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Antioxidant and In-vitro Antihyperglycemic Activity of *Cheilocostus speciosus* using Saccharomyces Glucose Absorption Assay

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Abstract: There has been a significant increasement of prevalence of diabetes over the world. A wide variety of new pharmaceuticals that are being developed rapidly for the diabetes but still with many side effects. Therefore, a huge attention has gone to the medicinal plants in searching of cure for diabetes. *Cheilocostus speciosus* has a rich history in traditional medicine systems such as Ayurveda. It has been recognized for its medicinal properties and has been traditionally used for the treatment of various illnesses, including diabetes. This study aims to evaluate free radical scavenging antioxidant activity and in-vitro antihyperglycemic activity using saccharomyces glucose inhibition of ethyl acetate extract of *Cheilocostus speciosus*. The ethyl acetate plant extract of *Cheilocostus speciosus* leaves were fractionated into 4 fractions as, ethyl acetate sub fraction, n-hexane sub fraction, n-butanol sub fraction and dichloromethane sub fraction. They have screened for the Antioxidant activity using Ferric Reducing Antioxidant Power Assay and In-vitro Antihyperglycemic activity using Saccharomyces Glucose Absorption Assay.

***IndexTerms* – Antioxidant, Antihyperglycemic, *Cheilocostus speciosus*, Diabetics**

1. INTRODUCTION

Diabetes is a chronic condition that develops when the body either cannot utilize the insulin the pancreas makes appropriately or does not create enough of it. A hormone called insulin controls blood sugar [1]. Too much blood sugar persists in the bloodstream when insufficient insulin or cells cease reacting to insulin. Over time can result in severe health issues like renal disease, eyesight loss, and heart disease [2].

Free radicals are unstable chemicals that the body creates in response to environmental and other stresses, and antioxidants are compounds that can stop or decrease the harm that free radicals cause to cells. "Free radical scavengers" is another name for antioxidants. Antioxidants can be found in both natural and synthetic sources. It is believed that some plant-based foods are high in antioxidants. Phytonutrients, or nutrients derived from plants, include plant-based antioxidants.

The development of diabetes complications, including microvascular and cardiovascular problems, is significantly influenced by oxidative stress [3]. Increased oxidative stress, which is generally acknowledged to play a role in the onset, progression, and development of diabetes as well as its complications, is defined as a persistent imbalance between the production of highly reactive molecular species (primarily nitrogen and oxygen) and antioxidant defenses [4].

According to statistics on the condition, there has been a rise in the number of diabetics in Sri Lanka. The International Diabetes Federation's most current figures show that 8.5% of people in Sri Lanka have diabetes. Currently, one in twelve individuals in the nation, or 1.16 million, have diabetes [5]. WHO estimates that there are 422 million people with diabetes globally, most of whom reside in low- and middle-income nations. Diabetes is responsible for 1.5 million daily fatalities [2].

The use of medicinal plants in antidiabetic treatments has been reported to be effective in treating diabetes. For treating diabetes, the usage of extracted bioactive components from medicinal plant extracts has dramatically risen in Sri Lanka. Many native and foreign medicinal plants are produced in Sri Lanka. Ayurveda, Siddha, Unani, and Deshiya chikithsa are four traditional medical systems that utilize herbal preparations. Traditional Sri Lankan doctors can use herbal remedies to treat several pathogenic illnesses, including diabetes mellitus [6].

We selected *Cheilocostus speciosus* (Thebu) plant for this study and examined the plant's leaves to determine the antioxidant and in-vitro antihyperglycemic activity of *Cheilocostus speciosus* leaves using FRAP assay and Saccharomyces glucose absorption assay [7].

The outcomes of this research hold significant promise for understanding the potential health benefits of *Cheilocostus speciosus* as a natural source of antihyperglycemic and antioxidant agents. The findings may contribute to the development of alternative or

complementary approaches for diabetes management, reducing the risk of associated complications. Additionally, understanding the underlying mechanisms of action can facilitate the discovery of bioactive compounds and aid in the development of novel therapeutic strategies.

2. RESEARCH METHODOLOGY

2.1 Collection and authentication

Cheilocostus speciosus plants were collected from an estate in Galle in southern Province, Sri Lanka. Plants which collected were identified and authenticated at Bandaranaike Memorial Ayurvedic Research Institute, Department of Ayurveda, Navinna, Maharagama, Sri Lanka.

2.2 Preparation of plant leaves

Leaves were separated from the collected *C. speciosus* plants. Then they were washed first using tap water and followed by distilled water. Washed leaves were air dried in shade. After that it was grind into a powder.

2.3 Extraction of plant leaves

The 100 g of air-dried powder of *C. speciosus* leaves was extracted by refluxing in 800 ml of ethyl acetate (EtOAc) at 50^0C for 30 minutes. The extractant solvent was evaporated in a water bath at 40^0C. 1.91g of the residue crude obtained from extract was stored in 4^0C freezer in the laboratory for further tests.

2.4 Fractionation

The fractionation of ethyl acetate crude extract was done using an activity guided solvent-solvent partitioning using a separating funnel. First the crude was dissolved in 50ml of EtOAc until it becomes saturated. After becoming dissolved it was filtered and collected a clear solution. It was then added to the separating funnel followed with n-hexane equal volume of 50ml. After mixing it up and settled, the two layers were separated. The n-hexane layer was again added to the separating funnel with 50ml of Dichloromethane (DCM). After mixing it up and settled, the two layers were again separated. The DCM layer was added to the separating funnel with 50ml of n-butanol. After separating the two layers as previously mentioned, both DCM layer and n-butanol layers were separately collected. Then the 4 sub fractions which collected were evaporated at 40^0C in a water bath. Bioactivity testing were done to the crude and 4 sub fractions (EtOAc, n-hexane, N-butanol, DCM) of EtOAc extracted crude.

2.5 Determination of Antioxidant activity by FRAP Assay

This assay was performed according to the procedure mentioned by M. Vijayalakshmi (2016) [8]. A dilution series from the crude of *C. speciosus* leaves was prepared (15,10,5,2.5,1.25,0.625,0.039 mg/ml) where the volume of each dilution was made up to 1ml using ethyl acetate. 2.5ml of PBS (6.6 pH) was added to all the tubes and mixed well. Then 2.5ml of 1% potassium ferricyanide solution was added and mixed well by vortexing. They were incubated at 50°C for 20 minutes. After incubating 2.5 ml of 10% TCA was added into it and mixed well. Then the tubes were centrifuged at 3000rpm for 10 minutes. Next 2.5ml of supernatant was collected from each tube and transferred them to separate test tubes. 2.5ml of ionized water was added into each test tube and mixed well. Then 0.5 ml of ferric chloride was added into it and mixed resulting a bluish color formation. The absorbance was taken at 700nm using UV double beam spectrophotometer. Ascorbic acid used as the positive control and distilled water as the negative control. The above same procedure was followed for the sub fractions where the concentrations of the dilution series taken as 1.7mg/ml, 1.6mg/ml and 1.4mg/ml (concentrations were taken according to the IC^{50} value calculated for the crude) and volume of each dilution was made up to 1ml using their extracted solvent. FRAP assay was done to the standard Diclofenac to compare the results of sub fractions with the standard. In here, the same procedure was followed with diclofenac dilutions of 0.6mg/ml, 0.5 mg/ml and 0.4 mg/ml diluted with distilled water. The free radical scavenging antioxidant activity was expressed as a percentage of inhibition which was calculated according to the following equation.

$$\%\ \text{Inhibition of FRAP activity} = [(A1 - Ao)/ (Ac\text{-}Ao)] \times 2 \times 100$$

Where A1 is the absorbance of the sample; Ao is the absorbance of the negative control; Ac is the absorbance of the positive control.

2.6 Determination of In-vitro Antihyperglycemic activity using Saccharomyces Glucose Absorption Assay

This assay was conducted according to the well-defined procedure mentioned by G. Rehman (2018)[11] with slight modifications. First 20% yeast cell suspension was prepared. Commercial baker's yeast (1 g) was dissolved in 5 ml of distilled water. It was mixed properly by vortexing at the speed of 2000 rpm. Then the mixture was centrifuged at 3800 rpm for 5 minutes. After centrifugation, the turbid supernatant was discarded and sedimented yeast cells were mixed again with 5 ml of distilled water. The same procedure was repeated until a clear supernatant was obtained. To prepare the 20% of yeast cell suspension, 20 parts of the clear supernatant fluid were diluted with 80 parts of distilled water.

The stock solution of crude extract was prepared at 250 µg/ml w/v by mixing in ethyl acetate. The dilution series was prepared using the crude and the sub fractions with their respective solvents at different concentrations of 1.7 mg/ml, 1.6 mg/ml, and 1.4 mg/ml w/v. 1ml of each concentration of the extracts were supplemented with 1 ml of several different glucose solutions (15 mmol/l, 25 mmol/l, 30 mmol/l) separately. The well-mixed solution was incubated for 10 minutes at 37 °C. Then 100 µl of 20% yeast cell suspension was added to each tube and mixed. All tubes were incubated for 1 h at 37 °C in the water bath. After the incubation, the tubes were centrifuged at 3800 rpm for 5 minutes. The absorbance of the final glucose solution was assessed by the glucose oxidase method using a UV double beam spectrophotometer at 520 nm. The same procedure was followed with the standard drug Metformin

along with extract. The negative controls were prepared with only the solvents. The percentage increase of glucose uptake by yeast cells was determined using the following formula [9].

$$\% \text{ Increase in glucose uptake} = (Ac - A1) / Ac \times 100$$

Where Ac is the absorbance of control; A1 is the absorbance of sample.

3. RESULTS AND DISCUSSION

3.1Determination of Antioxidant activity

In current study, first we have found the antioxidant activity of ethyl acetate crude extract using frap assay. We have used concentration gradient and calculated antioxidant activities as percentage (Table 1).

Table 1; FRAP assay results of ethyl acetate crude extract

Concentration (µg/ml)	**39**	**625**	**1250**	**2500**	**5000**	**10000**	**15000**
Antioxidant activity	5.71	5.10	31.61	79.83	141.24	165.17	195.73

By using concentration against percentage inhibition graph mentioned in figure 1, we have found the IC_{50} value of the ethyl acetate crude extract as 1.597mg/ml. IC_{50} value provides valuable insights into the potency of the plant extract in combating oxidative stress, a key contributor to diabetes complications. IC_{50} represents the concentration at which a substance exerts half of its maximal inhibitory effect.

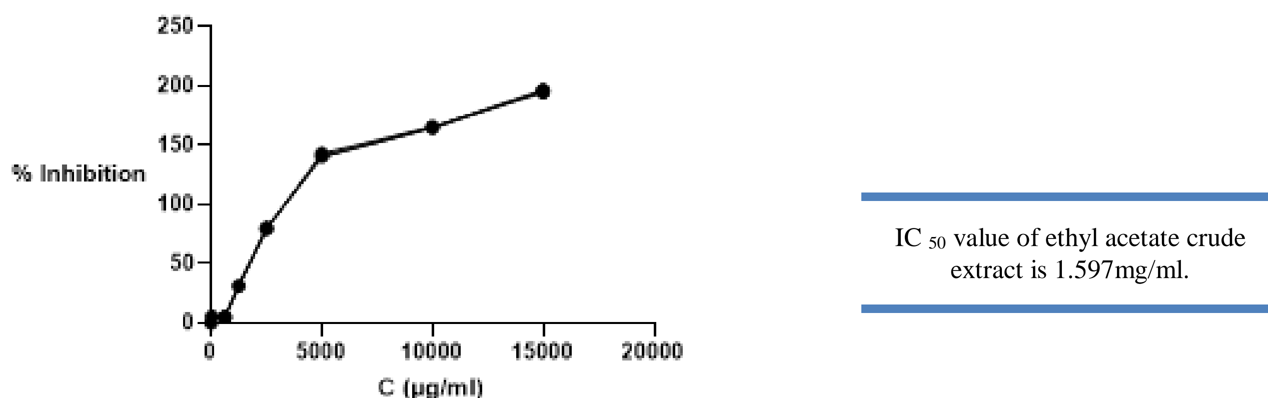

Figure 1; concentration vs percentage inhibition graph

As mentioned in methodology we have done FRAP assay for sub fractions (ethyl acetate sub fraction, n-hexane sub fraction, n-butanol sub fraction, DCM sub fraction) and the results were given by compared to the optimum antioxidant value of diclofenac standard of 0.5mg/ml concentration (Table 2).

1.7mg/ml ethyl acetate sub fraction had 71.66±0.01% antioxidant activity, 1.6mg/ml ethyl acetate sub fraction had 70.74±0.01% antioxidant activity, 1.4mg/ml ethyl acetate sub fraction had 59.97±0.02% antioxidant activity also 1.7mg/ml n-hexane sub fraction had 72.71±0.02%, 1.6mg/ml n-hexane sub fraction had 61.21±0.01% and 1.4mg/ml n-hexane sub fraction had 59.31±0.01% antioxidant activity as shown in Table 3.

n-butanol subfraction continuously showed low antioxidant activities in all three concentrations and in 1.7mg/ml DCM sub fraction showed antioxidant activity which is lower than 50%. By doing FRAP assay for sub fractions we have found that the ethyl acetate sub fraction and n-hexane sub fractions shows the highest antioxidant activities.

Table 2; Antioxidant activities of Diclofenac standard

	Concentration (mg/ml)	**Antioxidant activity**
Diclofenac Standard	0.6	2.0133
	0.5	2.2754
	0.4	2.1458

Table 3; FRAP assay results of sub fractions

	Concentration (mg/ml)	% Activity
Ethyl acetate fraction	1.7	71.66±0.01
	1.6	70.74±0.01
	1.4	59.97±0.02
n-Hexane fraction	1.7	72.71±0.02
	1.6	61.21±0.01
	1.4	59.31±0.01
n-Butanol fraction	1.7	45.64±0.02
	1.4	33.64±0.01
DCM fraction	1.7	39.95±0.02
	1.6	87.60±0.01
	1.4	75.67±0.01

3.2Determination of In-vitro Antihyperglycemic activity

The results of the present study revealed that Saccharomyces glucose uptake activity is increased at high glucose concentrations and high ethyl acetate crude extract concentrations (Table 4)

Table 4; results of Saccharomyces glucose absorption assay in crude

Glucose Concentration (mmol/l)	**15**			**25**			**30**		
Ethyl acetate crude conc. (mg/ml)	1.7	1.6	1.4	1.7	1.6	1.4	1.7	1.6	1.4
Glucose uptake activity	**5.77±0.04**	**14.71±0.04**	**11.65±0.13**	**10.74±0.14**	**8.61±1.80**	**13.74±2.64**	**14.90±0.07**	**13.53±0.25**	**6.52±0.14**

Table 5; results of Saccharomyces glucose absorption assay in ethyl acetate sub fraction

Glucose Concentration (mmol/l)	**30**		
Ethyl acetate fraction conc. (mg/ml)	1.7	1.6	1.4
Glucose uptake activity	**4.61±0.21**	**0.56±0.15**	**3.16±0.26**

Table 6; Results of Saccharomyces glucose absorption assay in n-Hexane sub fraction

Glucose Concentration (mmol/l)	**30**		
n-Hexane fraction conc. (mg/ml)	1.7	1.6	1.4
Glucose uptake activity	**14.60±0.09**	**15.74±0.17**	**3.79±0.05**

Table 7; Results of Saccharomyces glucose absorption assay in DCM sub fraction

Glucose Concentration (mmol/l)	**30**		
DCM fraction conc. (mg/ml)	1.7	1.6	1.4
Glucose uptake activity	**7.15±0.10**	**1.87±0.04**	**11.80±0.08**

Table 8; Results of Saccharomyces glucose absorption assay in n- Butanol sub fraction

Glucose Concentration (mmol/l)	**30**		
n-Butanol fraction conc. (mg/ml)	1.7	1.6	1.4
Glucose uptake activity	**10.28±0.14**	**18.63±0.01**	**8.51±0.12**

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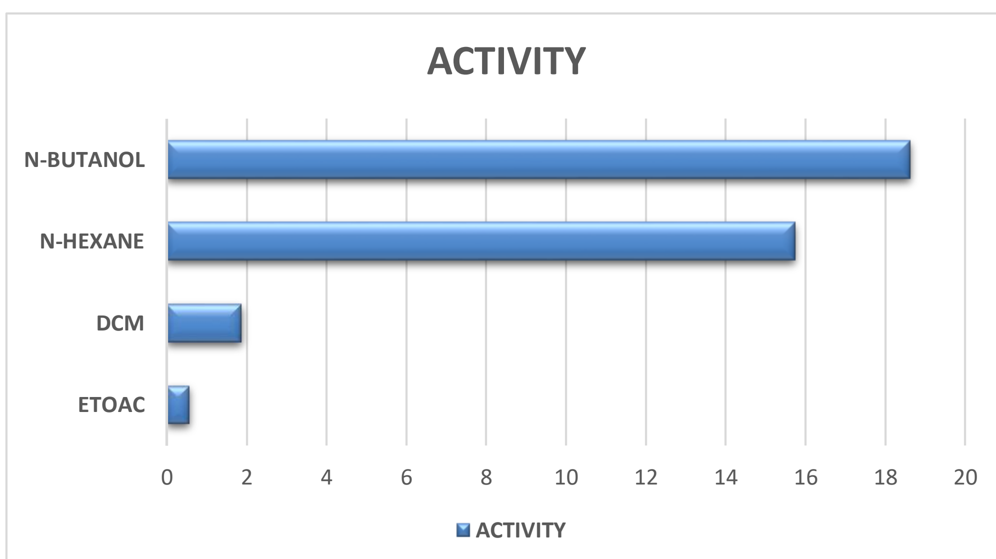

Figure 2; Summary of saccharomyces glucose uptake activity of sub fractions

The above bar chart shows a summary of recent study's results of Saccharomyces glucose absorption assay of sub fractions from activity guided solvent-solvent partitioning. According to the results of this current study, n-butanol sub fraction has the highest glucose uptake activity (18.63) while the Ethyl acetate sub fraction has the lowest glucose uptake activity (0.56).

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