In Vitro Antibacterial, Anticancer and Antidiabetic Potential of Freeze-dried Aqueous *Borassus flabellifer* L. Seed Powder Extract

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Banu *et al.*: Therapeutic analysis of *Borassus flabellifer* L. Seed Powder

The *Borassus flabellifer* fruit which is one of the summer fruits of India can be considered a promising therapeutic food as it is a good source of bioactive compounds. The aim of this study was to evaluate the antibacterial, anticancer and antidiabetic activity of the locally available aqueous freeze-dried seed powder extract of *Borassus flabellifer*. Antibacterial activity was evaluated using agar well diffusion assay against gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) and gram-negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*). Anticancer activity was studied using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay against HeLa and Vero cells. Antidiabetic activity of the sample was evaluated using alpha amylase and alpha glucosidase inhibition assays. Results revealed that the sample displayed maximum antibacterial activity against gram-negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*); showed satisfactory anticancer activity against the HeLa cells and exhibited potent alpha-amylase and alpha-glucosidase inhibitory activity, thus indicating the use of the *Borassus flabellifer* seed powder as a potential therapeutic agent for the development of drugs and functional foods.

Key words: Antibacterial, anticancer, antidiabetic, *Borassus flabellifer* L., freeze-dried

Non-Communicable Diseases (NCDs) have turned out to be the foremost reason of human mortality and morbidity in many countries. NCDs comprise of diabetes, Cardiovascular Disease (CVD), cancer, chronic respiratory disease and mental health conditions; obesity and overweight being related to one or more of these NCDs. NCDs lead to chronic metabolic syndrome due to a combination of factors that include genetics, physiology, lifestyle, environment, food and behaviour patterns[1]. Bray *et al.*[2] predicted that, cancer would be the major cause of death in the 21st century. There are 36 different kinds of cancer affecting both men and women. Although there are conventional and modern methods to treat cancer, all of them have side effects[3]. On the other hand, Szablewski[4] reported that, India has the second largest diabetic population in the world. Diabetes is characterized by increased blood sugar levels and associated complications such as kidney and cardiovascular malfunction[5]. Although a number of hypoglycemic agents are available to treat diabetes, these synthetic drugs have many side effects such as gastrointestinal disorders, nausea and leave a metallic taste in the mouth[6]. Another major public health concern in recent times is antibiotic resistance. Bacterial infections are constantly developing and re-emerging, leading to an alarming fear and desperate need to treat them[7]. An analysis on global burden of diseases, has projected that, diets low in fruits and vegetables were accountable for 1.8 and 3.4 million deaths in 2013[8], showing that poor dietary habits lead to a range of NCDs.

Fruits occupy a major part in the functional food category owing to the high amounts of bioactive compounds. These compounds have the ability to lower the risk of a wide number of diseases[9]. Researchers are continuously exploring natural agricultural products for pharmaceutical purposes[10] since natural sources are widely available, lower in cost and have no side effects when compared to

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modern therapeutic drugs[11]. Secondary metabolites such as flavonoids are capable of exerting biological activity and thereby exhibit pharmacological properties such as antimicrobial, anti-inflammatory, antidiabetic, anticholesterolemic, antioxidant and anticancer. A high flavonoid content is directly associated with a high total phenolic content in foods[12]. Hence consuming phenolic-rich fruits can positively impact human health.

Among the different variety of fruits of South India, *Borassus flabellifer* (*B. flabellifer*) of Arecaceae family, is a plant native to Tamil Nadu and bears fruits commonly called "Nungu" in Tamil and "Palmyra palm" in English. These fruits are widely distributed and consumed throughout Tamil Nadu during summer season. This plant is highly versatile and capable of exerting a number of pharmacological benefits such as anti-inflammatory, anticancer, antioxidant, antidiabetic, antibacterial, antifungal, anthelmintic activity, hemolytic activity, analgesic activity[13], wound healing, immunomodulatory, diuretic and anti-malarial activity[14]. The tender seed found within the seed coat is fondly consumed because of its natural cooling property. In addition, the tender seed has the ability to treat dermatitis, nausea and vomiting and is known to possess antioxidant and anti-inflammatory potential[15].

Different parts of *B. flabellifer* are known to contain abundant phytochemicals[16]. Muthulakshmi *et al.*[17] carried out a qualitative phytochemical analysis on the *B. flabellifer* seed coat and identified the presence of flavonoids, tannins, saponins, alkaloids, carbohydrates, phenolic compounds, terpenoids and glycosides, recommending its usage for drug formulation and medicinal application.

The thin outer covering of *B. flabellifer* called the seed coat is peeled off and often discarded as waste; this has been utilized in this study along with the tender seed to investigate the different therapeutic properties such as antibacterial, anticancer and anti-diabetic effects of *B. flabellifer* seed powder to obtain maximum medicinal benefits.

MATERIALS AND METHODS

Procurement of materials:

B. flabellifer fruits were procured from a local farm in Kancheepuram district, Tamil Nadu during summer months. Fruits of the same size and maturity level were selected. Both seed and seed coat were utilized in this study. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was procured from Sigma-Aldrich. Other chemicals and reagents used in the study were procured from SRL chemicals, Sigma-Aldrich, MP Biomedicals, India, Research Laboratory Corporations, Pune and Himedia, Mumbai, India. The instruments used in the study were Carbon dioxide (CO_2) incubator (BPN 50CH (UV)), multimode microplate reader (Synergy™ LX), refrigerated centrifuge (C-24 Plus), 96-well plate and serological pipette.

Preparation of extract:

The fibrous outer portion of the fruits was removed and the tender seed with seed coat found within, was used in the present study. The sample was prepared by subjecting the seeds and seed coats to washing with distilled water, pulping and freeze-drying in a lyophilizer (LSI 30). The sample was freeze-dried at -20° and dried at 65°. The prepared sample was stored in aluminum pouches maintained at 4° for further analysis. The sample extract was prepared by dissolving 200 mg extract in 20 ml distilled water to arrive at a concentration of 10 mg/ml.

In vitro antibacterial activity:

Test microorganisms: Gram-positive strains such as *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 96) and gram-negative strains such as *Escherichia coli* (MTCC 443), *Klebsiella pneumoniae* (MTCC 109) were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India.

Agar well diffusion assay: The antibacterial activity of aqueous *B. flabellifer* seed powder extract was analysed using agar well diffusion method[18]. About 25 ml of molten Mueller Hinton agar was poured into sterile petri plates and allowed to solidify. 100 µl of 18 h grown test organisms were streaked on the surface of the solidified agar medium using an L-rod spreader and was left to set for 5 min. 5 mm wells were made in all plates using a sterile cork borer. Different concentrations of the aqueous seed powder extract (50-200 µg/well) were poured into the wells. Solvent saline loaded well served as negative control and azithromycin well (30 µg/ml) served as positive control. Finally, the inoculated plates were incubated at 37° for 24 h, after which the zone of inhibition diameter around the wells was measured using an antibiotic zone scale.

In vitro anticancer activity:

The aqueous *B. flabellifer* seed powder extract was evaluated for its cell growth inhibition property on African Green Monkey kidney cell line Vero and cervical carcinoma cell line HeLa.

Cell culture: HeLa and Vero cells were purchased from National Centre for Cell Science (NCCS), Pune. The cell lines were grown in T25 tissue culture flasks containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % Fetal Bovine Serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin obtained from Genetix Biotech, India and Sigma-Aldrich. All cell cultures were maintained at 37° in a humidified atmosphere of 95 % air and 5 % CO_2. Cells were regularly passaged and maintained until commencement of analysis.

Cell growth inhibition by MTT assay: Cell proliferation (MTT) assay was performed according to the method described by Dantu *et al.*[19]. A monolayer of HeLa and Vero cells were separated and suspensions of single cell were prepared with trypsin-Ethylenediaminetetraacetic Acid (EDTA). Using a haemocytometer, the viable cells were counted and a cell suspension of final density of 1×10^5 cells/ml was prepared with Dimethylsulfoxide (DMSO) medium containing 5 % FBS. A density of 3000 cells/well (100 µl per well) of cell suspension were seeded in 96-well plates and incubated for attachment at 37° for 24 h. A working stock of aqueous *B. flabellifer* seed powder extract of 2× (25, 50, 100, 200, 300 and 400 µg/ml) concentration was prepared in complete DMEM supplemented with 10 % FBS and antibiotics. 100 µl volume of 2× stock was transferred to respective wells to maintain the final concentration range of 12.5, 25, 50, 100, 150 and 200 µg/ml and the plate was further incubated for 48 h at 37°, 5% CO_2 in the incubator. Standard mefloquine was used as positive control in the concentration similar to that of samples (12.5, 25, 50, 100, 150 and 200 µg/ml). The medium without samples served as blank and triplicates were maintained for all concentrations.

After 48 h of incubation, 15 µl of MTT (5 mg/ml) in Phosphate Buffered Saline (PBS) was added to each well and incubated at 37° for 3 h. The medium that did not react with MTT was decanted, while the formazan crystals that were formed were solubilized in 100 µl of DMSO. The plates were read at 570 nm using Synergy HT Microplate Reader and the percentage growth was calculated using the formula

Growth percentage (%)= $[(T-T_0)/(C-T_0)]\times100$

Where, T=optical density of test, C=optical density of untreated control and T_0=optical density at time zero (at the time of compound addition).

From the percentage growth, a dose response curve was generated and Half Maximal Inhibitory Concentration (IC_{50}) values were interpolated from the growth curves.

In vitro antidiabetic activity:

Alpha amylase inhibition assay: Alpha amylase inhibition assay was studied according to the method described by Telagari and Hullatti[20]. Different concentrations (100, 200, 300, 400 and 500 µg/ml) of the sample were added to 200 µl of 0.02 M sodium phosphate buffer. 20 µl of alpha amylase solution was added and incubated for 10 min at room temperature. Following this, 200 µl of soluble starch was added and incubated for 1 h. The enzymatic reaction was terminated by adding 400 µl of 3,5-Dinitrosalicylic acid (DNS) reagent and placing it in a boiling water bath for 5 min, followed by cooling and the addition of 15 ml distilled water. The color change was observed and the absorbance was read at 540 nm using a Ultraviolet-Visible (UV-Vis) spectrophotometer. Percent inhibition was calculated according to the formula

% Inhibition=Absorbance (control)-Absorbance (sample)/ Absorbance (control)×100

Alpha glucosidase inhibition assay: Alpha glucosidase inhibition assay was studied according to the method described by Watanabe *et al.*[21]. 0.7 U/ml of yeast alpha glucosidase was dissolved in 100 mm phosphate buffer (pH 7.0) and used as enzyme extract. P-nitrophenyl alpha-D-glucopyranoside (pH 7.0) was used as the substrate. 100 µl of different concentrations (100, 200, 300, 400 and 500 µg/ml) of the seed powder extract were mixed with 1000 µl of phosphate buffer at 37° for 5 min. Following this, 50 µl of substrate solution was added and incubated for another 5 min at 37°. The absorbance was read at 405 nm using a UV visible spectrophotometer and percentage inhibition was calculated according to the formula

% Inhibition=Absorbance (control)-Absorbance (sample)/ Absorbance (control)×100

Statistical analysis:

The study data was analysed using Statistical Package for the Social Sciences (SPSS) software version 7. Results are expressed as mean±Standard Deviation (SD) of the triplicate values. Mean values were tested for significance using one-way Analysis of Variance (ANOVA) followed by Tukey's test. IC_{50} value was calculated using GraphPad Prism 5 software.

RESULTS AND DISCUSSION

The antibacterial potential of the aqueous sample extract was evaluated by measuring the zone of inhibition against gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and gram-negative (*Escherichia coli* and *Klebsiella pneumoniae*) bacteria as shown in fig. 1. The zone of inhibition was measured in mm and compared against the standard i.e., azithromycin (30 µg/ml). Results indicated that the inhibitory activity of *B. flabellifer* seed powder was dose-dependent as shown in Table 1. As the concentration of the sample increased, the diameter of zone of inhibition also increased. The sample exhibited maximum inhibition against gram negative bacteria *(Klebsiella pneumoniae*-20 mm and *Escherichia coli*-16 mm) and minimum inhibition was recorded against gram-positive bacteria (*Bacillus subtilis*-10 mm and *Staphylococcus aureus*-10 mm).

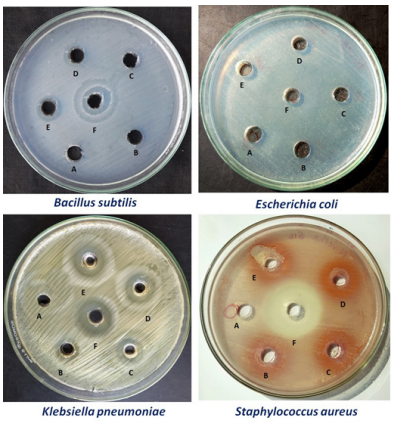

Fig. 1: *In vitro* antibacterial activity of freeze-dried aqueous *B. flabellifer* seed powder extract
Note: Concentration range, (A): Control; (B): 50 µg/ml; (C): 100 µg/ml; (D):150 µg/ml; (E): 200 µg/ml; (F): 30 µg/ml and azithromycin was used as standard

TABLE 1: ANTIBACTERIAL ACTIVITY OF FREEZE-DRIED AQUEOUS *B. flabellifer* SEED POWDER EXTRACT

Bacterial pathogens		Zone of inhibition (mm)				
		50 µg/ml	100 µg/ml	150 µg/ml	200 µg/ml	Standard 30 µg/ml (azithromycin)
Gram positive	*Bacillus subtilis*	-	6	8	10	20
Gram negative	*Escherichia coli*	8	9	14	16	26
Gram negative	*Klebsiella pneumoniae*	8	11	18	20	23
Gram positive	*Staphylococcus aureus*	6	7	8	10	25

Note: Values of single experiment

It can be seen from Table 1 that, although there was an inhibitory effect against all bacteria, the aqueous freeze-dried seed powder extract exhibited maximum inhibitory activity against gram negative bacteria, *Klebsiella pneumoniae* at 200 µg/ml (20 mm) and *Escherichia coli* at 200 µg/ml (16 mm). Gram negative bacteria possess an outer membrane which act as a barrier and prevent the entry of antibiotics thus making it a challenge for antibiotics to pass through[22]. Antibiotic resistance has become a serious concern as certain bacterial strains have developed resistance to almost all available antibiotics. In 2017, the World Health Organization (WHO) published a list of priority pathogens that are antibiotic resistant, appealing for the development of new antibiotics to reduce mortality and morbidity worldwide. Forming a majority of the list was, gram-negative bacteria due to their distinctive structure[23]. Some severe infections like intra-abdominal infections, urinary tract infections, ventilator-associated pneumonia and bacteremia are caused by gram-negative bacteria[24]. One promising way to fight these infections and overcome antibiotic resistance is to identify natural sources exhibiting antibacterial effect and in this search, the *B. flabellifer* fruit has been identified as one such. This can be supported by a study conducted by Vijayakumari *et al.*[25] where ethanolic *B. flabellifer* fruit pulp powder extract was analysed for the presence of phytochemicals and it was found to contain flavonoids, saponins, phenolic compounds and glycosides.

The presence of these bioactive compounds was correlated to the antibacterial effect exhibited by the fruit pulp powder against pathogens such as *Escherichia coli* and *Staphylococcus aureus*. In another study, Gummadi *et al.*[26] observed significant antibacterial activity of methanolic *B. flabellifer* seed coat extract against both gram-positive and gram-negative bacteria. Since freeze-dried *B. flabellifer* seed powder showed good inhibitory activity against the gram-negative bacteria, the present study suggests that, it can be considered as a potential ingredient in the development of antibiotics.

The aqueous freeze-dried *B. flabellifer* seed powder extract was tested against Vero cell line to evaluate its cell growth inhibition property and the results are presented in Table 2. Since the freeze-dried *B. flabellifer* seed powder can be considered as an essential ingredient in food product development, testing the safety of the compound and ruling out toxicity on normal cells is important. Thus, the cytotoxicity activity of the sample was tested on the Vero cells and a slight decrease in cell viability was seen with an IC_{50} value of 734 µg/ml. The dose response curve obtained for Vero cells was shown in fig. 2 and microscopic images of cytotoxicity activity

of the sample on Vero cells was shown in fig. 3.

TABLE 2: CYTOTOXICITY EFFECT OF FREEZE-DRIED AQUEOUS *B. flabellifer* SEED POWDER EXTRACT ON VERO CELL LINE

Concentration (µg/ml)	% Viability of Vero cells	
	Standard mefloquine	*B. flabellifer* seed powder extract
12.5	105.7±0.78[a]	105.8±0.1[a]
25	100.8±2.2[b]	104.5±0.5[a,b]
50	103.0±0.9[c]	101.7±4.6[c]
100	99.3±0.5[d]	96.5±3.8[d]
150	97.2±0.3d,[e]	95.1±8.6[d,e]
200	94.6±1.0[f]	92.8±4.0[f]

Note: Mean±SD of three independent estimations; [a-f]values followed by the different superscripts are significantly different within the column

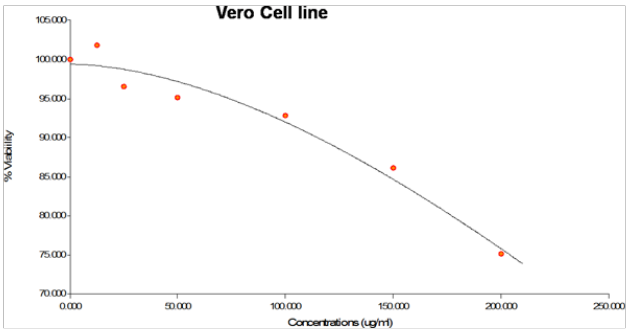

Fig. 2: Dose response curve for Vero cell line

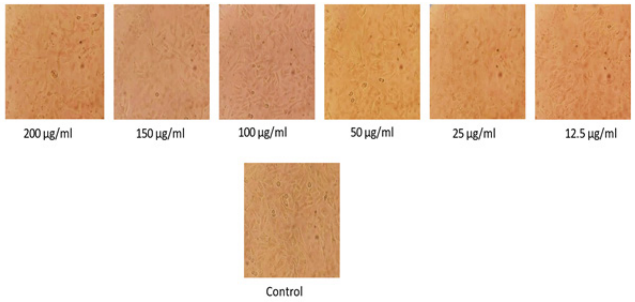

Fig. 3: Microscopic images of cytotoxicity effect of freeze-dried aqueous *B. flabellifer* seed powder extract on Vero normal kidney cells
Note: Scale bar-100 µm

The aqueous freeze-dried *B. flabellifer* seed powder extract was tested against HeLa cell line to evaluate its cell growth inhibition property and the results are presented in Table 3. The anti-cancer effect of *B. flabellifer* seed powder on HeLa cells indicates that with increasing concentration of the sample, the viability percentage of cancerous cells significantly ($p>0.05$) decreased by 30 % (from 102.7±3.4 to 73.6±4.1) indicating that the sample has the ability to kill cervical cancer cells *in vitro*. On the other hand, the viability percentage reduced from (75.7±0.4 to 12.6±0.55) in the presence of the standard mefloquine. The Half maximal Effective Concentration (EC_{50}) value for HeLa cell line was 137 µg/ml, which was calculated based on top-end bottom value along with the hill slope as shown in fig. 4. The EC_{50} value indicates the concentration of sample effective in inhibiting 50 % growth of cancer cells. The dose response curve obtained for HeLa cells was shown in fig. 4 and microscopic images of anti-cancer activity of the sample on HeLa cells was shown in fig. 5.

TABLE 3: ANTICANCER EFFECT OF FREEZE-DRIED AQUEOUS *B. flabellifer* SEED POWDER EXTRACT ON HELA CELL LINE

Concentration (µg/ml)	% Viability of HeLa cells	
	Standard mefloquine	*B. flabellifer* seed powder extract
12.5	75.7±0.4[a]	102.7±3.4[a]
25	62.09±0.6[b]	100.8±9.6[a,b]
50	44.0±0.25[c]	98.0±6.6[b,c]
100	28.9±0.9[d]	92.1±5.3[d]
150	20.2±2.4[d,e]	81.2±2.4[e]
200	12.6±0.55[f]	73.6±4.1[f]

Note: Mean±SD of three independent estimations; [a-f]values followed by the different superscripts are significantly different within the column

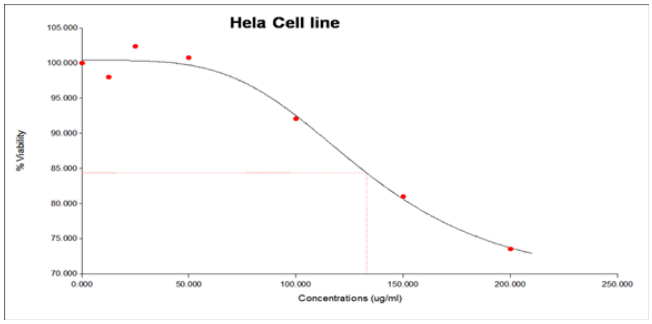

Fig. 4: Dose response curve for HeLa cell line

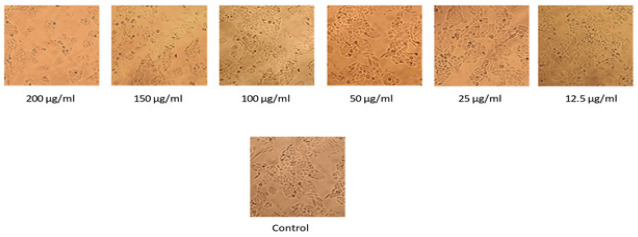

Fig. 5: Microscopic images of anti-cancer effect of freeze-dried aqueous *B. flabellifer* seed powder extract on HeLa cancer cells
Note: Scale bar–100 µm

Results in Table 2 and Table 3 indicate that, cell viability was inversely proportional to the sample concentration because as the sample concentration increased, there was a decline in the cell viability percentage. The proliferation of normal Vero cells was minimal (Table 2) and this demonstrates the *in vitro* safety of the freeze-dried *B. flabellifer* seed powder suggesting that it can be used as an ingredient in food product development once it is clinically tested on animals and humans. The

presence of phytochemicals could be the contributing factor to the anticancer potential of the sample and this is supported by the following studies. Mohan *et al.*[27], while evaluating the antimitotic activity of the methanolic *B. flabellifer* seed coat extract, identified the presence of phytoconstituents like carbohydrates, reducing sugars, triterpenoids, tannins and phenolic compounds and recommended its use for the treatment of cancer. Rao *et al.*[28] noticed significant cytotoxicity while testing the seed coat extract for its anticancer activity on the HeLa cell line and it was seen that, the seed coat extract inhibited cancer cell growth even at lower concentrations (32 µg/ml). It has been reported that approximately 1000 plant species on earth have anticancer properties[15] and the *in vitro* findings of the present study help us understand that the *B. flabellifer* seed powder could also be one among them.

The aqueous freeze-dried *B. flabellifer* seed powder extract was evaluated for its antidiabetic activity using the alpha amylase and alpha glucosidase assays. The experiment was performed in triplicates and the results are presented in Table 4. The maximum alpha amylase inhibition was 30.39±0.02 % at 500 µg/ml and the IC_{50} was 822 µg/ml, while maximum alpha glucosidase inhibition was 32.42±0.02 % at 500 µg/ml and IC_{50} value was 777 µg/ml. The results in Table 4 indicate that the inhibition of both enzymes by the sample extract was dose-dependent (100-500 µg/ml) and there was a significant effect ($p>0.05$) of the plant extract on the enzyme activities.

TABLE 4: ANTIDIABETIC ACTIVITY OF FREEZE-DRIED AQUEOUS *B. flabellifer* SEED POWDER EXTRACT

Concentration (µg/ml)	% Inhibition of alpha amylase	% Inhibition of alpha glucosidase
100	10.29±0.02[a]	13.65±0.01[a*]
200	14.71±0.01[a,b]	15.19±0.02[a*]
300	24.51±0.03[c*]	18.26±0.01[a,b]
400	26.47±0.02[c]	26.45±0.02[c]
500	30.39±0.02[d]	32.42±0.02[d*]

Note: Mean±SD of three independent estimations; [a-d]values followed by the different superscripts are significantly different within the column; *values are significantly different within the row, $p<0.05$

Diabetes, which is a worldwide prevalent metabolic disorder can be controlled by maintaining glucose homeostasis[5] and one such way is by inhibiting the carbohydrate metabolizing enzymes namely alpha amylase and alpha glucosidase[12]. Although synthetic anti-diabetic drugs are available, there is a huge demand for natural, non-toxic anti-diabetic agents since the former is expensive and causes side effects. Medicinal fruits stand a great chance in controlling blood sugar levels and exerting antidiabetic activity due to the pharmacologically active phytoconstituents present in them[29]. While looking for natural options, researchers stumbled upon the ethanolic extract of *B. flabellifer* flowers and observed a decrease in the blood glucose levels when administered to alloxan induced diabetic rats[30].

This paved way for this plant to be identified as a potential antidiabetic source. On evaluating the *in vitro* anti-diabetic activity of the aqueous *B. flabellifer* seed powder extract, it was observed that the sample had the ability to inhibit both enzymes thereby delaying glucose absorption. This is an indication for the pharmaceutical industry to consider using this seed coat powder in formulating a potent, natural anti-diabetic drug.

In conclusion, the results of the present study indicate that freeze-dried *B. flabellifer* seed powder has the ability to exhibit inhibitory activity against gram-negative bacteria, thereby helping to reduce the incidence of bacterial infections; can inhibit the growth of cervical cancer cells and is capable of inhibiting alpha amylase and alpha glucosidase enzymes. This proves that *B. flabellifer* possesses *in vitro* antibacterial, anticancer and antidiabetic activities. However, to be considered effective in the field of medicine, clinical tests involving animal and human trials should be conducted. If the *in vivo* findings match the *in vitro* results, this *B. flabellifer* seed powder can be considered a natural ingredient for therapeutic purposes.

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Conflict of interests:

The authors declared no conflicts of interest.

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