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ANTIDIABETIC POTENTIAL OF *BRACHYLAENA DISCOLOR*

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Abstract

Background: The traditional African herbal medicinal system has many reports of anti-diabetic food plants with no known side effects. Such plants and their products have been widely prescribed for diabetic treatment with little known mechanistic basis of their functioning. Therefore, these natural products need to be evaluated scientifically in order to confirm antidiabetic property claims.
Materials and Methods: In this study, leaves of *Brachylaena discolor* were evaluated for potential to inhibit α-amylase and α-glucosidase. The leaves were also screened for toxicity and free radical scavenging capacity.
Results: Results from the study show that the methanolic extract gave a higher α-glucosidase inhibition potential and was able to effectively scavenge free radicals better than the aqueous extract. The toxicity, cytotoxicity and mutagenicity screen also showed that both plant extracts are safe for use.
Conclusion: These results therefore indicate that *B. discolor* has the potential for use as a potential dietary adjunct or therapy for the treatment of diabetes.

Key words: *Brachylaena discolor*, α-amylase, α-glucosidase, DPPH, toxicity

Introduction

Diabetes mellitus is a metabolic disease which occurs as a result of insulin deficiency and/or insulin resistance and is a major cause of disability and hospitalization (Kamgang et al., 2008). Diabetes has become a major health concern in the world, with an expectation that diabetes and obesity will reach epidemic proportions, affecting developing countries to a far greater extent than the developed world (Rheeder, 2006). This may be attributed to the high number of African countries, which are undergoing a demographic transition and coming under increased western influence. Thus leading to an increase in the consumption of fat, salt and sugar, coupled with a rapid increase in urbanization in African countries and increase in nutritional deficiency thereby providing an elevated risk factor for the prevalence of diabetes (Colagiuri et al., 2006). Estimates from the world Health Organization have projected an increase from 171 million in the year 2000 to 366 million in 2030, with approximately 70% occurring in developing countries (Wild et al., 2004). Currently Type 2 diabetes is the most prevalent form of diabetes mellitus, however oral hypoglycemic drugs which are the main form of treatment, have been shown to have undesirable side-effects and high secondary failure rates (Moller, 2001). In addition to the lack of efficacy by pharmacological agents for the treatment of Type 2 diabetes there is also limited access to these drugs for people living in many rural areas as well as cost implications. These limitations have prompted research into traditional medicines as potential replacements for conventional pharmacological agents used in the treatment of diabetes (Grover et al., 2002).

There are many studies that focus on the scavenging activity of medicinal plants to evaluate the antioxidant activity because hydroxyl ions have a role to play in the pathogenesis of different diseases associated with diabetes mellitus. Free radicals are involved in many disorders such as neurodegenerative diseases, cancer and diabetes, therefore antioxidants through their scavenging power are useful for the management of those diseases (Rahimi et al., 2005). Antioxidants such as vitamins C and E have been shown to reduce oxidative stress as well as lowering glycosylated haemoglobin in diabetic patients through supplementation with Vitamin C (Clarke et al., 1996). Oxidative stress in diabetes coexist with a reduction in the antioxidant status which can result in an increase in the deleterious effects of free radicals (Sabu and Kuttan, 2002).

Botanical preparations including plant food supplements are widely used in both Eastern and Western diets. Due to the important biological functions of phytochemicals they are ubiquitous in all plants, and are therefore found in all food groups such as fruit and beverage products (Tiwari and Rao, 2002). Plant foods contain an array of bioactive compounds with natural antioxidants present in foods and other biological material having gained considerable interest in recent years due to their presumed safety and potential nutritional and therapeutic value. There is increasing concern with the growing use of plant food supplements, as these are generally not assessed for their safety before entering the market (Van den Berg et al., 2011). Ethnobotanical leads such as indigenous knowledge have already resulted in the discovery of an estimated 122 drugs from 94 plant species (Fennell et al., 2004).

Over the years, research has seen that many developing countries have resorted to easily accessible, effective traditional medicinal herbs such as *Syzygium cordatum, Allium sativum* and *Ficus thonningi* due to prevailing poor socio-economic conditions (Musabayane et al., 2005). These plants have been found to control diabetes mellitus by exhibiting hypoglycaemic and antioxidant effects and as a result, the WHO has supported the investigation of medicinal plants for underlying mechanisms for their hypoglycaemic effects (World Health Organisation, 2011). There is currently little regulation with regard to prescription and use of plant food supplements in South Africa increasing the likelihood of potentially toxic plants being applied.

The potential genotoxic effects that follow prolonged use of some of the more popular herbal remedies, are also cause for alarm (Fennell et al., 2004). It is therefore necessary to identify amylase inhibitors from natural sources which have less side effects. The traditional African herbal medicinal system practiced for thousands of years have reports of anti-diabetic plants with no known side effects. Such plants and their products have been widely pre-scribed for diabetes treatment all around the world with little known mechanistic basis of their functioning. Therefore, these natural products need to be evaluated scientifically in order to confirm claims for their anti-diabetic properties (Iwueke and Nwodo, 2008).

The plant chosen for this study was *B. discolor*, an evergreen shrub usually 4 – 10 m in height which is often multi-stemmed. There are three known varieties; var. *discolor* which is commonly known as the Coast Silver Oak or Coastal Silver Oak, var. *transvaalensis* commonly known as the Forest Silver Oak or Natal Silver Oak and var. *rotundata*. The leaf infusions of *B. discolor* are taken as tonics used to treat diabetes

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and renal conditions and in some cases the infusions are taken as purgatives against intestinal parasites (Hutchings, 1996). The dried milky infusion of the leaf is used as antihelmintics against roundworm (Palmer and Pitman, 1972). The aim of this study was to investigate the potential of *B. discolor* as a possible dietary adjunct or therapy for diabetes.

Materials and Methods

Plant Material

Leaves of *B. discolor* were collected in Durban, Province of Kwazulu Natal, South Africa, and identified by using available floral keys. The leaves were carefully examined for insect-damage or fungus-infection and those infected were discarded. Healthy leaves were dried at 40°C for 48 h in a convection oven. Once completely dry, plant material was ground to a fine powder and the dried plant material stored in amber Schott bottles at room temperature until required.

Preparation of Extracts

Aqueous extraction of the dried *B. discolor* plant material was carried out according to the procedure outlined by Jeremy and Whiteman (2003) with minor modifications. 50 g of the dried plant material was stirred for 24 h in 200 ml of distilled water. The slurry was centrifuged and the supernatant collected. This was then concentrated by placing it in a biofreezer (Snijders Scientific, Holland) at -70°C for 24 h, and then freeze-dried (Virtis Benchtop Freeze Dryer).
Methanolic extracts of the dried plant material were produced in a similar manner as the aqueous extraction procedure, except the sample was extracted in 80% methanol. The supernatant was concentrated using a Buchi RE Rotoevaporator including a Buchi 461 water bath set at a temperature of 45°C. The concentrate was placed in a biofreezer and freeze-dried. The freeze-dried material for both plant extracts were used as stock and working solutions were prepared for appropriate applications.

α-Amylase Inhibition Assay

The effect of *B. discolor* crude extract on α-amylase (EC 3.2.1.1) activity was determined according to the method described by Ali et al. (2006) with some modification. Preliminary experiments were conducted to establish optimal assay conditions such as temperature, substrate enzyme and inhibitor concentration. The antidiabetic activity was investigated through the inhibition of α-amylase, an enzyme active in the digestion of starch, which thus reduces the absorption of glucose. Briefly alpha amylase (1 U/ml) was pre-incubated with 1–5 mg/ml aqueous and methanol plant extract for 5 min. The reaction was started by addition of 1 ml of 0.2% potato starch dissolved in 20 mM phosphate buffer (pH 6.9). The reaction mixture was then incubated at 20°C for 3 minutes and terminated by addition of 1 ml of DNS reagent (1% 3,5-dinitrosalicylic acid, 12% sodium potassium tartrate in 0.4M NaOH). The reaction mixture was heated for 15 min at 100°C. Amylase activity was determined by measuring the absorbance at 540 nm and expressed as percentage of the blank control (without the extract). The α-amylase inhibition potential was expressed as a percentage of inhibition and calculated by the following equations:

Percentage inhibition = 100 − % reaction…….....(1)

% reaction = (mean maltose in sample/mean maltose in control) × 100…….....(2)

The concentration of the extract required to inhibit the enzyme activity by 50% (IC_{50}) was calculated using regression analysis. All experiments were performed in triplicate.

α - Glucosidase Inhibition Assay

The effect of *B. discolor* crude extract on α-glucosidase inhibition was determined according to the method described by Kim et al. (2000) with minor modification. Bakers yeast α-glucosidase was dissolved at a concentration of 0.1 U/ml in 100 mM phosphate buffer (pH 7.0). The enzyme source used for this assay was up from phosphate buffer containing bovine serum albumin (2 g/l) and sodium azide (0,2 g/l). The substrate used for the assay was p-nitrophenyl-α-D-glucopyranoside. Methanolic and aqueous plant extracts were made up to concentrations of 1 – 5 mg/ml in 100 mM phosphate buffer. The positive control used was acarbose at a concentration of 1 mg/ml of buffer. Briefly 10 µl of each of the *B. discolor* extracts were incubated for 5 min with 50 µl of the enzyme source in a 96 well microplate. After the incubation 50 µl of substrate was added and incubated for an additional 5 min at room temperature. Both the pre-substrate and the post substrate additions were measured at 405 nm on a Biohit ELx800 microplate reader. The percentage of α-glucosidase inhibition which occurred was measured using the following equation:

Percentage inhibition = [(1 – B)/A] × 100……...(3)

where A is the absorbance of the control and B is the absorbance of the samples containing extract. The concentration of extract required to inhibit the enzyme activity by 50% (IC50) was calculated using regression analysis. All experiments were performed in triplicate.

DPPH (1,1-diphenyl-2-picrylhydrazyl) Radical Scavenging Capacity

The anti-oxidative properties of the crude extracts of *B. discolor* were tested using the DPPH (1,1-diphenyl-2-picrylhydrazyl radical) photometric assay (Tomić et al., 2009). The freeze-dried aqueous and methanolic plant extracts were made up to final concentrations of 1 – 500 µg/ml in methanol respectively. Rutin (Sigma) found in the buckwheat plant *Fagopyrum* was used as a positive control. 1 ml of 0.3 mM DPPH in methanol was added to 1 ml of plant sample solution of different plant extract concentrations and allowed to react at room temperature for 30 min. Methanol was used as a blank and 1.0 ml methanol plus DPPH solution was used as a negative control. The positive control was DPPH solution (1 ml) plus 1 ml of a 1 mM Rutin solution.

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Each test was carried out in triplicate and results expressed as the mean and standard deviation of the mean. The absorbance values were measured in a Varian Cary 1E UV- visible spectrophotometer at 518 nm and the average absorbance values was converted into the percentage antioxidant activity, using the following equation:

$$\% \text{ Inhibition} = 100 - [((Abs_S - Abs_B)/Abs_N) \times 100] \quad \text{.....(4)}$$

where Abs_S is the absorbance of the test sample including DPPH, Abs_B is the absorbance of the sample blank (test sample excluding DPPH) and Abs_N is the negative control (containing all reagents except the test compound).

Cytotoxicity Testing using a Cell Line

The cell line used in this study was the HeLa cell line derived from a human cervix adenocarcinoma (Freshney, 2005). HeLa cells were received in an active state and incubated at 37°C in a humidified incubator (Snjiders Hepa, United Scientific group, Cape Town, South Africa) with 5% CO_2. When cells were 80% confluent, they were sub-cultured, and stock cultures were stored in a biofreezer (Snijders Scientific, Holland) at -70°C until required. Cells were regenerated as described by Freshney (2005). Cells were removed from the -70°C biofreezer, the vial was swabbed with 70% ethanol to sterilize and, rapidly thawed. The cells were then transferred to 20 ml of pre-warmed 10% cell culture media in 75 cm^2 tissue culture flasks and incubated at 37°C humidified incubator with a 5% CO_2 atmosphere.

The effect of extracts was assayed using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay according to protocols by Mosmann (1983). The assay was carried out in 96 well, flat bottomed microtitre plates (Costar, Corning, USA). Approximately 200 µl of 5.5×10^4 cells were added into each well, 20 µl of the crude plant extracts (10 – 1000 µg/ml) were added to the wells. In the control wells 20 µl, of 2% DMSO and 20 µl DMEM were added and the plate incubated in a 37°C humidified incubator with a 5% CO2 atmosphere for 20 hours. Then 20 µl of MTT reagent (Sigma, St Louis, USA) was added and the plates incubated for a further 4 hours at 37°C in the incubator with 5% CO_2 atmosphere. Subsequently 100 µl of 100% DMSO was added to each well and the plate incubated for an additional 1 h.
The absorbance was read at 450 nm with a reference wavelength of 630 nm on an ELISA microplate reader (Digital Analogue Systems, Italy). The percentage viability was determined using the formula below:

$$\% \text{ Cell viability} = (\text{absorbance treated cells} \times 100)/\text{absorbance untreated cells} \quad \text{..... (5)}$$

Toxicity Testing using Brine Shrimp

Toxicity of the aqueous and methanolic extract of *B. discolor* was evaluated using the brine shrimp lethality assay with minor modifications. 25 mg of *Artemia salina* (brine shrimp) eggs (Natures Petland, Durban, South Africa) were added to artificial salt water (23 g NaCl, 11 g $MgCl_2.6H_2O$, 4 g Na_2SO_4, 1.3 g $CaCl_2.2H_2O$, 0.7 g KCl in 1000 ml distilled water) and kept at room temperature. The pH was adjusted to 9.0 using Na_2CO_3 to avoid risk of death to the nauplii during incubation as a result of a decrease in pH at room temperature. After 24 h, 15 ml of yeast solution was added to the chamber for every liter of salt water in order to feed the larvae. 48 h after the eggs were incubated, the larvae were extracted by picking up the moving larvae and visibly counted. Each vial with 100 µl of plant sample at different concentration (10 - 1000 µg/ml of plant extract dissolved in 2% DMSO) contained 10 nauplii, including the control group, and was filled to 5 ml total volume with artificial salt water containing 3 mg of yeast. The vials were then incubated at 27°C for 24 h. After 24 h, dead larvae were counted and percentage death determined.

Ames Mutagenicity Test

The Salmonella mutagenicity assay was conducted according to the method described by Maron and Ames (1983) with minor modifications. Two cultures of the *S. typhimurium* were obtained from the South African Medical Research Council (MRC). Using a flamed bacteriological needle a colony was asceptically removed from the pure culture and inoculated into a sterile 250 ml Erlenmeyer flask containing 25 ml of nutrient broth (Oxoid) and 78 µl of 8 mg/ml Ampicillin (to maintain the stability of the plasmid). The flask was then incubated on a shaker at 37°C (150 rpm) for 16 h to obtain an optical density at 660 nm of between 1.2 and 1.4. In a sterile test tube, 100 µl of the broth culture was added to 2 ml of 0.05 mM histidine/0.05 mM biotin top agar, vortexed and plated onto a minimal glucose agar plate. The plate was then incubated at 37°C for 48 h. Broth cultures of *S. typhimurium* were made by inoculating nutrient broth with master plate colonies. These cultures were then incubated on a shaker at 37°C (150 rpm) for 24 h. The methanolic and aqueous crude extracts of *B. discolor* were dissolved in 2% DMSO to obtain concentrations of 100 – 10 000 µg/ml. Sterile distilled water was used as a negative control and sodium azide (NaN_3) which is highly mutagenic was used as a positive control. The NaN_3 was dissolved in 2% DMSO solution to obtain concentrations of 5 – 20 µg/ml. Three plates were prepared for each concentration of test compound. In a sterile test tube, 100 µl of bacterial culture, 100 µl of test compound and 2.9 ml of soft agar held at 45°C, were added. This was briefly mixed and poured onto glucose minimal agar plates. Once the agar overlay solidified, the plates were inverted and incubated at 37°C for 48 h, after which the number of revertant histidine dependant colonies were counted and the mutant frequency determined. The mutant frequency was determined using the following formula:

$$\text{Mutant frequency} = \text{Revertant number of colonies / negative control} \quad \text{.....(6)}$$

Results and Discussion

The main aim in the treatment of diabetes mellitus (DM) is to maintain normal blood glucose levels in both the fasting and postprandial states (Donga et al., 2011). One therapeutic approach to maintain normal blood glucose levels is by suppressing the production and/or absorption of glucose by inhibiting either the α-amylase or α-glucosidase enzymes. Alpha amylase breaks starch down into various oligosaccharides and disaccharides, which are further hydrolyzed by α-glucosidases to produce glucose and other monosaccharides, which are then absorbed in the small intestine (Ahmed et al., 2010). Experimental animal studies and clinical studies have both shown that inhibitors of α-amylase and α-glucosidase are capable of suppressing the production and absorption of glucose through the small intestine (Tarling et al., 2008). Inhibitors currently in clinical use are acarbose and miglitol which are use for the inhibition of α-amylase and α-glucosidase, while voglibose is used only

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for the inhibition of α-glucosidase (Sudha et al., 2011). To determine if *B. discolor* aqueous and methanolic extracts could act at this level they were evaluated for their potential to inhibit α-amylase and α-glucosidase. Aqueous and methanol extracts of the leaves of *B. discolor* were screened for their α-amylase inhibitory activity against *Bacillus* sp. (Sigma, A550) α-amylase. As shown in Figure 1 both extracts demonstrated inhibition amylase. However the methanolic extract was a stronger inhibitor with a maximum inhibition of 110 %. IC_{50} values of leaf extracts and the positive control acarbose and are shown in Table 1. The extracts were also screened for their α-glucosidase inhibitory. As shown in Figure 2, the aqueous extract demonstrated the highest percentage inhibition against *S. cerevisiae* α-glucosidase (48,55%). The IC_{50} values were not significantly different and are shown in Table 1. The results obtained for the inhibition of α-amylase indicated that *B. discolor* methanolic extract (IC_{50} = 1,81) and the aqueous extract (IC_{50} = 10,99) are capable of inhibiting the action of amylase. This suggests that both polar and nonpolar components of the leaf extract of *B. discolor* inhibited α-amylase. It is important to note the difference in IC_{50} values between the methanolic and aqueous extracts, which show that the methanolic extract is more effective at a much lower dose than the aqueous.

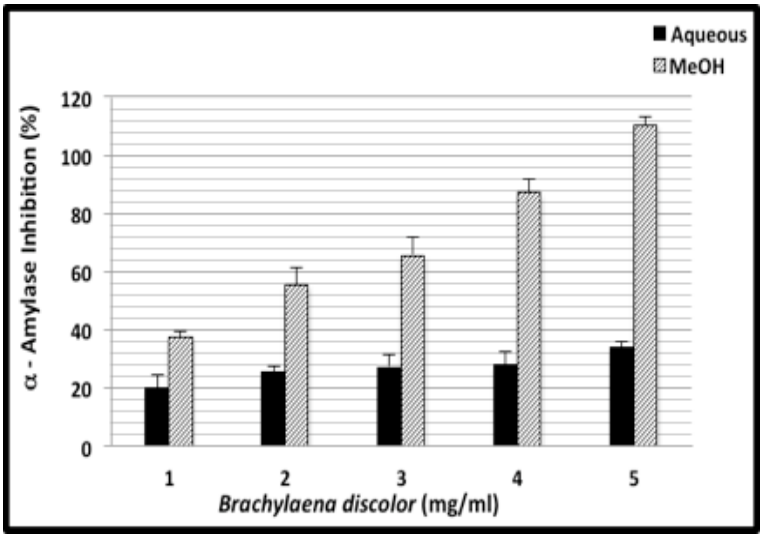

Figure 1: α-Amylase inhibition potential of aqueous and methanolic extracts of *B. discolor*. Each column represents mean ± SD (n=3).

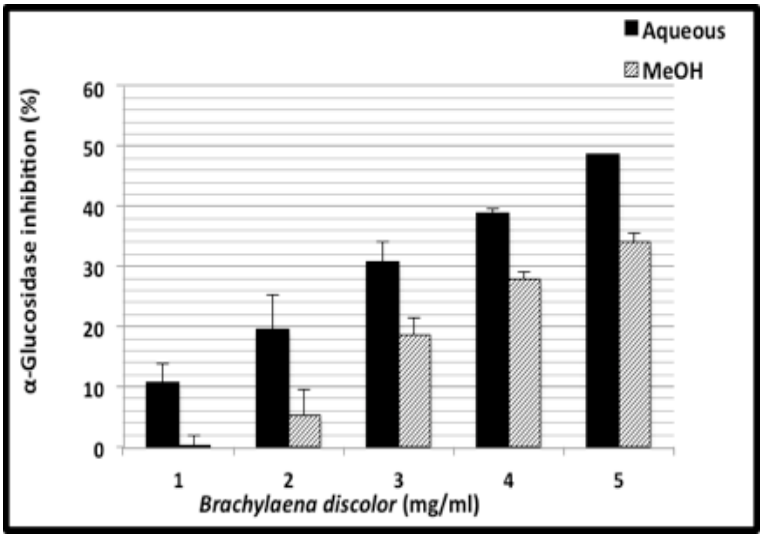

Figure 2: α-Glucosidase inhibition potential of aqueous and methanolic extracts of *B. discolor*. Each column represents mean ± SD (n=3).

Table 1: Inhibitory effect (IC_{50}) of aqueous and methanolic extracts of *B. discolor* and acarbose on α-amylase and α-glucosidase

Sample	α-amylase IC_{50} (mg/ml)	α-glucosidase IC_{50} (mg/ml)
***B. discolor* (Aqueous)**	10,99	5,11
***B. discolor* (Methanolic)**	1,81	7,18
Acarbose	0,03	1,20

In patients with diabetes there are multiple factors responsible for the increase in oxidative stress. Among these factors glucose autooxidation is the most dominant, leading to the production of free radicals. In this study *B. discolor* was evaluated for its potential antioxidant effect (Arulselvan and Subramanian, 2007). The DPPH assay was used as it determines the activities of both hydrophilic and lipophilic chemicals. The

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DPPH free radical scavenging capacity results of Rutin (positive control) and *B. discolor* extracts are expressed as a percentage of inhibition. Rutin, reduced 96 ± 0.18% whilst the *B. discolor* exhibited an increase in inhibition relative to the increase in extract concentration (Fig 3). The minimum concentration at which both extracts exhibited free radical scavenging activity was 1 µg/ml. There is substantial agreement among scientists that relatively higher levels of antioxidant secondary plant metabolites are produced by plants in response to biotic and abiotic stress (Mazid et al., 2011). The aqueous extract showed a higher inhibition than the methanolic extract at the 60 - 100 µg/ml range. The IC_{50} for the methanol and aqueous extracts were 92.3 µg/ml and 82.8 µg/ml respectively. The results indicate that there is no significant difference between the DPPH free radical scavenging capacity of the methanol and aqueous extract of *B. discolor* and the positive control (Rutin). Therefore the methanolic and aqueous extracts of *B. discolor* may act as a potential source of antioxidants. The scavenging ability of both the aqueous and methanolic extractions of *B. discolor* show potential use of their antioxidant mechanism in diabetes and related complications.

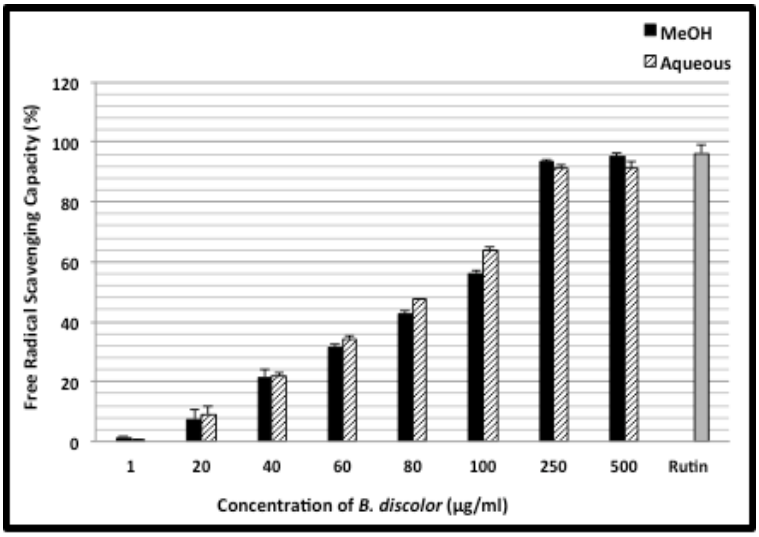

Figure 3: DPPH radical scavenging capacity of *B. discolor* leaf extracts [methanolic (MeOH) and aqueous (H_2O)]. Values are mean of triplicate analysis with Rutin (1 mM) serving as a positive control.

The MTT assay is a cytotoxicity test of metabolic competence based upon the assessment of mitochondrial performance (Mosmann, 1983). The cytotoxic effect of both extracts of *B. discolor* on the HeLa cell line is shown in Figure 4 using the MTT Assay. Both the aqueous and methanolic extracts stimulated the growth of the HeLa cell line with an increase in cell viability indicating a proliferative effect of the extracts on the cell line.

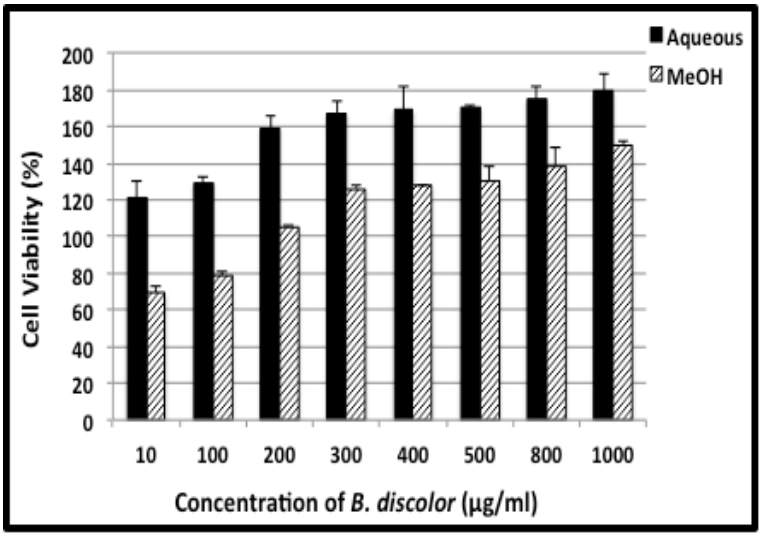

Figure 4: Cytotoxic effect of aqueous and methanolic (MeOH) extracts of *B. discolor* on the HeLa cell line. Each column represents mean ± SD (n = 3).

With regard to the Brine Shrimp Lethality Assay for a test compound to be considered highly toxic it needs to show shrimp death of 70% or more. For a compound to be considered slightly toxic it needs to show cell death of between 50 – 70%. None of the crude extracts in this assay showed levels of toxicity, with both the extracts showing 100% shrimp survival at the highest concentration tested which was 1000 µg/ml, whereas the positive control which was an organophosphate showed 100 % mortality.

The mutant frequency is expressed as the quotient of the number of revertant colonies over the number of colonies in the negative control. Table 3 shows the mutant frequency of the plant extract tested on the *S. typhimurium* TA 98 strain. The greater the number of revertant colonies, the greater the mutant frequency. According to Maron and Ames (1983) a mutagenic potential is assumed if the mutant frequency is greater than 2; a possible mutagenic potential is assumed if the mutant frequency ranges between 1.7 and 1.9; and no mutagenic potential is assumed if the mutant frequency is lower than 1.6. No mutagenic activity was exhibited by any of the plant extracts up to concentrations of 1000

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µg/ml. Sodium azide was the chosen mutagen used in this experiment which showed a mutagenic potential; as the concentration increased so did the number of revertant colonies. The methanolic extract of *B. discolor* only showed a mutagenic potential for the *S. typhimurium* TA 100 strain at a concentration of 1000 µg/ml. Sodium azide (NaN_3) was the chosen mutagen used in this experiment and showed a mutagenic potential with an increase in the number of revertant colonies directly proportional to the concentration.

Table 3: Mutant frequency of *S. typhimurium* TA 100 and TA 98 strains in agar plates with methanolic and aqueous extracts of *B. discolor*

Sample	Concentration (µg/ml)	Mutant frequency	
		TA 100	TA 98
Aqueous extract (*B. discolor*)	1000	0,147 ± 0,055	1,383 ± 0,525
	100	0,144 ± 0,031	0,432 ± 0,078
	10	0,131 ± 0,030	0
Methanolic extract (*B. discolor*)	1000	0,196 ± 0,052	0,907 ± 0,118
	100	0,118 ± 0,017	0,752 ± 0,158
	10	0,153 ± 0,043	0,253 ± 0,014
NaN_3	20	2,546 ± 0,215	1,561 ± 1,729
	10	2,112 ± 0,600	4,819 ± 1,341
	5	2,530 ± 0,480	1,448 ± 1,292

The results obtained in this study show that, *B. discolor* has α-amylase and glucosidase inhibition potential as well as high free radical scavenging capacity. This study suggests that *B. discolor* may be used to reduce the oxidative damage caused by free radical and serve as a potential functional food additive or nutraceutical component in the treatment of diabetes. Further studies are however necessary to identify the active compounds within *B. discolor*.

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