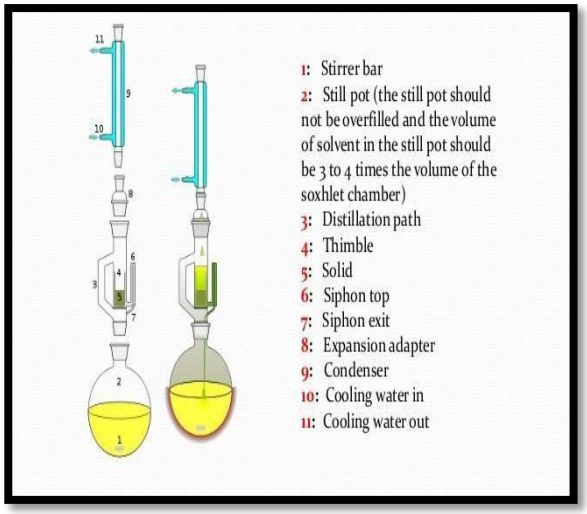
Kingdom:	Plantae
Clade:	Tracheophytes
Clade:	Angiosperms
Clade:	Eudicots
Clade:	Rosids
Order:	Myrtales
Family:	Lythraceae
Genus:	Punica
Species:	P. granatum

Pharmacological profile traditional use

The pomegranate tree was said to have flourished in the Garden of Eden and has been used extensively in the folk medicine of many cultures. The juicy pomegranate fruit with its multitudinous seeds was a popular symbol of fertility and fecundity in ancient times and it is counted among the seven kinds of produce with which the land is blessed. Doctors in Greece prescribed pomegranate juice as a remedy for inflammation, intestinal worms, persistent coughs, diarrhea, and dysentery [7,8].

Therapeutic Use

The pomegranate has been used in natural and holistic medicine to treat sore throats, coughs, urinary infections, digestive disorders, skin disorders, arthritis and to expel tapeworms. However, modern research suggests that pomegranates might to be useful in treating such serious conditions as prostate cancer, skin cancer, osteoarthritis and diabetes. Studies also show that pomegranate seeds might help rid the digestive system of fats. Clinical research shows that pomegranates, when part of a healthy diet, might help prevent heart disease, heart attacks and strokes. This is because pomegranates have the potential to thin the blood, increase blood flow to the heart, reduce blood pressure, reduce plaque in the arteries, and reduce bad cholesterol while increasing good cholesterol [9,10].



PUNICA GRANATUM

MATERIALS & METHODS

1. Collection of *Punica granatum*leaves
2. Extractionmethods:
   a) Soxhlet
   b) Maceration
   c) Preliminarymethods
   d) Activity

Materials: Punica granatum leaf, ethanol, water , Soxhlet

Methods: Maceration [11]

PROCEDURE:

The drug to be extracted is packed in a paper cylinder made from a filter paper and it is placed in the body of soxhlet extractor. The solvent is placed in the flask. The apparatus is then fitted as shown in the figure.

SOXHLET EXTRACTOR [12]:

When solvent is boiled on a heating flask it gets converted into vapours. These vapours enter into condenser through the side tube& get condensed into hot liquid which falls on the column of the drug. When the extractor gets filled with the solvent the level of siphon tube also raises upto its top. The solvent containing active ingredients of the drug in the siphon tube over and run into the flask. Thus emptying the body of extractor. This alternation of filling and emptying the body of extractor goes on continuously. The soluble activate constituents of drug remain in the flask while the solvent is repeatedly volatilized. The process of filling and emptying of extractor repeated until the drug is exhausted normally the process is repeated about 15 times for complete exhaustion ofdrug.

VACUUM EXTRACTION:

The process of extraction is done under reduced pressure in presence of vacuum for extracting active constituents present in the drug. It is costly process and not preferable in certain conditions [13].

DIGESTION:

The process in which the drug is extracted by heating at a particular pressure. This will increase the penetration power of men strum, so that there is complete extraction of drug.

INFUSION:

It consists of pouring over the drugs and allowed it to keep in a contact with eater for the stated period usually 15 min with occasional stirring and finally filtering off the liquid. The marc not pressed. The boiling water commonly used as a solvent, since it has a great solvent action than cold water [14].

DECOCTION:

In this process the drug is boiled with water for stated period generally 10 min after boiling the liquid is strained and water is passed through the content of strainer to make the required volume. This process is used for vegetable drugs of hard and woody materials[15].

EXPERIMENTAL WORK ON PUNICA GRANATUM

Leaf material collection:

The leaf material of *punica granatum* was collected from the ap. India in January 2020 and identity was confirmed by Dr. M. Venkaiah, associate professor, dep. of botany, University College of sciences and technology, Andhra University, Vishakhapatnam.

EXTRACTIONPROCESS:

The freshly collected leaf was dried and coarse powdered and passed through#20 the coarse powdered materials were extracted using various solvents like ethanol &water for about 8cycles bysoxhelation [16,17].

PROCEDURE [18,19,20]:

The powdered drug to be extracted is packed in a thimble form made of a filter paper and it is placed in the body of soxhlet extractor. The ethanol, water was placed in the flask and maintained temperature about71C. When solvent is boiled on heating the flask it gets converted into vapours. These vapours enter into the condenser through the side tube and get condensed into hot liquid which falls on the column of the drug. When the extractor gets filled with solvent, the level of siphon tube also raises upto its top the solvent containing active constituents of the drug in the siphon tube siphon over and run into the flask thus emptying the body of extractor. The alternation of filling and emptying the body of extractor goes on continuously. The soluble active constituents of the drug remain in the flask with the solvent is repeatedly volatilized. The process of filling and emptying of extractor is repeatedly until the drug is extracted. Leaf material is completely dried and kept for aqueousextraction. The crude extracts were concentrated

under vacuum. The extracts were stored in desiccators untol use.

Plant Material	Solvent used	Volume of the Solvent	Weight of the Extract	% Yield
Leaves (100 gm)	Ethanol	500ml	7.10gm	7.10
	Water	500ml	2.30gm	2.30

General Procedure for Phyto chemical Screening test [21]:

TEST FORALKALOIDS

Mayer's test: To small quantity of extract, Mayer's reagent was added. Cream or white Coloured

precipitate was formed, indicates the presence of alkaloids.

Dragandroff's test: To small quantity of extract dragandroff's reagent was added. Orange Brown precipitate was formed which indicates the presence ofalkaloids.

Wagner's test: To small quantity of extract Wagner's reagent was added. Presence of Reddish brown precipitate indicates the presence ofalkaloids.

Hager's test: To small quantity of extract, Hager's reagent was added. Formation of Yellow precipitate indicates the presence ofalkaloids.

TEST FORGLYCOSIDES[22]

Legal's test: To the hydrolysed 1ml of pyridine, few drops of sodium nitropruside were added. Then it was made alkaline with NaOH. Pink colour was observed which indicates the presence ofglycosides.

Baljet test: To the extract sodium pirate was added .yellow orange colour was observed which indicates the presence ofglycosides.

TEST FORTANNINS[23]

Ferric chloride test: To the extract add ferric chloride solution .Blue colour is formed for Hydrolysable tannins and green colour for condensedtannins.

Phenazone test: To the extract, add 0.5gms of sodium acid phosphate and filter. To the filtrate add 2% phenazone solution. Bully precipitate is formed which is oftencoloured.

Gelatin test: To the extract add 1% gelatine solution containing 10 % NaCl. Precipitate is formed.

S. no	Name of the test	Ethanol extract	Water extract
1	Liebermann Burchard (for terpenes &steroids)	+	+
2	Salkowski (for terpenes & steroids)	-	-
3	Mayers (for alkaloids)	+	-
4	Molish (for carbohydrates)	-	-
5	Fehlings (for carbohydrates)	-	-
6	Baljets (for cardiac glycosides)	-	+
7	Legal (for cardiac glycosides)	-	+
8	Test for phenolics (Fecl test)	+	+
9	Shinoda (for flavonoids)-	+	+

TEST FORFLAVONOIDS[24]

Shinoda test: To the extract, add few magnesium turnings and add concentrated Hcl drop wise. Pink scarlet crimson red or occasionally green to blue colour appears after few minutes indicate the presence offlavonoids.

Zinc hydrochloride test: To the extract add a mixture of zinc dust and concentrated Hcl. Red colour appears after few minutes indicates the presence of flavonoids.

Alkaline reagent test: To the extract, add few drops of sodium hydroxide solution. Intense yellow colour is formed which turns to colourless on addition of few drops of dilute acid indicates the presence offlavonoids.

TEST FORSTEROIDS

Salkowski test: Few drops of concentrated acid was added to the extract, shaken and on standing lower layer turns to red incolour.

Liedermann burchards test: To the extract few drops of acetic anhydride were added and mixed well. Few ml of concentrated sulphuric acid was added from the sides of the test tube. A reddish brown ring was formed at the junction of twolayers.

TEST FORSAPONINS

Foam test: Small amount of extract was shaken with little quantity of water .The foam persisted for10minutes it confirmed the presence of saponins.

TEST OFCARBOHYDRATES[25]

Molish test: (molish reagent) & conc. Sulphuric acid long the sides of the test tube. A reddish violet ring at the junction of two Liquids shows the presence ofcarbohydrates.

Fehling's test: The extract when heated with equal volumes of Fehling's A&B solutions gives a brick red precipitate showing the presence of reducingsugar.

TEST OFPROTEINS

Ninhydrin test: To the extract ninhydrine reagent was added and boiled. Purple colour was obtained indicates the presence ofproteins.

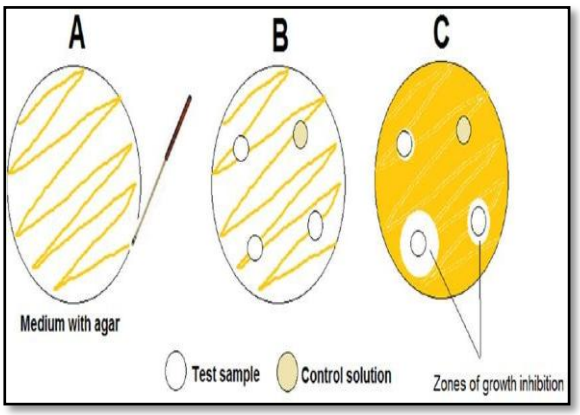
Biuret test: To the extract equal volume of 5% sodium hydroxide was added. To these 4 drops of 1% copper sulphate was added .violet colour obtained indicates presence of proteins.

SCREENING OF ANTI-MICROBIAL ACTIVITY:

ANTIMICROBIAL ACTIVITY:

Introduction:

The development of drug resistance in human pathogens against commonly used anti has necessitated a search for new Anti-microbial substances from other sources including plants. Plants are known to produce a variety of compounds to protect themselves against a variety of their pathogens and therefore considered as potential source to different classes of anti- microbial substance .Plants used in traditional medicine contain a wide range of substances that can be used to treat chronic as well as acute infectious diseases. The substances that can either inhibit the growth of micro-organisms or kill them are considered as candidates for developing new drugs for treatment of various



infectious diseases. The plants used in traditional system of medicine are well known as remedies for diseases in the rural areas of developing countries. Herbal medicines have been used in developing countries as an alternative to Allopathicmedicine.

Our extensive review of literature has revealed a variety of medicinal plants Possessing anti-inflammatory and hepato protective activity which also exhibited Good anti-microbial properties .On the basis of these reports the leaf extracts Were subjected for anti-microbial activity against variousmicrobes.

RESULTS & DISCUSSION

Methods employed for Anti microbial activity under present study:

Cylindrical plate method or cup plate method:

In the presence study, antimicrobial screening was carried out by cup plate method.

Test organism:Bacteria

Gram-positive organism:Bacillus subtilis

Gram negative organism:Escherichia coli

Fungi:-Aspergillus Niger

Standardization of micro-organisms:

One loop-full micro-organism were inoculated into 100 ml of sterile medium and incubated for 24 Hrs at 37C for bacterial culture.

After 24 Hrs incubation, 1 ml of broth containing micro organisms was added to 9 ml of peptone water. 10 fold serial dilutions were made in the range of 10 to 10. 100 µl of the dilutions ranging from 10-5 to 10-8 were spread over the sterile nutrient agar. (SDA) plates and kept at 37 and for 24 hr.

The number of colony forming units (CFU) was counted a Number of Micro- organisms per1 ml of stock culture was calculate.

Preparation of test and standard solutions:

The stock solution of test compounds was prepared by dissolving the dried extracts at a concentration of1mg/ml and pure compounds at a concentration of 1mg/ml in DMSO respectively. The stock solution of reference standards (streptomycin) was prepared at a concentration of 25 µg/ml sterile water. Anti microbial activity was screened by adding 0.05ml/50µl stock solution to each cup by micro pipette.

Culture medium:

The following media were used for our anti microbial studies.

Nutrient agar for bacteria:

Beef extract - 0.3%

Sodium chloride - 0.5%

Peptone - 0.5%

Agar - 2.0%

pH - 7.2-7.4

Sterilization:

Sterilization of the media ,water ,etc .,was carried out by auto claving at 15lbs/inch 2,121°C for 20 minutes .The glass ware like syringes , petridishes , pipettes ,or one hour The sterilized medium was cooled to 40° Cand poured into the petridishes to contain 6 mm thickness. The media was allowed to solidify at room temperature.

Stock solution:

Initially 10 mg extracts were weighed accurately and dissolved in 10ml ethanol to get concentration of 1000 µg/ml.

Principle:

Plant material	Dose (mg/ml)	Zone of inhibition(diameter in mm)		
		Gram(+)ve	Gram(-)ve	Fungi
		B.S	E.C	A.N
Ethanol	1000	12	12	14
	500	14	16	16
Aqueousextract	500	13	11	18
Standard:				
Streptomycin	25	16	18	15
Control:				
DMSO	50ml	-	-	-

The antimicrobial substance diffuses from the cup through a solidified Agar layer in a petridish or a plate to an extent so that the growth of added microorganism is in habited entirely in a circular area or zone around the cavity Containing the solution of a known quantity of antimicrobial substance. The Antimicrobial activity is expressed as the zone of inhibition in millimetres, which Is measured with a zone reader.

Determination of Zone of Inhibition by CUP-PLATE Method:

The anti microbial activity in terms of zone of inhibition of ethanol, and Aqueous Extracts of *Punica granatum,* was determined against4 different microorganisms and the results were compared with Streptomycin Standard. All the dilutions for the preparation of test (1000µg/ml) and standard (25µg/ml) drug were done in DMSO. The nutrient agar plates were prepared; the Inoculum was spread on the surface using a sterile cotton swab and using sterile Cork borer of 6 mm diameter made cups. Then the cups were filled accordingly .After introducing the sample, standard and control in the cups the petridishes were kept in refrigerator at 4º C for 2hrs, for diffusion and then incubated at 37ºCFor 24 hrs .The Anti- microbial activity was measured as a diameter in mm of Inhibitory zones on the agar Plates. The experiment was repeated in triplicate and the average value was written. The ethanol, Chloroform and Aqueous extracts of *Punica granatum* plant were screened for anti-microbial activity against a wide spectrum of microorganisms and the activity was compared with appropriate reference standards (streptomycin for both gram-positive and gram-negative organisms). Microorganisms were grown in nutrient agar medium. DMSO was used as control and the drug vehicles for the leaf extracts and reference standardsrespectively.

A= Standard

B= Sample (1000, 500)B=Ethanol extract

C= sample (1000, 500) C=Water extract

Measuring the zone of inhibition:

Inhibition zone of micro organisms:

The presence of definite zone of inhibition of any size around the cup Indicated antimicrobial activity .The solvent ethanol was run simultaneously to assess the activity of test drug which was used as a vehicle. The results were mentioned.

Allthe extracts at a concentration of 500 µg per each cup exhibited Anti-microbial activity against one or the other organisms in dose Dependent manner. *Punica granatum* leaf extracts of different polarities have shown good anti-microbial activity against Gram (+) ve and Gram (-) ve microorganisms. Ethanol extract, has exhibited inhibition against Gram (+) ve and Gram (-) ve. Ethanol and Aqueous extracts has exhibited moderate activity against Gram (+) ve and Gram (-) ve microorganisms. This is the first time report of Anti-microbial activity of *Punica granatum* extracts against these microorganisms.

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