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STANDARDIZATION AND ANTI BACTERIAL ACTIVITY OF *COUROUPITA GUIANENSIS* FRUIT SHELL EXTRACT

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Abstract: Cannon ball (*Couroupita guianensis*) ripen fruits were collected in month of May; outer shell was removed and dried under shade for 15 days. The dried shell was powdered and fine powder (#80) was extracted with alcohol (95%) by maceration method. Extract was filtered by vacuum filtration and filtrate was evaluated for pH, viscosity, fluorescence specific gravity. The liquid extract was evaporated and dried under reduced pressure and % yield was determined. Standardization of powder sample was done as per WHO guidelines. The powder sample showed extractive values for alcohol (2.11%) and water (19.5%), moisture content (7.55%), total ash value (14.01 %), acid insoluble ash value (12.56%) sulphated ash value (15.45%) water soluble ash value (2.38%). Phytochemical screening showed presence of tannins, sugars and polyphenolic compounds. Alcoholic extract was screened for antibacterial activity by cylindrical cup plate method using four standard bacterial cultures *E.coli, S.aureus, B.subtilis, P.aeruoginosa.* the most significant activity was found against *B. subtilis* at concentration 4mg as compare to other tested organism.

Keywords: Antibacterial activity, Cup plate method, Maceration method, Phytochemical screening, Polyphenolic compounds, Shell extract

INTRODUCTION

Many efforts have been made to discover new antimicrobial compounds from various kinds of sources such as microorganisms, animals, and plants. One of such resources is folk medicines. Systematic screening of them may result in the discovery of novel effective compounds (Tomoko *et al.* 2002)[1]. The increasing prevalence of multidrug resistant strains of bacteria and the recent appearance of strains with reduced susceptibility to antibiotics raises the specter of untreatable bacterial infections and adds urgency to the search for new infection-fighting strategies (Sieradski *et al.* 1999)[2]. Many microorganisms can cause several diseases and in spite of the tremendous advancement of medical science and technology, diseases are the leading health problem particularly in the under privileged population in the remote rural areas in the developing countries. Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources, many based on their use in traditional medicine. Medicinal plants are used locally in the treatment of infections caused by fungi, bacteria, viruses and parasites and over 60% of people in Nigeria rural areas depend on the traditional medicine for the treatment of their ailments (Ghani et al., 1989)[3]. Different plants have been used as a source of inspiration in the development of novel drugs (Robbers et al., 1996)[4]. Plant derived medicines are widely used because they are relatively safer than the synthetic alternatives, they are easily available and cheaper (Iwu et al., 1999)[5]. Many plant species have been evaluated for their antimicrobial activity in the past 20 years (Castello et al., 2000)[6]. Since then efficacy of many medicinal plants in the treatment of many diseases have been put to test in many laboratories (Shajahan and Ramesh, 2004)[7].

Couroupita guianensis (Cannon ball) is a large deciduous tropical tree 90' tall and indigenous to the Amazon rainforest. The leaves, up to 6" long, are simple with serrate margin; it flowers in racemes; the yellow, reddish and pink with stunning fragrant. Flowers are large 3" to 5" waxy aromatic smelling growing directly on the bark of the trunk (cauliflory). Fruits are large globose woody look like big rusty cannonballs hanging in clusters, like balls on a string. The fruit contains small seeds in a white, unpleasant smelling edible jelly. The hard shells are used to make containers and utensils. Cannon ball flowers are considered of special significance in Buddhist culture in Sri Lanka. In Tamil Nadu, it is called Nagalingam flower. The sivalingam shape is visible at the center of the flower and snake shaped pollen is the specialty of this flower and it has very good fragrance. This rare flower can be used for Shiva Pooja. Plant is indigenous to rainforest of the Guianas in Northeastern South America; a popular ornamental in Caribbean and SE Asian botanic gardens and listed as a rare tree and flower in India[8].

Plant is used primarily as ornamental; fruits edible and occasionally eaten, but smell of white flesh discourages most people; fruit shells sometimes used as utensils; some fractions of the stem, bark and flowers exhibit antimicrobial activity. Different parts of the plant are studied for antioxidant, leaves for antimicrobial. Traditionally leaves as used as antiseptic and toothache. The fruit pulp, bark and flowers are used for medicinal applications and have antimicrobial- and fungal activity. The Cannonball Tree possesses antibiotic, anti-fungal, antiseptic and analgesic qualities. The trees are used to cure colds and stomach aches. Juice made from the leaves is used to cure skin diseases, and shamans of South America have even used tree parts for treating malaria. The inside of the fruit can disinfect wounds and young leaves ease toothache[9, 10, 11]. In Ayurveda, it's used extensively as an anti-inflammatory medicine. The volatile oils from the flowers show anti-bacterial and anti-fungal properties. It is one of the ingredients in the many preparations which cure gastritis, scabies, bleeding piles, dysentery, scorpion poison and many more[12].

MATERIALS AND METHODS

Plant was identified and authenticated by Dr. Madhukar Bachulkar, Taxonomist and Principal, Ashokarao mane college of arts and science, Pethvadagaon, Dist- Kolhapur, Maharashtra. Sample was collected in month of May. Fresh fruits after removing outer shell were used in experiment. Outer shell was dried and powdered. Ethanolic extract was concentrated in rotary film evaporator and dried by vacuum dryer. Microbial culture was obtained from NCIM, Pune. Standards ciprofloxacin, doxycyclin and fluconazole tablets were purchased locally. All other solvent and chemicals were of AR grade and procured from Loba chem.

Experimental:

Collection of raw material: fresh fruit were collected from Mahavir Garden, near Collector Office, Kolhapur (MS).

Preparation of extract[13]: Outer shell of fruit was removed by sharp knives. Shell was made into small pieces and dried under sunlight for 15 days. Then shell was put on maceration with the aqueous alcohol (70%) at room temperature for 24h. Extract was filter through muslin cloth and filtrate was screened for phytoconstituents and physical constants. Further filtrate was evaporated by rotary film evaporator and dried using vacuum dryer. A percentage yield was calculated. Dry extract was screened for antibacterial activity using agar diffusion method.

Evaluation of liquid extract: Liquid extract was subjected for physical evaluation[14]. The different parameters were checked such as color, pH, fluorescence, specific gravity, density and viscosity. The fluorescence study was made by using about 10 ml of extract in glass test tube along with solvent blank and observed in day light, short (254nm) and long (366nm) in UV cabinet. Specific gravity and density was determined by method described in[15] Martin using sp. gr. bottle. Viscosity was determined by using Oswald viscometer and time taken by water and each extract from A to B were recorded. Viscosity was determined by using formula

$$\eta_2 = \frac{\text{Density of extract x time taken by extract from A to B}}{\text{Density of water x time taken by water from A to B}} \times \text{Viscosity of water}$$

Where, η_2 is the viscosity of extract

The pH of extract was determined by indicator paper. After completion of physical analysis all the extracts were concentrated in rotary vacuum film evaporator and dried in vacuum dryer. The nature of residue obtained and yield was recorded. (Table No. 1) The dried extract was then stored in vacuum desiccators along with dehydrating agents like anhydrous $CaCl_2$ and silica sachets for further studies.

Table no.1: Physical evaluation of fruit shell extract

Sample	Color	Density	Sp.gr.	Viscosity	pH	Fluorescence		Nature of solid extract
						S	L	
Fruit shell	BB	0.773	0.8041	1.0236	4.5	LG	SG	Waxy

BB- Brownish Black, LG- Light Green, SG-Slight Green

Phytochemical screening of extract: About 500 mg of dried extract was dissolved in 100 ml of respective solvent and solution obtained was subjected for phytochemical screening using different specific and general reagents Samples were prepared as per the requirement of procedure and tests were repeated for final confirmation of phytoconstituents. (Table No. 2)

Table no.2:Phytochemical screening of extract

Carbohydrates	Proteins	Fixed oils	Glycosides	Tannins	Alkaloids	Steroids	Triterpenes
+	+	-	+	+	+	-	-

+ = present, - = absent

Standardization of dried fruit shell:

Determination of extractive value[16]: 2g powder sample drug was macerated with 100ml each of pure solvent for 24h. Maceration was carried out in 250 ml stoppered iodine flasks in rotary orbital shaker with 60 rpm at room temperature. After completion of maceration, samples were filtered using oil free vacuum pump and extractive values were determined by withdrawing 20ml of filtrate and evaporating to dryness at 45-50°C on thermostatic water bath. Five such determinations were made and average values are reported.

Determination of moisture content: 2g air dried powder sample was accurately weighed using electronic balance in a previously weighed porcelain dish and heated in a hot air oven at 105°C until concordant weights were obtained. The difference between final and initial weight was considered as moisture content. Five such determinations were made at identical conditions and average % of moisture content (w/w) was obtained

Determination of ash value:

a) Total ash value: 2g air dried powder sample was accurately weighed in previously weighed ash crucible. Each crucible was then kept on porcelain tripod stand inside the muffle furnace. The switch was made on and temperature was adjusted to 100°C for first half an hour with vent open. Then vent was closed and temperature was slowly increased up to 450°C. The appearance of red hot crucible or formation of white ash was considered as end time for experiment. Allowed the crucible to cool to room temperature and was removed from furnace and weighed. Difference in final weight to that of initial empty crucible weight was considered as ash value. Five similar determinations were made and % total ash value for each tested sample was obtained.

b) Acid insoluble ash value: The total ash obtained from the above experiment was used for determination of acid insoluble ash value. The total ash was dissolved in 25ml of 0.1N HCl in beaker and boiled for 5-10 minutes. The cooled sample was filtered through ash less Whatman filter paper (No.1) and residue was dried thoroughly in air. The residue along with filter paper was then subjected for re-ignition at 450°C in muffle furnace and upon cooling to room temperature the weight of residue left was considered as acid insoluble ash. Five such determinations were made and average acid insoluble ash value was determined.

c) Sulphated ash value: Air dried powder sample (2g) was accurately weighed in tarred ash crucible and moistened with 2ml of 0.1N H_2SO_4. Moist samples were kept in muffle furnace and heated initially at 100°C and then temperature was raised slowly up to 450°C till redness was obtained. The crucibles were cooled to room temp and residue was weighed. Difference between final and initial weight was considered as sulphated ash value. Average of five determinations was made and reported as sulphated ash value.

d) Water soluble ash value: The experiment was carried out similar to the determination of total ash value. The total ash obtained was dissolved in 25 ml of glass distilled water and filtered through ash less filter paper. The residue left out was dried in air and reignited in muffle furnace at 450°C. After cooling to normal temperature the residue was weighed. The difference between total ash and water insoluble ash was calculated and values were considered as water soluble ash. Five such determinations were made and % of different ash values were reported in (Table No. 3)

Table no.3 Standardization of dried fruit shell

Sr. No.	Extractive value*		% Ash value*				% Moisture Content*
	Alcohol	Water	Total	Acid Insoluble	Sulphated	Water soluble	
1	2.11	19.5	14.01	12.53	15.45	2.38	7.55

*Average of five determinations.

Screening of antimicrobial activity:

Bacterial and fungal strains: The test organisms were purchased from NCIM, NCL Pune. The organisms were sub-cultured in the media specified. The organisms, their ATCC code, media in which they are sub-cultured are given in Table No.4. Bacteria were incubated at 37°C in incubator for 24h. They were further stored at 4°C in the refrigerator to maintain stock culture.

Table No.4: Microorganisms with their ATCC Codes and media used for subculture

Sr. No.	Name of microorganism	ATCC Code	Media
01	*Escherichia coli*	8739	Nutrient Agar
02	*Bacillus subtilis*	6633	Nutrient Agar
03	*Pseudomonas aeruginosa*	27853	Nutrient Agar
04	*Staphylococcus aureus*	25923	Nutrient Agar

Antibacterial activity

Preparation of media:

Nutrient Agar: Accurately weighed 28g of nutrient agar was dissolved in the 1000 ml of distilled water by heating with frequent agitation. The media was finally sterilized in autoclave at 121°C for 15 min.

Preparation of test and standard drug solutions:

Preparation of test extract: Test extract was prepared freshly by dissolving 1g of previously dried extract in the 10 ml of respective solvent. This gives the 100 mg ml^{-1} of stock solution. From which 0.1, 0.2 and 0.4ml was diluted to 1 ml each and 0.1ml was used for testing the activity.

Preparation of normal saline solution: Accurately weighed 0.9g sodium chloride was dissolved in 100 ml of distilled water. Normal saline was sterilized before preparation of microbial suspension at 121°C for 15 min. in autoclave.

Preparation of standard drug solutions:

Doxycyclin: Weighed accurately 100 mg Doxycyclin, and dissolved in 100 ml of 0.1M hydrochloric acid to get 1000µg/ml stock solution. This was then diluted further with distilled water to get solution of 10µg/ml and 0.1ml was used in well.

Ciprofloxacin: Weighed accurately 100 mg Ciprofloxacin, and dissolved in 100 ml 0.1M hydrochloric

acid to get 1000µg/ml stock solution. This was then diluted further with distilled water to get solution of 10µg/ml and 0.1 ml was used in well.

Fluconazole: Weighed accurately 100 mg Fluconazole, and dissolved in 100 ml of DMF (5% in normal saline) to give 1 mg/ml stock solution. This solution was further diluted with buffer (which was prepared by dissolving 2g of dipotassium hydrogen phosphate and 8g of potassium dihydrogen phosphate in distilled water to produce 100 ml) to get solution of 10µg/ml. Working solution 0.1 ml of each was used in the well as positive control which will have test concentration of 1µg each of Doxycyclin, Ciprofloxacin and Fluconazole in the well.

Sterilization of equipments and media:

Dry heat sterilization: All the glass wares previously washed were sterilized in hot air oven. Petri-dishes, pipettes, test tubes were wrapped separately in the paper and kept in the hot air oven for sterilization at 180°C for 1h.

Moist heat sterilization: Normal saline solution and nutrient Medias were sterilized in autoclave at 121°C for 15 min.

Preparation of microbial suspension: Microbial suspensions were prepared by transferring one loop full of stock culture to the 10 ml of normal saline solution. All the procedure was conducted in the laminar air flow in aseptic area.

Antimicrobial activity:

Cylinder-plate or cup-plate method[17-21]: All the sterilized materials were kept in the aseptic area in the Ultra-Violet laminar air flow. Bacterial suspensions (3ml) were then poured in the petriplates. As soon as nutrient agar attained 50°C temperature, 20 ml media was poured in to the petriplates containing bacterial suspension and plates were rotated to mix the suspension with media. When the agar got solidified bores were made in the plate with sterile borer of 8 mm diameter. In each plate six bores were made. Out of which one is meant for addition of standard, two for negative control of blank solvents of standard and sample and remaining three bores for addition of same concentrations of sample. 0.1 ml of sample was added in each cylinder. The plates were kept to allow diffusion at room temperature for three hours and then incubated in the upright position in incubator at 37°C for about 21h for bacterial growth. The diameter of zone of inhibition was accurately measured for bacterial growth in each treated plate as shown in (Table No.5.) The zone of inhibition of bacterial growth by the test solution was compared with the zone of inhibition by the standard at tested concentrations.

Table no.5: Antibacterial activity of *Couroupita guianensis* fruit shell extract

Organism used	*Zone of Inhibition(mm)				
	Std1	Std2	Conc. of extract		
			1mg	2mg	4mg
E. coli	31	20	10	13	13.5
B. subtalis	27	23	11	13	16
S. aureous	36	18	12	13	14.5
P. aeuroginosa	37	26	10	12	15.5

* Average of triplicate, std1- Ciprofloxacin, std2- Doxycyclin

RESULT AND DISCUSSION

The fruit shell was observed to become greenish from inner side after exposing to atmosphere due to presence of sulphur as that of fruit pulp and idea was generated to screen for its antimicrobial activity. The shell has shown better extractive value in alcohol and standardized for moisture content, ash value, extractive value, pH, viscosity and florescence studies. Ethanolic extract has shown significant antibacterial activity as compare to control and standards used at 4mg concentration. Many plants have been screened recently for antimicrobial activity to search the lead moiety from plants[22-24]. From the study it was confirmed that fruit shell contains more sulphated ash value and has very good antimicrobial activity against *B. subtilis* and needs further investigation to confirm the active constituent responsible.

CONCLUSION

From the above study it was confirmed that the fruit shell has very good antimicrobial activity may be because of polyphenols and needs further study to find exact constituents responsible for activity[25]. Standard values are helpful in identification and authentication of plant material.

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