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PHYTOCHEMICAL, ANTIOXIDANT AND ANTIMICROBIAL STUDY ON RIND OF THE FRUIT EXTRACT OF *Punica granatum* Linn.

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ABSTRACT

Punica is a small genus of fruit bearing deciduous small tree. According to legend, pomegranates grew in the Garden of Eden, and the fruit has been used as a folk medicine for thousands of years.[1] Pomegranate fruit peel is a rich source of polyphenoles, and also contain potentially pharmacologically active chemicals including the sapogenin steroid, Chlorogenin, flavonoids etc.[2] Peel contain phenolic punicalagins, gallic acid and other fatty acids, catechine and some flavonoids, flavons and anthocyanins.[3] This present study reveals an account of updated information about some of the physicochemical characters, phytochemical nature, antimicrobial activity and antioxidant activity of aqueous and alcoholic extract of the fruit rind. Here the physicochemical parameters like ash values, extractive values and total fiber contents were estimated. The total phenol and flavonoids content, inorganic minerals like sodium and potassium were estimated. The flavonoid present in the rind was isolated and its R_f values were calculated by HPTLC method, and it was compared with that of the standard epicatechine. The antioxidant activity of the alcoholic extract was evaluated by Reducing power ability, DPPH method and Hydrogen peroxide methods. Aqueous and alcoholic extracts were screened for their antibacterial and anti fungal activities and compared with that of the standards like Amikacin and Ketakonazole. The alcoholic extract shows significant antioxidant activity and both extracts shows good antimicrobial activity, but the aqueous extract is more active towards all bacteria and fungi organisms. This plant drug consist of polyphenols and flavonoids, it may have the antimicrobial and antioxidant activity, it has been used for many applications in food preservations, pharmaceuticals, Neutraceuticals and natural therapies.

KEYWORDS: epicatechine, pharmacognosy, Neutraceuticals, Amikacin, Ketakonazole.

1. INTRODUCTION

Punica is a small genus of fruit bearing deciduous small tree (*Punica granatum)* and it belongs to the family Punicacea. Pomegranate is a symbol of life pomegranate is originated from Persia and has been cultivated in central Asia, Georgina, America and the Meditaranian region for its medicinal value.[4] The fruit of the *Punica granatum* tree, has a storied history. According to legend, pomegranates grew in the Garden of Eden, and the fruit has been used as a folk medicine for thousands of years. More recently, it has been promoted as a "super food" that can relieve symptoms of many diseases.[5] In addition, there is some concern that pomegranate juice might interact with medications and making some less effective. Pomegranate fruit peel extract is a rich source of polyphenoles, chemicals in plants that provide their flavor and color.[6] The juice and rind have antioxidant properties than red wine and green tea, while the juice, rind, and oil from seeds contain isoflavones. Polyphenoles are also antioxidants, meaning they help protect cells from damage and may lower inflammation in the body.[7] The bark, fruit, root, and rind of the fruit, are used medically in Asia and the Middle East, but in the West the fruit and its juice are more often the parts being studied. They are useful in vitiated conditions of tridosha, diabetes, cough, asthma, bronchitis, cephalalgia, ophthalmopathy, dyspepsia, colic, flatulence, hyperacidity, peptic ulcer, erysipelas, skin diseases, leprosy, haematogenesis, anemia, emaciation, hepatopathy, jaundice, strangury, diarrhoea, dysentery, hemorrhages, leucorrhoea, menorrhagia, cardiac disorders, intermittent fevers and greyness of hair.[8]

Generation of free radicals or reactive oxygen species (ROS) during metabolism and other activities beyond the antioxidant capacity of a biological system gives rise to oxidative stress. Oxidative stress plays a role in heart diseases, neurodegenerative diseases, cancer and in the aging process.[9] This concept is supported by increasing evidence that oxidative damage plays a role in the development of chronic, age-related degenerative diseases, and that dietary antioxidants oppose this and lower risk of disease. Antioxidants are the substance that when present in low concentrations compared to those of an oxidisable substrate significantly delays or prevents oxidation of that substance. Apart from their role of

health benefactors, antioxidants are added in foods to prevent or delay oxidation of food, initiated by free radicals formed during their exposure to environmental factors such as air, light and temperature.[10] At present most of the antioxidants are manufactured synthetically. They belong to the class of synthetic antioxidants. The main disadvantage with the synthetic antioxidants is the side effects when taken in vivo. Strict governmental rules regarding the safety of the food has necessitated the search for alternatives as food and preservatives.

2. MATERIALS AND METHODS

2.1. Chemicals

1,1-diphenyl-2-picryl hydrazyl (DPPH), Ascorbic acid were purchased from Sigma-Aldrich, India. Hydrogen peroxide, trichloroacetic acid, ferric chloride, potassium dihydrogen phosphate, sodium hydroxide, potassium ferricyanide were purchased from Merck, Mumbai, India. Solvents and all the reagents used were of analytical grade.

2.2. Plant materials

Pomegranate fruits were collected in Coimbatore District, Tamilnadu, India and authenticated by Dr. G.V.S. Moorthy, Botanical survey of India, Coimbatore. Its voucher Number is BSI/SRC/5/23/2012-13/Tech.747. The voucher specimen has been submitted and preserved in herbarium for future reference. The fruit peels were separated from the fruit, collected, cleaned and shade dried. Then powdered and passed through mesh size 80 and stored in an air tight container.

2.3. Preparation of extract

About 1kg of drugs powdered and extracted with methanol and water separately by cold maceration method for 7 days. Then the extracts were filtered and the last traces of the solvent were evaporated under reduced pressure in a rotary evaporator. The yield of the dry extracts was calculated.[11]

2.4. Determination of ash values

About 2 g accurately weighed powdered drug was incinerated in a silica crucible at a temperature not exceeding 450^{o}C for 4 hours in a muffle furnace until free from carbon. It was then cooled and weighed. The % w/w of ash with reference to the air-dried drug was calculated. The acid insoluble ash, water soluble ash and sulphated ash was done according to the standard procedure. Average of the triplicate values was calculated.[12]

2.5. Determination of extractive value

Accurately weighed 5 g of air-dried powdered drug was macerated with 100 ml of 90% alcohol of the specified strength in a closed flask for 24 hrs, shaken frequently during first 6 hrs and allowed to stand for 18 h. It was then filtered rapidly, taking precautions against loss of the solvent and 25 ml of the filtrate were evaporated to dryness in a tared flat-bottomed shallow dish and dried at 100^{o}C to constant weight. The % w/w of alcohol soluble extractive value was calculated with reference to the air-dried drug .The same procedure was repeated with different solvents like chloroform, petroleum ether, benzene and water according to the standard procedure.[12]

2.6. Determination of Fibre content

3gm of the finally powdered crude drug wasweighed and extracted with petroleum ether at room temperature. Then the drug was dried from that 2gm of drug was taken for the estimation. The drugs were boiled separately with 300ml of dilute sulphuric acid for 30 minute. Filter the extract material through a muslin cloth and wash with boiling water. Then boil the material with 200ml dilute sodium hydroxide for 30 minutes. Filter through muslin cloth and wash with boiled water 25ml of alcohol successively. After washing the residue transfer to silica crucible, which was previously weighed (W1). Dry the residue for 2 to 3 hours for 130^{o} C and cool the crucible in the desiccators and weigh again (W2). Incinerate the residue for 30 minute at 100 ^{o} C and cool it to room temperature in desiccators and weigh again (W3). Then the total fiber content was calculated by using the following formula.

(W2-W1) - (W3-W1) / weight of sample x 100.[12]

2.7. Preliminary phytochemical analysis

The extracts were prepared with methanol and water as solvents and taken for the preliminary photochemical evaluation by standard procedure used to detect the nature of phytoconstituents present in them.[12]

2.8. Estimation of total Phenols

The total phenol content was determined according to the method described by (Siddhuraju and Becker, 2003). Ten micro litter aliquots of the extracts (2mg/2ml) were taken in test tubes and made up to the volume of 1 ml with distilled water. Then 0.5 ml of Folin-Ciocalteu phenol reagent (1:1 with water) and 2.5 ml of sodium carbonate solution (20%) were added sequentially in each tube. Soon after vortexing the reaction mixture, the test tubes were placed in dark for 40 min and the absorbance was recorded at 725nm against the reagent blank. The analysis was performed in triplicate and the results were expressed as tannic acid equivalents. Values are means of three independent analyses ± standard deviation (n = 3) TAE – Tannic acid equivalent.[13,14]

2.9. Estimation of total lipid content

Estimation of total lipid content was determined by (Chung et al., 1980,) method. About 10g of the samples was used to extract lipids with 150 ml of petroleum ether for 16 hr, at a solvent condensation rate of 2–3 drops/sec according to AACC Approved Method 30-25 with minor modifications of sample size and extraction time. The obtained extract was concentrated and evaporated at room temperature to dryness. The weight of extract gives the total lipid content which was expressed as mg/g dry matter.[15,16]

2.10. Estimation of total flavonoids

The flavonoid content was determined by the use of a slightly modified colorimetric method described previously by (Zhishen et al. (1999). A 0.5ml aliquot of appropriately (2mg/2ml) diluted sample solution was mixed with 2ml of distilled water and subsequently with 0.15ml of 5% $NaNO_2$ solution. After 6 min, 0.15 ml of 10% $AlCl_3$ solution was added and allowed to stand for 6 min, and then 2ml of 4% NaOH solution was added to the mixture. Immediately, water was added to bring the final volume to 5ml, and then the mixture was thoroughly mixed and allowed to stand for another 15min. Absorbance of the mixture was determined at 510 nm versus water blank. The analysis was performed in triplicate and the results were expressed as rutin equivalent. Values are means of three independent analyses ± standard deviation (n = 3) RE – Rutin equivalent.[17]

2.11. Determination of sodium, potassium and calcium by flame photometer

An accurately weighed amount of the ash of the plant was digested with 5 mL of 10% HCl and filtered through Whatman No.4 filter paper. The residue was washed with hot water, cooled and made to volume. The sample solution was then compared in the flame photometer against standard solutions of NaCl, KCl and $CaCO_{31}$ containing the same amount of HCl. The concentrations of the sodium, potassium and calcium ions were calculated by extrapolation method.[18,19]

2.12. Phytochemical screening and high performance thin layer chromatography (HPTLC)

The methanolic extract was subjected to preliminary phytochemical screening to identify the presence of various phytoconstituents present in the extract. Commercially available precoated HPTLC plates of Silica gel 60 F_{254} (Merck, India) were used for the study. The solutions of the three extracts were applied on the respective HPTLC plates using Linomat IV applicator. The plates were dried after application. Twenty microlitres of methanolic fraction of the extracts were spotted in the form of a band and also ascorbic acid and epicatechin also spoted using Linomat IV Sample Applicator (Camag, Switzerland).TLC pattern was developed using Toluene: ethyleacetete (9:1). Then the plates were scanned in Camag Scanner at a wavelength of 365nm. Peak areas and peak heights were recorded from which percentage of separated compounds were determined.[20,21]

2.13. ANTIOXIDANT ACTIVITY

2.13.1. DPPH radical scavenging activity

The free radical scavenging activity was measured by (Kumaran A. 2007) method, the decrease in absorbance of methanolic solution of DPPH. A stock solution of DPPH (33mgL-1) was prepared in methanol and 5ml of this stock solution was added to 1ml of the plant extract solutions at different concentrations (25,50,75,100,150,200, 250µg/ml-1). After 30min, absorbance was measured at 517nm and compared with the standard ascorbic acid (10-50µgml-1) pH 7.4.Percentage of DPPH scavenging activity of the plant extracts and the standard was calculated. The percentage extract of inhibition was calculated by the formula [(Ao-A1)/Ao] x100, when Ao is the absorbance of the control & A1 is the absorbance of the extract/standard.[22]

2.13.2. Reducing power determination

The reducing power of the extracts was determined by (Oyaizu.M.1986) method with different concentrations of extracts/standard (50-250mgm/ml) in methanol were mixed with phosphate buffer (PH 6.6) and incubated with (2.5ml) of potassium ferricyanide solution (1%w/v) at 50^{o}C for 20 min. Then 2.5 ml of trichloro acetic acid was added to the mixture and which was then centrifuged for 10min. The supernatant (2.5ml) was mixed with distilled water (2.5ml) and ferric chloride (0.5ml) and the absorbance was measured at 700nm. Increased absorbance of the reaction mixture indicates increased reducing power.[23]

2.13.3. Scavenging of hydrogen peroxide

The ability of three extracts to scavenge hydrogen peroxide was determined (Ruch. R.J.1984) by a solution of hydrogen peroxide (2mol/l) was prepared in phosphate buffer (PH 7.4). Hydrogen peroxide concentration was determined by spectrometrically absorbance at 230nm.Extracts were prepared at the concentration of 50-250mgm/ml and added to the hydrogen peroxide solution (0.6ml). Blank solution contains phosphate buffer without hydrogen peroxide. For each concentration a separate blank sample was used for background subtraction.[24] The % of inhibition activity was calculated from the formula [(A0 – A1)/A0] X 100.Where A0 is the absorbance of the control and A1is the absorbance of extract/standard.

Statistical analysis

Values were represented as mean + SD of threeparallel measurements and data were analysed using the t-test.

2.14. ANTI MICROBIAL ACTIVITY

The antimicrobial activity of aqueous and alcohol extracts were determined by disc diffusion method.[25] The test organism used for the screening of anti bacterial activity four gram +ve bacteria(*Staphylococcus aureus, Staphylococcus pyogens, Bacillus subtilis, Entrococcus facecales*) and three gram –ve bacteria (*Salmonella typhi, Serratia, Proteus Mirabitis* and *Psudomonasaureginosa)* and the test organisms used for the screening of anti-fungal activity is (*Aspergillus nigar, Candia albicans*). These above mentioned organisms were identified and procured from Microbiology Department, Karpagam University, Coimbatore. The stock cultures were maintained in nutrient agar slant at 4°C and sub-cultured. Working cultures were prepared by inoculating a loopful of each test microorganism in 3ml of nutrient broth from nutrient agar slants. Broths were incubated at 37°C for

12 hours. The suspension was diluted with sterile distilled water to obtain approximately 106 CFU/ml.[26]

Disk diffusion method

The In vitro antibacterial screening of the crude extracts was carried out by the disc diffusion method (Bauer AW, KIRBET). Disk diffusion method is equally suited to screening of antibiotics or the products of plant evaluation and is highly effective for rapidly growing microorganisms and the activities of the test compounds are expressed by measuring the diameter of the zone of inhibition.[27] In this method the compounds are applied to the agar medium by using paper disc. This method is semi quantitative test to find out the organism as susceptible, intermediate or resistance to the test materials as well as bacteriostatic or bactericidal activity of the compound.

The extract was dissolved in millipore water and methanol to produce a concentration of 100µg/ml and 200µg/ml. The sterile discs were prepared by impregnating in the above different extracts and slightly dried to evaporate the solvent. The disk was completely saturated with the extract and allowed to dry. Mueller Hinton (MH) agar plates were swabbed with test bacteria and extract disks with one of the standard positive control disks (Amikacine) and (Ketoconazole) as a standard for antibacterial and anti fungal respectively was placed on the MH agar plate. DMSO was taken as the negative control. Plates were incubated overnight at 37°C.The zone of inhibition for the extracts against each organism is recorded in terms of millimeter (mm).[28,29]

3. RESULT AND DISCUSSION

3.1 Physiochemical Parameters

The physiochemical parameters like Ash values, extractive values, and fiber contentof the drug sample is calculated with reference to the weight of air dried powdered drug and the values are given in table 1.

Table 1: Physico chemical evaluation *of Punica granatum*.

S.NO	Parameters	Punica Powder
A	ASH VALUES	
1.	Total ash	9.7 % w/w
2.	Acid insoluble ash	3.2 % w/w
3.	Water soluble ash	3.78 % w/w
4.	Sulphated ash	5.45 % w/w
B	EXTRACTIVE VALUES	
1.	Methanol	17.23 % w/w
2.	Chloroform	0.42 % w/w
3.	Petroleum ether	0.93 % w/w
4.	Water	21.16 % w/w
C	Fiber content	0.49% w/w

3.2. Phytochemical study

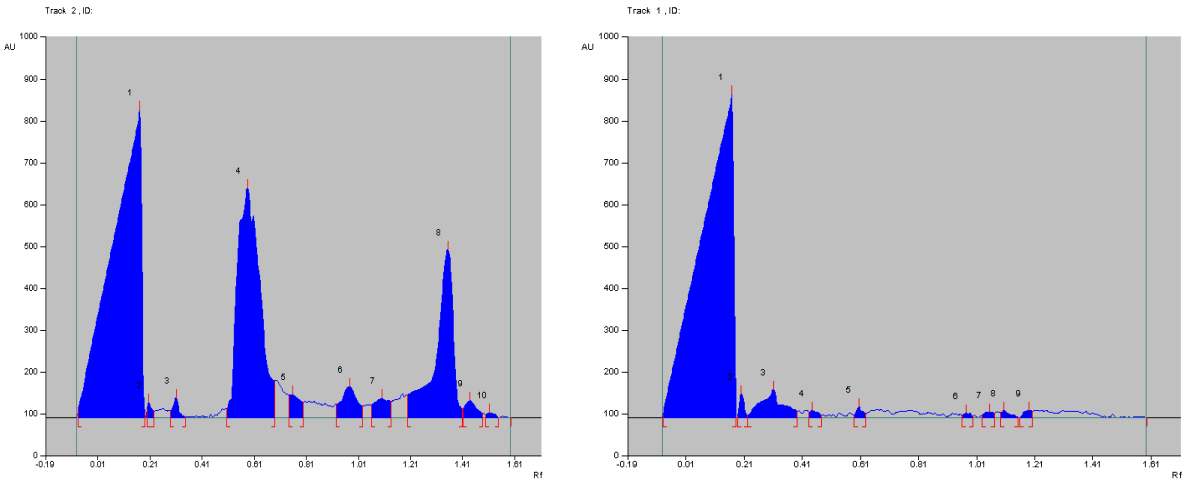
The qualitative phytochemical analysis of *Punica granatum* extracts showed the presence of majority of the compounds including alkaloids, carbohydrates, proteins & free amino acids, tannins, phenolic compounds, phytosterols and flavanoids. It is given in the table 2. The quantitative estimation quantifies the amount present in the extract of phenols, flavonoids, lipids and the minerals like sodium, potassium and calcium. The results were given in the table 3. Presence of flavonoid was identified by chemical test and it was estimated for quantification and the individual components were isolated and confirmed by HPTLC method. The isolated compounds R_f values were calculated. The results were mentioned in the table 4.

Table 2: Preliminary Phytochemical Analysis.

S.No	Chemical Test	Methanol	Acetone	PetroleumEther	Chloroform	Water
A	Alkaloids	+	+	+	+	+
B	Carbohydrates	+	+	+	+	+
C	Proteins and Free Amino acids	+	+	+	+	+
D	Tannins and Phenolic Compounds	+	+	+	+	+
E	Phytosterols	+	+	+	+	+
F	Flavanoids	+	+	+	+	+
G	Saponins	–	–	–	–	–

Table 3: HPTLC analysis of methanol extracts of the plant.

Sl.No	Particulars	No of Peaks	RF Value	Area	Area %
1)	Alcoholic extract of *Punica granatum*	9	0.17,**0.20**,0.31,0.59,0.76,0.98,1.10, 1.35,1.44,1.57	39507.5 292.8 183.6	48.44% 0.36% 0.23%
2)	Epicatechin	1	**0.20**	450.8	0.99%



SAMPLE **STANDARD**

Fig. 1: HPTLC Fingerprint.

Table 4: Quantitative estimation.

S.No	Parameters	quantity
1	Total Lipid (mg/g)Dry matter	12.3±0.08
2	Total Phenols(mg TAE/g extract)	32.56±2.50
3	Flavonoid content(mg RE/g)	3.62±0.20
4.	Sodium	56ppm
5.	Potassium	65ppm
6.	Calcium	113ppm

3.3. Antioxidant Activity

Free radicals, mainly the Reaction Oxygen Species are involved in initiation, promotion and progression of carcinogenesis. Reactive Oxygen Species induce oxidative damage of DNA and other cellular components leading to cancer related mutations. Consequently antioxidants play an important role in the protection of human body against damage by reactive oxygen species and also the intakes of natural antioxidants has been associated with reduced risks of cancer and other diseases related with oxidative damages. The selected plant is possessing radical scavenging activity which is mostly related to the phenolic compound and the phenolic hydroxyl group.[30] The concentration of hydrogen peroxide in water varies according to the phenolic compound. Since phenolic compounds present in the extract are good electron donors, they may accelerate the conversion of H_2O_2-H_20.Hydrogen peroxide is mainly produced by enzymatic reaction.[31] These enzymes are located in microsomes, peroxysomes and mitochondria. In plant and animals cells super oxide dismutaseis able to produce hydrogen peroxide by dismutation of oxygen, thus contributing to the lowering of oxidative reactions. The natural combination of dismutase and catalase contributes to remove hydrogen peroxide and thus has a true cellular antioxidant activity. Hydrogen peroxide is also able to diffuse easily through cell membrane.[32,33] The generation of hydrogen peroxide by activated phagocytes is known to play an important part in the killing of several bacterial and fungal strains. The ability of the plant extracts to scavenge hydrogen peroxide is followed by decay in hydrogen peroxide concentration. By Hydrogen peroxide method the drug shows the IC_{50}120µg/ml when compared with the standard ascorbic acid40µg/ml and scavenging ability of drug was evaluated by DPPH method, it shows the IC_{50} value of 140µg/ml when compared with the standard epicatechin 48µg/ml. It is noticed that the extract is capable of scavenging hydrogen peroxide in an amount dependent manner. The drug extract shows significant effect by reducing power determination method and shows the IC_{50} values of 135 µg/ml compared with the standard 37µg/ml. The results of antioxidant activity of the plant and their I.C.$_{50}$ values are shown in the Figure 2, 3, and 4. The result of free radicals scavenging activity of ethanol extract of the plants and the positive control ascorbic acid, in hydrogen peroxide free radical system and by DPPH scavenging method the extract showed potent antioxidant activity with reference IC_{50} value of the standard.

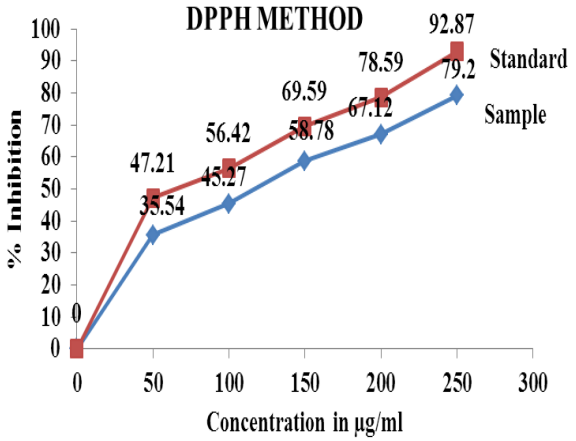


Fig.2

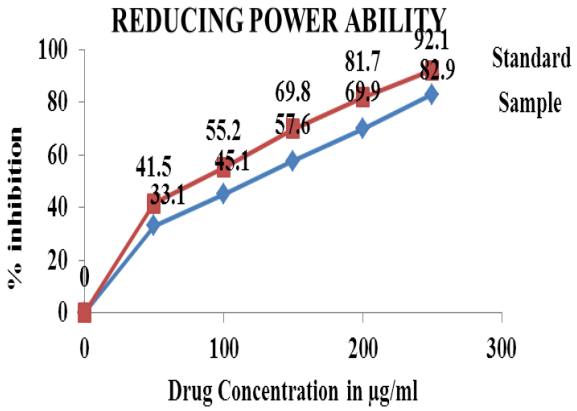


Fig.4

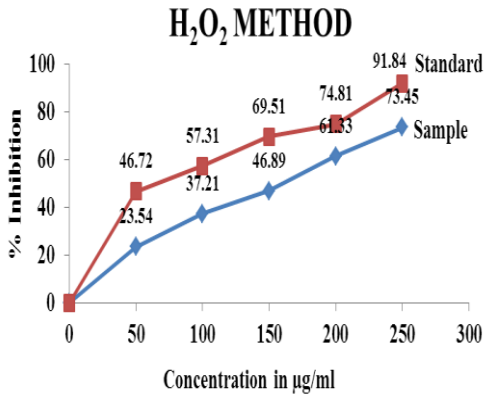


Fig.3

3.4 Anti Microbial Activity

The antimicrobial activity of the drug in aqueous and alcohol extracts was determined by disc diffusion method. The zone of inhibition obtained is given in the table no. 5.

Table 5: Antimicrobial activity of extracts

Sl. No.	Name of the Organism	*Punica granatum Zone of inhibition in mm*					
		WATER			ALCOHOL		
	BACTERIA	**100µg**	**200µg**	**Standard**	**100µg**	**200µg**	**Standard**
1	*Staphylococcus aureus* (+ve)	20	25	18	22	26	18
2	*Streptococcus pyogenes* (+ve)	21	23	17	25	27	17
3	*Bacillus subtilis* (+ve)	20	25	20	22	25	20
4	*Entrococcus facecales* (+ve)	22	27	17	27	30	17
5	*Salmonella typhi* (-ve)	21	26	17	25	29	17
6	*Serratia* (-ve)	21	26	19	25	29	17
7	*Psuddomonas aeruginosa* (-ve)	17	22	18	19	26	18
8	*Proteus mirabitis* (-ve)	18	22	18	25	28	18
	FUNGUS						
9	*Aspergillus flavus*	22	24	23	25	30	23
10	*Candida albicans*	20	26	20	25	30	20

Bacteria Standard: Amikacin
Fungal Standard: Ketokonazole

The extracts showed better activity and the higher diffusion rate or the degree of sensitivity of the tested microorganisms. [34] The extract contain more phyto chemicals that can be used to control diseases caused by these organisms. The different rate of inhibition could be due to the quantity of the phytochemical compounds present in the extracts. The extracts of the *Punica granatam* rind of fruit showed more pronounced activity towards gram negative and gram positive organisms. The phytochemical analysis of the extracts also justifies the presence of bioactive compounds alkaloids, terpenoids, Phenols, flavonoids and steroids, which are responsible for antimicrobial activity and also that more highly oxidized phenols are having more inhibitory to micro organisms.

CONCLUSION

The rind of the pomegranate fruit is more used in traditional system of medicine. Anyway in this study it was confirmed the presence of phenols and flavonoid. The antioxidant and Antimicrobial activity of the extracts were confirmed, where both of the extracts are having good activity but more in alcoholic extract. It can be used as a home medicine, simply as a decoction whenever necessary or it can be prepared as pickle. Rind powder can also be used in herbal face pack.

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