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Research Article

IN VITRO DOSE DEPENDENT STUDY ON ANTI HUMAN PATHOGENIC BACTERIAL AND FREE RADICAL SCAVENGING ACTIVITIES OF METHANOLIC SEED COAT EXTRACT OF *BORASSUS FLABELLIFER* L.

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ABSTRACT

The study aimed to evaluate the anti bacterial activity against human pathogenic organisms and free radical scavenging activities of methanolic seed coat extract of *Borassus flabellifer* . The anti bacterial activity were tested against human pathogenic bacteria sps like *Salmonella typhi, Vibrio cholerae, Shigella dysenteriae,* and *Enterococcus faecalis* which are clinical isolates collected at department of microbiology, King Goerge hospital of Andhra medical college, Visakhapatnam. The methanolic seed coat extract was showed significant inhibitory activity. The highest inhibitory activity was showed against *Vibrio cholerae* . The MIC of seed coat extract was found to be the range between 0.1-10 mg/ml. 0.1 mg/ml was the lowest MIC to *Vibrio cholerae* 1, 10 & 10 mg/ml were the MIC values to *Shigella dysenteriae, salmonella typhi and Enterococcus faecalis* respectively. Radical scavenging activity of methanolic seed coat extract of *B. flabellifer* was carried out using DPPH, ABTS employing as acorbic acid as standard drug. 2.85, 7.90µg/ml were the IC_{50} values of seed coat extract to ABTS, DPPH radical scavenging activities respectively. The anti bacterial as well as radical scavenging activities dose dependently increased with seed coat extract. The present study revealed that seed coat of *Borassus flabellifer* has shown significant anti-bacterial and radical scavenging (anti-oxidant) activities.

Keywords: *Borassus flabellifer* L., Seed coat, anti- human pathogenic bacterial activity, DPPH, ABTS, free radical scavenging activity, Zone of inhibition, MIC.

INTRODUCTION

Screening of the plants for their biological activity is done on the basis of either their chemotaxonomic investigation or ethno botanical knowledge for a particular disease. Identification of a particular compound against a specific disease is a challenging long process. Importance of the plant lies in their biologically active principles. There are two types of plant chemicals, primary metabolites such as sugars, proteins, amino acids, chlorophylls etc. The other category of chemicals is called secondary metabolites, which includes alkaloids, terpenoids, saponins and phenolic compounds. These chemicals exert a significant physiological effect on the mammalian system. Due to the side effects of the present day antimicrobial compounds and emerging antibiotic resistance, the need for developing the newer antimicrobial compounds has been gaining momentum. The ethno medicinal plants provide an immense scope to explore novel antimicrobial compounds [1] all over the world.

The methanol extract of *Terminalia bellerica* was more effective than crude extract against most of the microbes tested except *E. coli* (enteropathogen) and *P. aeruginosa* [2]. It has been reported that the ethanol and methanol extracts of *Aloe vera* gel showed higher activity while acetone extract, showed least or no activity against most of the tested pathogens [3]. The antibacterial activity is exhibited by aqueous and organic extracts of *Thymus capitatus* L. (Lamiaceae) leaves and stems [4]. The antimicrobial activity of diethyl ether extract of *Cassia auriculata* and *Emblica fischeri* showed better promising results in controlling the bacterial growth [5]. The petroleum ether extract of *Digera muricata* (L.) Mart. (Amaranthaceae) showed inhibition against *V. cholerae* [6]. The maximum inhibitory activity of *Cassia auriculata* flowers was observed against all organisms except *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* [7]. Organic solvent leaf extracts of *Eucalyptus* have great potential as antimicrobial agents in the treatment of infectious organisms [8].

Active oxygen and related species: superoxide anion (O2.-), hydroxyl radical (OH.), nitric oxide (NO.), hydrogen peroxide (H2O2), lipid radical (L.), lipid peroxyl radical (LO2 .) and lipid alkoxyl radical (LO.) play a vital role in biological processes of energy production, phagocytosis and signal transduction [9]. There is increasing evidence to show that active oxygen species may also play a causative role in various diseases such as atherosclerosis, ischemia reperfusion injury, inflammation, carcinogenesis, cataracts, brain dysfunction, immune-system decline, cardiovascular disease, and rheumatoid arthritis [10], Endogenous antioxidant enzymes, catalase, superoxide dismutase and glutathione peroxidase defend against oxidative damage caused by active oxygen and related radicals. In addition to the enzymatic antioxidant defenses, nutritional antioxidants in the diets may have protective effects to prevent oxidative stress related diseases. Low dietary intake of fruits and vegetables doubles the risk of most types of cancer as compared to high intake [11], and also markedly increases the risk of heart disease and cataracts [12].

Borassus flabellifer L, belongs to family Arecaceae, commonly known as Palmyra palm or Asian toddy palm is a native of tropical Africa but cultivated and naturalized throughout India. The coconut-like fruits are three-sided when young, becoming rounded or more or less oval, 12-15 cm wide, and capped at the base with overlapping sepals. When the fruit is very young, this kernel is hollow, soft as jelly, and translucent like ice, and is accompanied by a watery liquid, sweetish and potable. The different parts of the *Borassus flabellifer* are being used for medicinal properties viz., male flowers are used for anti-inflammatory activity.[13] The juice from flowering stalk used for diadetes[14]. Oral feeding of mice with palmryah flour induced generation of T suppressor cells which are able to suppress the DTH response to SRBC. The plant has also been used for treatment of gonorrhea and respiratory ailments, Leaf juice used for hiccups, gastric ailments, Bloom on base of leaves used as styptic for external wounds, Juice from flowering stalks used for diabetes, used with rice as a poultice, fermented, and used for gangrenous and indolent ulcers and abscesses[15]. Earlier we were reported anti bacterial activity against general lab experimental non-pathogenic micro-organisms and anti-tumor activity of *Borassus flabellifer* Seed coat.Reports are not available on anti oxidant activity of the seed coat of *B. flabellifer.* Therefore, the present study has been undertaken to investigate the anti bacterial activity against human pathogens and anti oxidant activity through the assessment of DPPH, ABTS radical scavenging activity.

MATERIALS AND METHODS

Plant material and preparation of Plant Extract

Borassus flabellifer tender seeds locally termed as '*Thati munjelu*' were obtained from local market in the summer season from

Visakhapatnam, Andhra Pradesh. Tender seed coat of *Borassus flabellifer* is removed and air dried then ground into powder which was dissolved in methanol so as to make 40% methanol extract. The extract is kept in orbital shaking incubator for 3 days and then centrifuged to remove the debris. Finally clear methanol extract was collected and then the solvent is removed by using rotavapour to get the dried powder of methanol dissolved components of the seed coat of the *Borassus flabellifer*. The dried rotavapoured powder appropriately dissolved in appropriate solvent and used in the study.

Test Microorganisms

Salmonella typhi, Vibrio cholerae, Shigella dysenteriae, Enterococcus faecalis which are clinical isolates of human pathogenic bacteria collected at King George hospital of Andhra medical college, Visakhapatnam, Andhra Pradesh (India).

Chemicals

ABTS (2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium) and 2,2 diphenyl-1-picrylhydrazyl (DPPH) reagents was obtained from Merck (India). Muller-Hinton media (HiMedia Pvt. Ltd., Mumbai, India), Ampicillin(Dr. Reddys laboratory, India). All reagents were of analytical reagent (AR) grade.

Anti bacterial activity by Agar Well Diffusion Method

The bacteria were grown in Muller-Hinton media (HiMedia Pvt. Ltd., Mumbai, India) at 37^{0}C and maintained on nutrient agar slants at 4^{0}C and stored at -20^{0}C. Inoculum of test organisms was prepared by growing pure isolate in nutrient broth at 37^{0}C for overnight. The overnight broth cultures was sub-cultured in fresh nutrient broth and grown for 3hrs to obtain log phase culture. The agar plates were prepared by pour plate method using 20ml M-H medium. The sterile M-H agar medium is cooled to 45^0 C and mixed thoroughly with 1ml of growth culture of concerned test organism (1 x 108 cells) and then poured into the sterile petri dishes and allowed to solidify. Wells of 6 mm size were made with sterile cork borer and test extracts were added. The agar plates were incubated at 37^0 C for 24hrs. The diameter of zones of inhibition was measured in mm using HiMedia zone reader [16].

Determination the MIC of the Methanol Extract by Broth Dilution Assay

The minimum inhibitory concentration of the methanol extract was determined using broth dilution assay[17]. The medium containing different concentrations of methanol extract of seed coat of *Borassus flabellifer* viz., 10, 1, 0.1, 0.01, 0.001 mg/ml prepared by serial dilution. After inoculation, the tubes were incubated for 24 hours at 37^{o}C. The MIC of each sample was determined by measuring the optical density in the spectrophotometer at 620 nm and compared with the OD value control in which contain the non-inoculated broth contain same concentration of extract.

Radical Scavenging Activity

ABTS radical scavenging activity

ABTS was dissolved in water to 7Mm concentration. ABTS radicl cation (ABTS*) was produced by reacting ABTS stock solution with 2.45 Mm potassium persulfate (final concentration) and allowing the mixture to stand in dark at room temperature for 12-16 hours before use. Because ABTS and persulfate react stoichiometrically at ratio 1:0.5, this will result incomplete oxidation of the ABTS. Oxidation of the ABTS commenced immediately, but the absorbance was not maximal and stable until more than 6h had elapsed. The radical was stable in this form for more than two days when stored in the dark at room temperature. For the study of extracts, the ABTS* solution diluted with ethanol to get the absorbance 0.70-0.80 at 734nm and equilibrate at 30^{0}C Stock solutions. Different concentrations of extract added to 1ml of ABTS* solution for assay. Blank also prepared using solvent [18, 19].

DPPH radical scavenging activity

DPPH (1, 1-diphenyl -2picryl –hydrazyl) free radical scavenging activity of the methanolic seed coat extract of *B. flabellifer* was determined by the method of Lamaison et al., which depends on scavenging of coloured free radical (DPPH) in methanol solution by seed extract of *B. flabellifer*. The reaction mixture contains DPPH and extract in a final concentration in 3ml. Absorption of DPPH at its absorption maximum 516nm is inversely proportional to the concentration of extract which depends on scavenging potential of extract. The activity was expressed as inhibitory concentration 50 (IC_{50}) i.e. the concentration of extract required to give 50% reduction in absorbance of test solution compared to that of blank solution[19, 20].

% inhibition =

[(Absorbance of Control – Absorbance of Test)/Absorbance of Control] x100

RESULTS AND DISCUSSION

Anti-Bacterial activity against human pathogens

The methanolic seed coat extract of *Borassus flabellifer* were studied against human pathogenic bacterial sps. viz i,e. *Salmonella typhi, Vibrio cholerae, Shigella dysenteriae, Enterococcus faecalis* . The study was done at different concentrations of extract was screened by agar well diffusion technique and the zone of inhibition was measured in mm in diameter.The results are given in Table1. The dose dependent study of methanolic seed coat extract was carried out at different concentrations viz. i.e. 10, 20, 30, 40 & 50 µg/µl. The zone of inhibition was dose dependently varied (Image.1). The inhibitory zone size was increased with the concentration of extract to all tested pathogenic bacterial Sps (Fig.1).

Fig1 Dose dependent effect of methanolic extract of Borassus flabellifer on different human pathogenic bacterial sps.

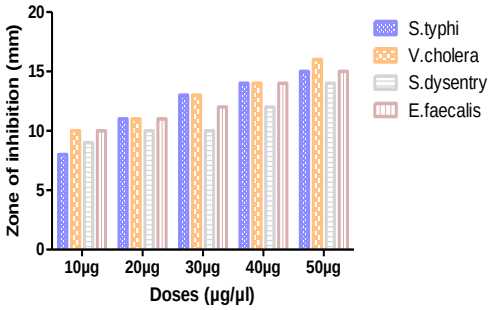


Table 1: Dose dependent inhibitory effect of methanolic seed coat extract of *B. flabellifer* on various human pathogenic bacterial sps.

Type of human pathogenic bacterial sps	Causing disease	Zone of inhibition at different doses(mm)					Ampicillin
		10µg	20µg	30µg	40µg	50µg	30µg
1. *Salmonella typhi*	*Typhiod fever*	8	11	13	14	15	18
2. *Vibrio cholerae*	Cholera	10	11	13	14	16	17
3. Shigella dysenteriae	Dysentery	9	10	10	12	14	15
4.Enterococcus faecalis	Gastro intestinal infections	10	11	12	14	15	15

Vibrio cholerae was showed high sensitivity to the extract among all other tested bacterial sps. 16mm was the highest zone of inhibition occurred against V.cholerae at 50 µg/µl concentration. The inhibitory zone values are comparable to Ampicillin (Antibiotic) used as standard (30 µg/µl). The MIC values of seed coat extract were found to be the range between 0.1-10 mg/ml concentration (Table 2). 0.1 mg was the lowest MIC to *Vibrio cholerae.* 1, 10 & 10 mg/ml were the MIC values of extract to *Shigella dysenteriae, Salmonella typhi and Enterococcus faecalis* respectively.

Radical Scavenging activity

ABTS radical scavenging activity

Methanolic seed coat extract of *B. flabellifer* showed potential ABTS radical scavenging activity. 34, 43, 51 percent of inhibition occurred to 1, 2.5, 5µg/ml concentrations of seed coat extract (Figure 3 & Table 3). IC_{50} value of seed coat extract found to be 2.85µg/ml comparable to Ascorbic acid (standard) IC_{50} value was 2.64µg/ml.

Table 2: The Minimum Inhibitory Concentration of methanolic sedd coat extract of *B. flabellifer* to different human pathogenic bacterial sps.

Type of pathogenic bacterial species	MIC (mg/ml)
1. *Salmonella typhi*	**10**
2. *Vibrio cholerae*	**0.1**
3. Shigella dysenteriae	**1**
4. Enterococcus faecalis	**10**

Table 3: Free radical scavenging activity of the methanolic seed coat extract of *Borassus flabellifer* L.

Sample	Concentration of extract ((µg/ml)	Percent of inhibition	IC50(µg/ml)
	DPPH radical scavenging activity		
Seed coat extract of *B. flabellifer*	2.5	23.74	7.90
	5	38.25	
	10	59.17	
Ascorbic acid (Vit-C) (Standard)	1	16.26	4.21
	2.5	32.90	
	5	57.89	
	ABTS radical scavenging activity		
Seed coat extract of *B. flabellifer*	1	34.39	2.58
	2.5	43.67	
	5	71.40	
Ascorbic acid (Vit-C) (Standard)	1	33.06	2.64
	2.5	61.81	
	5	98.53	

Fig 3. DPPH radical scavenging activity of methanolic seed coat extract of *B. flabellifer*

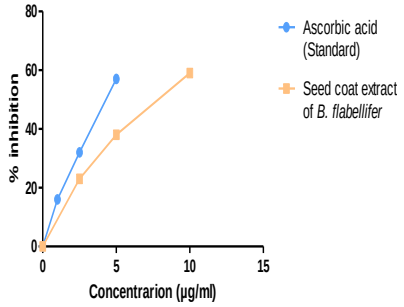
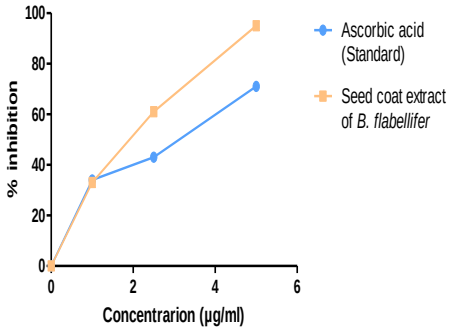


Fig 2. ABTS radical scavenging activity of methanolic seed coat extract of *B. flabellifer*



DPPH radical scavenging activity

Methanolic seed coat extract of *B. flabellifer* showed significant DPPH radical scavenging activity. 23, 38, 59percent of inhibition occurred to 2.5, 5, 10µg/ml concentrations of seed coat extract (Figure 2 & Table 3.). IC_{50} value of seed coat extract found to be 7.90µg/ml while 4.21µg/ml to Ascorbic acid which was used as standard.

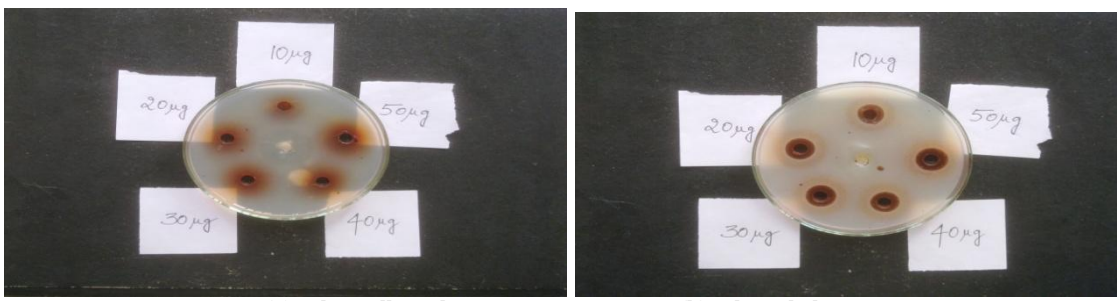
DISCUSSION

In spite of tremendous development in the field of synthetic drugs during recent era, they are found to have some side effects, whereas plants still hold their own unique place, by the way of having no side effects. Therefore, a systematic approach should be made to find out the efficacy of bioactive compounds from plants against pathogenic organisms. Some drugs are showed potential against general laboratory used test micro organisms but less effective against pathogenic organisms. Seed coat extract of *B. flabellifer* was showed significant activity not only general lab test bacterial but also on clinically isolated human pathogenic organisms but also anti oxidant potential through DPPH and ABTS free radical scavenging activities. These results showed the ability to reduce free radicals of extracts which may stop the free radical initiation or retard free radical chain reaction in the propagation of the oxidation mechanism.

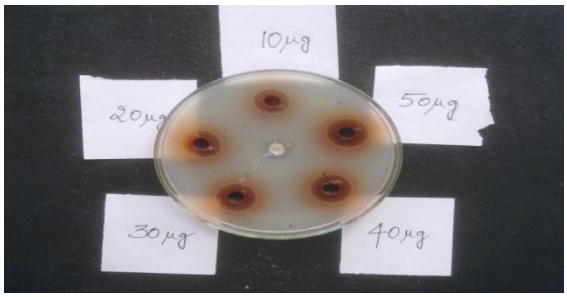
CONCLUSION

Seed coat of *Borassus flabellifer* has showing both anti bacterial and free radical scavenging activities. Further investigation is in progress to identify and isolate bioactive compounds which are present in the seed coat of *Borassus flabellifer.*

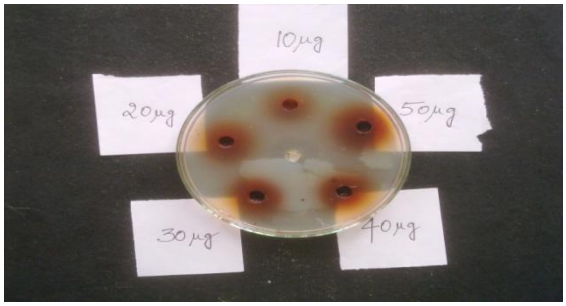
Image 1: Dose dependent inhibitory zones of methanolic seed coat extract of *borassus flabellifer* against different human pathogenic bacterial sps. on agar well plates.



(a) Salmonella typhi **(b) *Vibrio cholerae***



(c) Shigella dysenteriae



(E) Eenterococcus faecalis

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