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Original Article

EVALUATION OF BANANA (*MUSA* SP. VAR. NANJANGUD RASA BALE) FLOWER AND PSEUDOSTEM EXTRACTS ON ANTIMICROBIAL, CYTOTOXICITY AND THROMBOLYTIC ACTIVITIES

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ABSTRACT

Objectives: The present study is centered on potential utilization of banana flower (FB) and pseudostem (PB), as a source of antimicrobial, cytotoxic and thrombolytic contributor, which otherwise is discarded as waste or burnt.

Methods: FB and PB, the by-products of banana cultivation were extracted sequentially using various solvents *viz*., ether: chloroform (1:1), ethyl acetate, acetone, methanol, ethanol and water. *In vitro* antimicrobial activity of the extracts was tested against six bacterial strains using standard disc diffusion method and the minimal inhibitory concentration (MIC) was performed by microdilution method. Further to validate the safe consumption, extracts were assessed for toxicity evaluation in cell culture against 3T3-L1 cell line (obtained from adipose tissues) using MTT assay. Also, the thrombolytic activity was performed by clot disruption method.

Results: Phytochemical analysis demonstrated that FB and PB were a rich source of polyphenols (saponins, terpenoids, flavonoids, coumarins), cardiac glycosides and steroids. Extracts possessed antimicrobial activities against all the microorganisms tested, with MIC values in the range between 1.2 to 2.5 mg/ml. The investigation on thrombolytic activity by the aqueous extracts of FB (18%) and PB (13%) expressed a significant percentage of clot lysis with reference to Streptokinase (64%). Also, all the extracts of FB and PB exhibited no cytotoxic effect against 3T3L1 cell line.

Conclusion: The present work demonstrates the antimicrobial, cytotoxic and thrombolytic activities of FB and PB extracts. The activities exhibited could be the basis for their alleged health promoting abilities and serve as new source of natural nutraceutical with potential applications.

Keywords: Banana flower, Banana pseudostem, MIC, 3T3L1 cell lines, Clot disruption.

INTRODUCTION

Traditional medicines or precisely herbal medicines are gaining substantial importance over the past few decades for their enormous nutritive and therapeutic potentials. The long used allopathic medicines are effectively being replaced by herbal formulations for their rich source of natural bioactives [1]. As a part of the metabolism, plants produce an array of chemical entities which are classified under primary and secondary metabolites. The secondary metabolites are of less importance to the plants itself and are generally produced as a response to certain biotic and abiotic stress. However, these derive great pharmacological significance for their antimicrobial, antioxidant, anti allergic, hypoglycemic and anti cancerous properties [2, 3].

Ever increasing infectious and metabolic disorders pose challenges to the pharmaceutical industries in identifying newer lead molecules which have the potential to control these diseases. Infectious diseases are the world's leading cause of premature death which are presently being treated using synthetic antimicrobial agents. These drugs also induce certain complications like abdominal pain, diarrhea, cramping, headache, nausea and vomiting. More recently, drug resistance exhibited by various human pathogenic bacteria is reported all over the world. These factors promote the use of natural antimicrobial agents which prove safer and more acceptable alternatives for synthetic drugs [1, 4-5]. Another major concern leading to increasing death annually is the cardiovascular disorder caused primarily due to thrombosis diseases which are associated with blood coagulation and endothelial lesions [6]. Some of the synthetic platelet inhibitors like heparin and ticlopidine are widely used which are partially effective in treating thrombus formation. Also, like most synthetic agents, they lead to some complications like internal bleeding and prolonged clotting time during injuries and so on. Alternatively, considerable research in terms of identifying plant and animal sources of antiplatelet, anticoagulant, antithrombotic, and thrombolytic agents have led to discovery of wide resources with potential properties [7].

Banana plant, a member of the *musaceae* family, is used in traditional medicine for treatment of peptic ulcer, constipation, celiac disease, and diabetes. The fruit is relished worldwide while the secondary parts namely the banana flower (FB) and pseudostem (PB) are used as vegetables in India. Previous studies revealed FB and PB as potent antihyperglycaemic agents as well as effective in management of several diabetic complications [8-10]. However, scientific evidence of the antimicrobial, cytotoxic and thrombolytic effects of FB and PB has not been investigated. Therefore, the aim of the present study was to evaluate the *in vitro* antimicrobial, cytotoxic and thrombolytic potentials of various solvent extracts of FB and PB and assess their phytochemical constituents.

MATERIALS AND METHODS

Chemicals

Reagents and solvents used for extraction were procured from Merck (Mumbai, India). All of the other reagents were of analytical grade. The standard drugs were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, USA).

Maintenance and usage of 3T3L1 cell lines

The 3T3-L1 mouse embryo fibroblasts cell lines were procured from NCCS, Pune, India and cultured as per the protocol of Nahid et al. with slight modifications [11]. The 3T3-L1 pre-adipocytes were cultured in T25 tissue culture flask having Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine

serum (FBS) and penicillin/streptomycin (100 IU, 100 mg/ml, respectively) in a humidified atmosphere of 5% CO_2 at 37°C. The optimization of the cell concentration was carried out as described by Jaganathan [12]. All experiments were performed using the cells in the passage 20 in three independent sets.

Bacterial strains, culture medium and inocula preparation

Six species of test microorganisms selected for the study were obtained from Microbial type culture collection (MTCC), Chandigarh, India. The following cultures were considered for the current study. The Gram positive species were *Staphylococcus aureus* (MTCC 96), *Bacillus subtilis* (MTCC 121), *Bacillus cereus* (MTCC 430) and Gram negative species were *Escherichia coli* (MTCC 1304), *Klebsiella pneumonia* (MTCC 2275) and *Shigella flexneri* (MTCC 1457). All the bacterial strains were sub cultured on a fresh nutrient agar plate 24 h prior to antimicrobial test. Inocula were prepared by transferring several single colonies of microbes to a sterile 5 ml broth and the suspension was mixed to homogeneity to give a final density of 5 x 10^5 CFU/ml.

Plant material and preparation of extracts

Fresh FB and PB of *Musa* sp. var. Nanjangud rasa bale were collected from banana cultivating farms of Nanjangud, Karnataka, India. The specimen was identified by the department of Horticulture, Government of Karnataka, Mysore, India. Thick outer leaf sheath of the tender pseudostem was peeled off and the inner pith region was collected. Flowers were separated from the inflorescence by discarding the spathe. Both pseudostem and flower were cleaned, cut into small pieces and were dried at 40^{0}C in an oven.

This was powdered using a homogenizer and stored at 4^{0}C until use. The course powder was subjected to successive extraction with solvents ranging from non-polar to highly polar in series, namely ether: chloroform (1:1), ethyl acetate, acetone, methanol, ethanol and water using Soxhlet apparatus. Extraction was done for fifteen cycles with each solvent (300 mL). Solvents of individual extracts were filtered and the filtrate was concentrated under vacuum using rotary evaporator (Rotavapor R-200, Buchi, Switzerland) to remove the organic solvent. The stock solution (samples) was prepared by suspending each of the extract with their respective solvent such that the final concentration of the solvent was 0.5% (v/v).

Phytochemical screening

The powdered specimen and the aqueous extracts were subjected to several chemical tests to identify the constituents present in both FB and PB [13]. The chemical analysis was based on the visual qualitative observation of colour change or formation of a precipitate/turbidity after the addition of specific reagents.

Antibacterial activity

Disc diffusion and microdilution techniques on nutrient agar medium were used to evaluate the antibacterial properties of distinct extracts of FB and PB as described by Emmanuel et al with slight modifications [14]. The nutrient agar medium was inoculated with six different pathogenic bacterial strains mentioned above. Sterile discs (6 mm diameter) bearing successive extracts of FB and PB (50 µg) were made to placed on the inoculated nutrient agar media. Positive and negative control was also prepared by using Gentamicin and the individual solvents respectively. The impregnated discs were allowed to dry and further placed on inoculated plates. Finally the plates were kept for incubation at 37^{0}C for 24 to 48 h and the diameter of the inhibition zones around the disc was measured in millimetres. The lowest concentration of the extract needed to inhibit the growth of the organism was evaluated by the minimum inhibitory concentration (MIC).

Cytotoxicity screening

The *in vitro* cytotoxicity was assessed in 3T3L1 cell line using diverse extracts of FB and PB by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay with minor modifications [15]. Briefly, after being harvested from culture flasks, the cells were seeded at 1 x 10^6 cells in each well of sterile 96-well microtitre plate containing 100 µl of fresh growth medium per well and the cells were allowed to adhere for 24 h. The cells treated with the diverse extracts of both FB and PB were serially diluted with growth medium to obtain 11 various concentrations (500, 250, 125, 100, 50, 25, 12.5, 10, 6.25, 3.125, 1.25 µg/ml). A 100 µl of each concentration was added to each well and kept for incubation overnight. After 24 h of incubation at 37°C, 5% CO_2 and relative humidity of 95%, the medium was aspirated and the cells were washed once with sterile phosphate buffered saline (PBS). MTT solution (5 mg/mlin PBS, pH 7.2) was added to each well and the plates were incubated at 37°C in 5% CO_2 for 2-4 h, until a purple precipitate was clearly visible under the microscope. After 4 h of incubation, 100 µl of DMSO solution was added to each well, to solubilise MTT crystals and gently shook for 5 min. The plates were again incubated for 10 min and the absorbance was measured at 540 nm on a microtitre plate reader.

Thrombolytic activity

The thrombolytic activity was performed as per the procedure described by Sweta et al. [16] with minor modifications. 5 ml of blood was drawn from healthy volunteers. This was transferred in volumes of 0.5 ml each to previously weighed microfuge tubes. The tubes were incubated at 37 °C for 45 min for the formation of clot. The top layer containing serum was carefully eliminated without disturbing the clot and the tubes were weighed again. To each tube, 100 µL of the aqueous extract of FB and PB with predetermined concentrations were added and incubated again at 37°C for 90 min along with a positive control with Streptokinase (1 500 000 IU) and negative control with 0.9 % saline. The fluid obtained after incubation was eliminated and the clot lysis was estimated by weighing the microfuge tubes once again after the fluid being removed. The difference in weight before and after clot lysis was expressed in terms of percentage that determines the thrombolytic activity of the extract.

Statistical analysis

The experiments were done in triplicates. Results were expressed as Mean ± SE. Graph pad PRISM software (version 4.03) was used for calculating IC_{50} values.

Table 1: Exhibiting the presence/absence of phytochemicals in banana flower (FB) and pseudostem (PB)

S. No.	Phytochemicals	FB	PS
1.	Tannins	++	-
2.	Saponins	++	++
3.	Phlobatannins	-	-
4.	Terpenoids	++	++
5.	Flavonoids	+	+
6.	Cardiac glycosides	++	++
7.	Combined anthraquinones	-	-
8.	Free anthraquinones	-	-
9.	Carotenoids	-	-
10.	Reducing compounds	++	-
11.	Alkaloids	+	+
12.	coumarins	++	++
13.	Steroids	++	++

(**++**): indicates high content; (**+**): indicates moderate content; (**-**): indicates nil.

Table 2: Antibacterial activity through disc diffusion at 2.5 mg/ml concentration of banana flower (FB) and pseudostem (PB) extracts

	Extracts	Zone of inhibition [x] (in "mm")					
		Gram positive bacteria			Gram negative bacteria		
		Staphylococcus aureus	*Bacillus cereus*	*Bacillus subtilis*	*Escherichia coli*	*Klebsiella pneumonia*	*Shigella flexneri*
FB	E: C	9.39±0.53	11.64±0.68	10.78±0.22	18.6±0.37	11.35±0.58	18.58±0.52
	Ea.	-	-	12.08±0.38	13.83±0.52	-	12.56±0.44
	Ac.	12.54±0.47	7.82±0.21	8.71±0.64	8.92±0.95	6.03±0.50	8.14±0.77
	Me.	12.11±1.19	10.53±0.45	9.05±0.82	15.80±0.49	10.50±0.47	11.70±0.61
	Et.	15.24±0.28	10.84±0.19	14.53±0.43	17.99±0.19	12.41±0.52	21.72±0.44
	Aq.	4.66±0.40	-	7.19±0.17	11.5±0.5	-	7.53±0.41
PB	E: C	12.75±0.66	9.84±0.35	13.14±0.38	16.84±0.57	9.64±0.57	14.9±0.40
	Ea.	7.16±0.63	-	9.63±0.64	12.43±0.70	8.51±0.47	-
	Ac.	-	6.74±0.98	-	8.29±0.50	10.31±0.59	4.57±0.43
	Me.	10.41±0.51	9.51±0.57	11.21±0.75	16.04±0.18	9.03±0.20	10.00±1.0
	Et.	14.84±0.77	11.70±0.54	12.08±0.24	15.21±0.37	10.79±1.26	18.48±0.46
	Aq.	-	5.41±0.52	-	8.52±0.50	-	9.07±0.53
	Std.	17.76±1.08	11.81±0.21	14.33±0.58	22.14±0.89	12.41±0.62	24.77±0.29

[x] Values are expressed as mean ± SE. (E: C): chloroform: ether (1:1) extract; (Ea.): ethyl acetate; (Ac.): acetone; (Me.): methanol; (Et.): ethanol; (Aq.): aqueous; (-): inactive; (Std.): Gentamicin.

Table 3: The minimum inhibitory concentration (MIC) of banana flower (FB) and pseudostem (PB) extracts

	Extracts	MIC [x] (in "mg/ml")					
		Gram positive bacteria			Gram negative bacteria		
		Staphylococcus aureus	*Bacillus cereus*	*Bacillus subtilis*	*Escherichia coli*	*Klebsiella pneumonia*	*Shigella flexneri*
FB	E: C	1.86±0.11	1.53±0.13	2.42±0.13	1.43±0.09	1.75±0.10	1.51±0.10
	Ea.	-	-	2.30±0.22	1.95±0.06	-	2.45±0.09
	Ac.	2.27±0.19	NT	NT	NT	NT	NT
	Me.	1.87±0.09	2.54±0.08	NT	1.65±0.09	2.10±0.11	2.42±0.15
	Et.	1.49±0.02	2.14±0.25	1.64±0.06	1.38±0.05	1.96±0.06	1.14±0.06
	Aq.	NT	-	NT	2.55±0.10	-	NT
PB	E: C	2.23±0.11	NT	2.05±0.05	1.23±0.09	NT	1.75±0.10
	Ea.	NT	-	NT	2.44±0.10	NT	-
	Ac.	-	NT	-	NT	2.00±0.10	NT
	Me.	1.93±0.07	NT	1.91±0.13	1.74±0.08	NT	NT
	Et.	1.60±0.04	1.67±0.09	1.87±0.10	1.92±0.15	2.25±0.10	1.38±0.15
	Aq.	-	NT	-	NT	-	NT
	Std.	0.17±0.06	1.54±0.32	0.61±0.08	0.55±0.10	1.04±0.06	0.28±0.06

[x] Values are expressed as mean ± SE. (E: C): chloroform: ether (1:1) extract; (Ea.): ethyl acetate; (Ac.): acetone; (Me.): methanol; (Et.): ethanol; (Aq.): aqueous; (-): inactive; (NT): Not tested since extracts showed inhibitory effect with inhibition zone≤1 0 mm by disc diffusion method; (Std.): Gentamicin.

Table 4: *In vitro* cytotoxic activity of banana flower (FB) and pseudostem (PB) extracts tested against 3T3-L1 cell lines

	IC_{50} [x,y] in "µg/ml"					
	E: C	**Ea.**	**Ac.**	**Me.**	**Et.**	**Aq.**
FB	24.53±1.26	35.48±1.27	39.41±0.67	62.16±1.08	142.2±1.56	137.7±0.91
PB	45.69±0.65	24.17±0.69	47.7±0.48	70.22±0.71	128.3±1.02	108.4±0.59

[x]Values are expressed as mean ± SE. (E: C): chloroform: ether (1:1) extract; (Ea.): ethyl acetate; (Ac.): acetone; (Me.): methanol; (Et.): ethanol; (Aq.): aqueous; (-): inactive; (NT): Not tested since extracts showed inhibitory effect with inhibition zone≤1 0 mm by disc diffusion method; (Std.): Gentamicin.

[y]The IC_{50} value is defined as the inhibitor concentration to inhibit 50% of cell proliferation under assay conditions

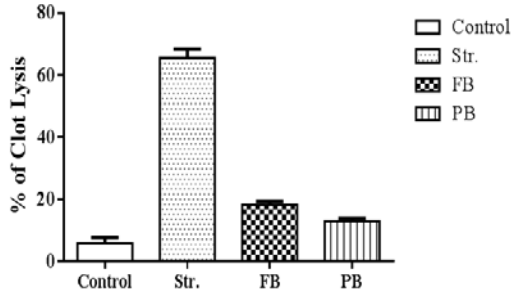

Fig. 1: Effect of banana flower (FB), Pseudostem (PB) extracts and Streptokinase (Str.) on *in vitro* clot lysis. Data are expressed as mean± SE, n=10

RESULTS

Phytochemical screening

The preliminary qualitative analysis of FB revealed a high presence of tannins, saponins, coumarins, terpenoids, cardiac glycosides, steroids and reducing compounds (table 1). Also, moderate presence of flavonoids and alkaloids was observed with no traces of carotenoids. Meanwhile, PB was deficient of carotenoids along with the tannins and reducing compounds. The other secondary metabolites like saponins, coumarins, terpenoids, cardiac glycosides, steroids were present in high contents with flavonoids and alkaloids in moderate levels.

Antibacterial activity

In the present study, distinct extracts of FB and PB were assayed for its *in vitro* antimicrobial activity against six microbial strains and their potential activity were qualitatively and quantitatively assessed by the presence or absence of inhibition zones and MIC values. The standard antibiotic gentamicin was used as positive control. Impregnated paper discs containing only solvents used as negative control did not show any inhibition zone. As shown in table 2 and 3, extracts of FB and PB displayed antimicrobial activity on different tested strains with variable sensitivity against the six extracts. Gram negative bacteria *K. pneumonia, S. flexneri and Escherichia coli* were inhibited by almost all the extracts of FB and PB, except the aqueous and ethyl acetate extract of FB which failed to show inhibition for *K. Pneumonia*. The Gram positive bacteria *S. aureus, B. cereus* and *B. subtilis* were also sensitive to all of the extracts. Among the two Gram positive bacteria, *B. cereus* was found to be more sensitive than *S. aureus* and *B. subtilis*. On the whole, the MIC values ranged from 1.2 mg/ml up to 2.5 mg/ml. Ethanol extracts of FB and PB exhibited higher antibacterial effects than the corresponding solvents extracts.

Cytotoxicity screening

The IC_{50} values (table 4) from MTT assay indicated that distinct extracts of FB and PB were not toxic to 3T3L1 cells. The ethyl acetate extract of FB and PB had a higher cytotoxicity. It is clear that 3T3L1 was most prone to the cytotoxic effects of mid polar solvent extracts of FB and PB, whereas the polar solvent extracts were insensitive.

Thrombolytic activity

As shown in fig. 1, FB and PB extracts exhibited moderate clot lysis activity of 18.30% and 13.04% respectively as compared to the standard (Streptokinase, a positive control,1 500 000 IU) that showed 65.51%. The clots treated with 100 µl of saline (negative control) showed only trifling clot lysis (5%).

DISCUSSION

Infectious diseases are among the major cause of morbidity and/or mortality worldwide and the use of antibiotics are the popular method of treatment against them. More recently, several reports suggest that a very negligible number of antibiotics exist for which the infectious microorganisms have not developed resistance, and thus, an efficient antimicrobial agent with effective inhibitory potential is very crucial [14, 17]. Among the alternative medications, herbal medicines are the most ubiquitous and thus declared by the World Health Organization as the surest means to achieve total health care coverage worldwide [18]. Herbal medicines are not only potent pharmacologically but also are less toxic and economically feasible and thus are extensively researched. Their rich nutraceutical values add on to their potential in disease management and thus are used as functional foods [16].

Most Asian countries use different parts of banana as food. FB and PB have also been used in some of the traditional medicinal formulations. Though, the ethnopharmacological application of banana has been in management of various diseases, there are several unexplored aspects to the secondary products FB and PB [9, 10]. In our study, we chose the popular variety of banana grown in different regions of Nanjangud (Mysore district) in Karnataka, India. A major characteristic of the fruit from this cultivar is its long shelf life, as it survives for around a fortnight after it starts ripening. Thus, the present study was outlined to evaluate the effects of various bioactive rich extracts serially extracted from FB and PB on antimicrobial, cytotoxic and thrombolytic potentials.

Our results were exhibited that all the extracts show a broad range of antimicrobial activity by inhibiting the growth of human pathogenic bacteria (Gram positive and Gram negative). The phytochemical screening showed abundance of various phytocomponents, which further confirmed our results thereby providing a baseline for future study on potentials of FB and PB as antimicrobial contributors. The inhibitory activity varied significantly among the isolates with an MIC value ranging from 1.2 – 2.5 mg/ml. The observed effects can be due to the alkaloid, saponins, flavonoids, tannin, glycosides and phenols found in crude extract which are known to possess antimicrobial compounds [19, 20].

To address the issue of safety of FB and PB we also carried out MTT assay on 3T3-L1 preadipocytes and the results suggested that the extracts were not toxic to 3T3-L1 preadipocyte cells with IC_{50} values greater than 24 µg/ml. The results are in agreement with the US NCI plant screening standards which declare crude extracts with IC_{50} values less than 20 µg/ml is cytotoxic [21]. We also assessed the effects of FB and PB for its potential as thrombolytic agents which activate plasminogen and facilitate in the treatment of myocardial infarction, deep vein thrombosis and thromboembolic strokes [22]. It was worth noting that up on treatment of clots with aqueous extracts of FB and PB, 18.30% and 13.04% of clot lysis occurred respectively. These findings suggest the potential of FB and PB in treatment of thrombosis diseases.

In summary, the extraction procedure of both FB and PB using non polar to polar solvents also play a major role in the activity witnessed. In our study, in agreement with the use of aqueous and alcoholic decoctions in traditional medicines for various health benefits, the best results were obtained in the ethanolic extracts of both FB and PB. Another influence on the availability of bioactive molecules is the climatic factor. Since most of the bioactive molecules are secondary metabolites produced by the plants as a result of various external factors, climatic conditions play a vital role in the presence of those active molecules [23]. Thus from our findings, it is concluded that the FB and PB serve to be a potential source of useful antibacterial drug along with thrombolytic activities with minimal cytotoxicity. Further purification and characterization of the active principles from the crude extracts will provide a better understanding of the antimicrobial mechanism and serves as a tool for potential lead compounds for microbial infectious diseases

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CONFLICT OF INTERESTS

The authors declare there is no conflict of interest

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