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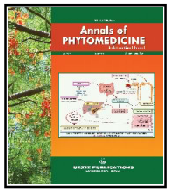

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In vitro antioxidant and antidiabetic activity in leaf extracts of *Syzygium zeylanicum* (L.) DC.

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Abstract

Herbal remedies serve as complementary and alternative treatments to allopathic medications, which are thought to have unfavourable side effects. Through lipid peroxidation, oxidation is thought to be a key factor in the pathophysiology of diabetes mellitus. Damage to DNA and proteins can result in vascular problems such as nephropathy, neuropathy, retinopathy, and coronary heart disease. Therefore, the current study was aimed to assess the ethyl acetate, ethanol and aqueous extracts of *Syzygium zeylanicum* (L.) DC. For *in vitro* antioxidant and antidiabetic potential by DPPH, ABTS, ferric oxide reducing assay (FRAP), total antioxidant assay, α-amylase assay and α-glucosidase assay. For antioxidant activity, ethanolic extract showed significant IC_{50} values (56.12 µg/ml for DPPH assay and 60.43 µg/ml for ABTS) comparing ethyl acetate and aqueous extracts. The ethanolic fraction had a greater α-glucosidase inhibitory and α-amylase inhibition effect (IC_{50} values 69.92 µg/ml and 65.48 µg/ml, respectively) than other extracts when it came to antidiabetic activity. Based on the findings, *S. zeylanicum* leaves have antioxidant and antidiabetic properties. Additionally, ethanolic extract of *S. zeylanicum* may have more antioxidant and potential ingredients in diabetes prevention than other extracts.

1. Introduction

Diabetes mellitus, a long-term metabolic disease caused by abnormalities in insulin secretion, actions, or both, is typified by hyperglycemia, hypertriglyceridemia, and hypercholesterolemia (Alam and Khan, 2020). The incidence of diabetes mellitus is continuously increasing worldwide, with India housing close to 20% of all diabetic individuals. This makes India the global centre for diabetes. By 2030, 79.4 million Indians are expected to suffer this illness (Kaveeshwar and Cornwall, 2014; Swetha and Malarkodi, 2023). Diabetic neuropathy, coronary artery and peripheral vascular disease, stroke, ulcer, amputation risk, renal failure, sexual dysfunction, and blindness are among the consequences associated with diabetes. According to Dastjerdi *et al*. (2015), these consequences are the cause of the increased disability, decreased life expectancy, and high health expenses linked to diabetes in practically every society. Numerous studies have indicated that oxidative stress is a major cause of hyperglycemia. Herbal medicines may have an edge over other treatments since they contain a variety of secondary metabolites that have bioactive chemicals that can operate through different pathways (Parra *et al*., 2017). Medicinal plants have been utilized for a wide range of ailments since ancient times. According to Koneri *et al*. (2014), phytochemicals found in the plant, such as flavonoids, phenols, and saponin, may have antioxidant and antidiabetic properties.

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Syzygium zeylanicum (L.) DC. (*S. zeylanicum*) is a member of the Myrtaceae family. It may grow up to two metres tall and has spreading branches and a soft, woody stem. There are white berries and dark green, lanceolate leaves with yellow blossoms (Anoop and Bindu, 2014). Recent results suggest that *S. zeylanicum* effect on oxidation and capacity to block enzymes that hydrolyze starch make it a promising candidate for use in the treatment of diabetes. According to Mai *et al*. (2007), *S. zeylanicum* leaf extract is a unique and promising material that exhibits remarkable performance in suppressing α-glucosidase and has antioxidant capabilities. Bioactive compounds like phenols and flavonoids generally dissolve in polar solvents. Hence, according to the increased polarity, petroleum ether, chloroform, ethyl acetate, ethanol and water were used to carry out cold maceration. Among the five extracts, ethyl acetate, ethanol and aqueous extracts were chosen with respect to yield obtained. The objective of this work is to evaluate and compare the antioxidant and antidiabetic properties of several *S. zeylanicum* crude extracts *in vitro*. Various *in vitro* antioxidant methods used were DPPH, ABTS, FRAP and total antioxidant assay. α-amylase and α-glucosidase are the most important carbohydrate-digesting enzymes which is responsible for increasing blood glucose levels. *In vitro* α-amylase and α-glucosidase inhibitory assay was carried out.

2. Materials and Methods

2.1 Collection of plant

The fresh plant samples were collected in the month of October from the Mahatma Gandhi University Campus, Kottayam district, Kerala, India. They were botanically authenticated by Dr. Sreekumar V.B, Senior Scientist, Forest Botany Department, Kerala Forest Research Institute, Peechi, Thrissur, Kerala. The specimen is

accessed to the KFRI herbarium (KFRI herbarium accession number 18370). After the plant samples were properly cleaned under running water to get rid of any remaining dust, they were blotted dry in the shade for about two weeks. They were then crushed into a powder and kept in an airtight container for later research.

2.2 Preparation of extracts

For the sequential solvent extraction (2500 ml) with increasing order of polarity, such as petroleum ether, chloroform, ethyl acetate, ethanol, and water, the powdered leaf samples (450 g) are utilized. Petroleum ether will be used to first defatten the powdered leaves. After that, it will remain for 48 h in an orbital shaker. The extract was concentrated using a rotary flask evaporator after the supernatant was collected and filtered through Whatman No. 1 filter paper. Every time, the residue is completely dried to eliminate the solvent used, before extracting with the subsequent solvent. For sequential solvent extraction, petroleum ether, chloroform, ethyl acetate, ethanol, and water are the solvents utilized. After being properly weighed, the dried extract will be kept in tiny vials for subsequent studies (Aggarwal *et al*., 2022; Mamarasulov *et al*., 2020).

2.3 *In vitro* antioxidant assay

2.3.1 DPPH radical scavenging assay

100 ml of ethanol was mixed with 4 mg of DPPH (2, 2-diphenyl-1-picrylhydrazyl) to create a 0.1 mM DPPH solution. After preparing sample extracts in DMSO at various concentrations up to 40 µl, 2.96 ml of DPPH (0.1 mM) solution was added. The reaction mixture was incubated at room temperature in the dark for 20 min. After 20 min, the absorbance was measured at 517 nm with a UV-Vis spectrophotometer. 3 ml of DPPH served as the control:

$$\%\text{RSA} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

RSA stands for radical scavenging activity. The absorbance of DPPH radical + ethanol is known as Abs control, whereas the absorbance of DPPH radical + sample extract is known as Abs sample (Punit *et al*., 2019).

2.3.2 ABTS decolorization assay

245 µl of potassium persulfate (100 mM) and 9.5 ml of ABTS (2.2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) were reacted to make the working solution of the ABTS•+ radical. The volume was then increased to 10 ml using distilled water. After being left at room temperature in the dark for 18 h, the solution was diluted with potassium phosphate buffer (0.1 M, pH 7.4) until it had an absorbance of 0.70 (± 0.02) at 734 nm. Samples in varying dilutions (µg/ml) were produced in methanol. A test tube containing 10 µl of the sample was filled with 2.99 ml of the ABTS radical working solution and thoroughly mixed. The resultant clear mixture's absorbance was measured at 734 nm. The following formula was used to determine the percentage radical scavenging assay of antioxidant activity:

$$\%\ \text{RSA} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

where Abs control is the absorbance of ABTS radical in methanol; Abs sample is the absorbance of ABTS radical solution mixed with sample extract/standard. The control was prepared by adding 10 µl of methanol in place of the sample (Rahman *et al*., 2016).

2.3.3 Ferric reducing antioxidant potential (FRAP)

The FRAP reagent was made by combining 2.5 ml of 2,4,6- tris (2-pyridyl)-s-triazine (TPTZ) solution (10 mM), 2.5 ml of ferric chloride solution (20 mM), and 25 ml of acetate buffer (30 mM; pH 3.6). Before using, the mixture was incubated for 15 min at 37°C. In this test, ascorbic acid, often known as vitamin C, was used as a standard. The calibration curve was prepared from varying concentrations of ascorbic acid. Different concentrations of the sample extract (made in ethanol) or standard were added to 2.85 ml of FRAP reagent in a test tube. After 30 min of dark incubation, the absorbance of the reaction mixture was measured at 593 nm. Rather than the test sample, the blank contained an equivalent volume of methanol. The results were reported as µg of ascorbic acid equivalents (AAE) per gram (Benzie and Strain, 1996).

2.3.4 Total antioxidant capacity assay

The phosphomolybdenum technique was used to assess the sample extract's overall antioxidant capacity in accordance with protocol. 3 ml of the reagent solution was mixed with 0.3 ml of the extract (0.6 M sulfuric acid, 4 M ammonium molybdate, and 28 M sodium phosphate). For 90 min, the reaction solutions were incubated at 95°C. 0.3 ml solution of blank was also used. The quantity of grams of ascorbic acid equivalent is used to indicate the total antioxidant activity. The calibration curve was prepared by using standard ascorbic acid (Arnao *et al*., 2001).

2.4 *In vitro* antidiabetic activity

2.4.1 *In vitro* α-amylase inhibition assay

100 µl of 0.02 M sodium phosphate buffer (pH 6.9) and 100 µl of α-amylase solution (4.5 units/ml/min) were added to a different quantity of sample extract, and the mixture was pre-incubated for 10 min at 25°C. Subsequently, 1.0 ml of dinitrosalicylic acid reagent was added to stop the reaction, and 100 µl of 1% starch solution was added. This mixture was then incubated at 25°C for 30 min. After 5 min of incubation in a boiling water bath, the test tubes were allowed to cool to room temperature. The reaction mixture was diluted with distilled water and absorbance at 540 nm was determined. The proportion of α-amylase enzyme inhibition was estimated by comparing the readings to the control, which had buffer instead of extract:

$$\%\ \text{Inhibition} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

where Abs control is the absorbance of control; Abs sample is the absorbance of test sample extract/standard (Prieto *et al*., 1999; Lordan *et al*., 2013).

2.4.2 *In vitro* α-glucosidase inhibition assay

100 µl 0.1 M phosphate buffer (pH 6.9) and 100 µl of α-glucosidase solution (1 unit/ml/min) were added to different concentrations of

the sample extract, and they were preincubated for 5 min at 25°C. Next, 100 µl of 5 mM p-nitrophenyl-α-D-glucopyranoside was added, and it was incubated for 10 min at 25°C. Following the incubation period, absorbance measurements were taken at 405 nm and compared to a control sample that was replaced with 100 µl of buffer. The results have been estimated and expressed in the form of percentage inhibition:

$$\% \text{ Inhibition} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

where Abs control is the absorbance of control; Abs sample is the absorbance of test sample extract/standard (Gazali *et al*., 2023).

3. Results

3.1 *In vitro* antioxidant assay

3.1.1 DPPH assay

The DPPH test was performed on the ethyl acetate, ethanol, and aqueous extracts of *S. zeylanicum*, at different concentrations ranging between 20 µg/ml to 100 µg/ml, and the results were compared to the standard (ascorbic acid). As seen in Figure 1, the percentage inhibition values for the standard ascorbic acid, ethyl acetate, ethanol, and aqueous extracts at 100 µg/ml were. 89.56, 76.43, 88.23, and 43.72%, respectively. In Table 1, the ethanol extract had the best scavenging effectiveness (IC_{50} = 56.12 µg/ml) in comparison to the other ethyl acetate extract (IC_{50}= 65.63 µg/ml) and the aqueous fraction (IC_{50}=113.5 µg/ml). When compared to an aqueous extract, the ethanol and ethyl acetate extracts demonstrated a considerable increase in DPPH radical scavenging.

Table 1: IC_{50} values of DPPH and ABTS radical scavenging effects of various extracts of *S. zeylanicum* leaves and ascorbic acid (standard)

Extracts	IC_{50} (µg/ml)	
	DPPH assay	ABTS assay
Ethylacetate	65.63	68.73
Ethanol	56.12	60.43
Aqueous	113.50	124.96
Ascorbic acid	13.85	14.34

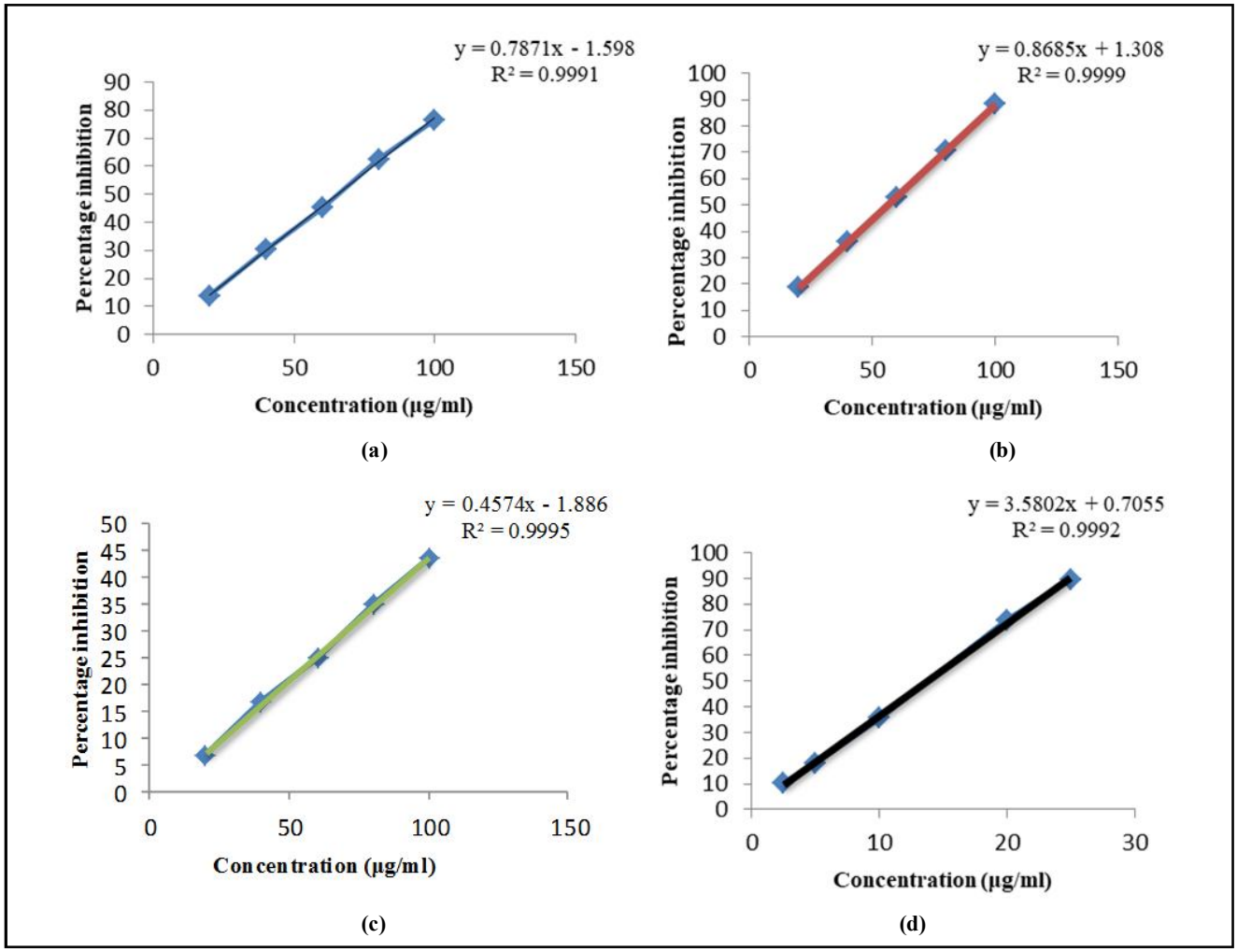

Figure 1: DPPH inhibitory assay of (a) ethylacetate, (b) ethanol, (c) aqueous extracts of *S. zeylanicum* leaves and (d) ascorbic acid.

3.1.2 ABTS^{++} Decolorization Assay

The ability of the extracts to scavenge the ABTS cation was demonstrated in Figure 2. The percentage inhibition values at 100 µg/ml for ethyl acetate, ethanol and aqueous extracts were 73.43, 83.62, and 39.95%, respectively, compared to the standard ascorbic acid, which exhibited an inhibition of 88.12%. Out of all the extracts, ethanolic extracts exhibit the highest scavenging efficacy against ABTS radicals (IC_{50} = 60.43 µg/ml). Table 1 data indicate that the ethanolic extract (IC_{50}=60.43 µg/ml) of *S. zeylanicum* has the highest scavenging activity, followed by the ethyl acetate (IC_{50}=68.73 µg/ml) and aqueous (IC_{50}=124.96 µg/ml) extracts. The antioxidant activity of ABTS in the ethanol fraction was significantly higher than that of ethylacetate and aqueous extract.

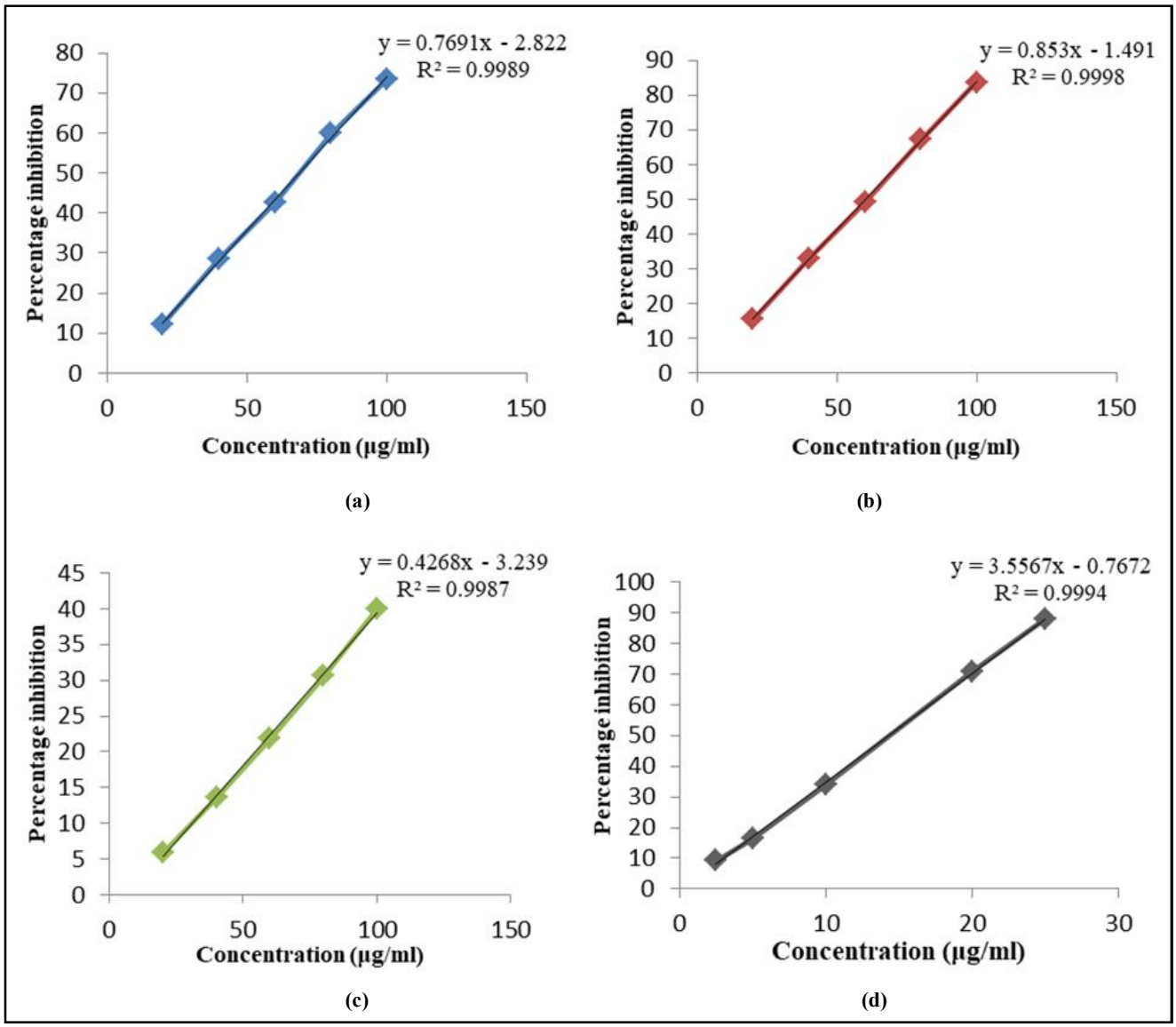

Figure 2: ABTS inhibitory assay of (a) ethylacetate, (b) ethanol, (c) aqueous extracts of *S. zeylanicum* leaves and (d) ascorbic acid.

3.1.3 FRAP and Total antioxidant capacity

As measured in mg of ascorbic acid equivalents (AAE)/g of extract, Figure 3 shows the reducing ability of the three extracts. All values increased with increasing concentrations (10-50 µg/ml). The ethanolic extract was shown to have the highest reducing ability among the three extracts. With 370.23 mg equivalents of ascorbic acid, the ethanolic extract exhibited the highest overall antioxidant activity comparing ethyl acetate, at 300.67 mg equivalents and aqueous extracts, at 180.92 mg equivalents.

3.1.4 *In vitro* antidiabetic activity

For ethyl acetate, ethanol, and aqueous extract, the percentage inhibition of α-amylase at higher concentrations of each fraction was determined to be 60.86, 78.42, and 51.46%, respectively, in Figure 4. The percentage inhibition obtained for extracts was compared with the acarbose standard (82.92%). The IC_{50} values for ethyl acetate, ethanol and aqueous extract were determined to be 84.12, 69.92, and 99.17 µg/ml, respectively, as indicated in Table. 2. The IC_{50} value of acarbose was found to be 49.67 µg/ml. Out of all,

the ethanolic extract has the most inhibitory effect on α-amylase when compared to standard. The leaf extracts exhibited α-glucosidase inhibitory activity at 20, 40, 60, 80, and 100 µg/ml doses (Figure 5). However, the ethanolic extract exhibited the highest percentage of inhibition (80.1%) among all fractions. The sequence of IC_{50} obtained is ethanol extract (65.43 µg/ml) > ethyl acetate extract (78.48 µg/ml) > aqueous extract (89.38 µg/ml) indicates that the ethanol fraction exhibited the highest inhibitory efficacy (Table 2).

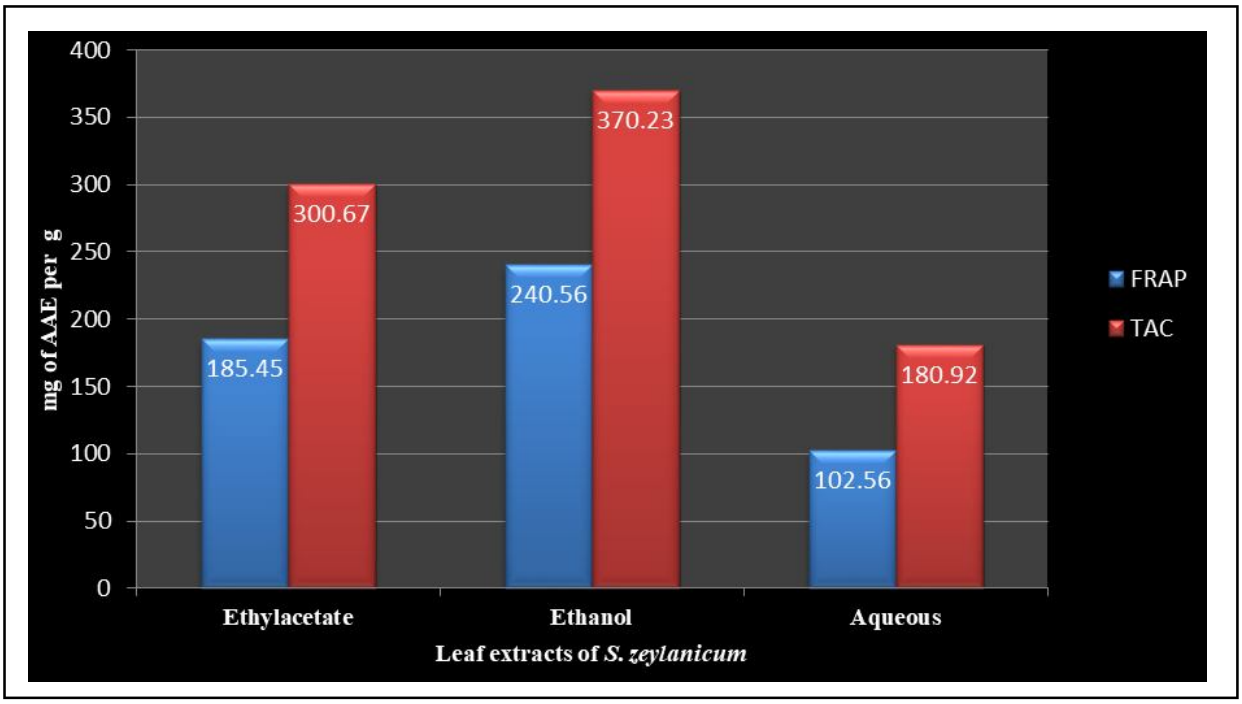

Figure 3: FRAP and total antioxidant assay (TAC) of ethyl acetate, ethanol, aqueous leaf extracts of *S. zeylanicum*.

Table 2: IC_{50} values of various extracts on *in vitro* α-amylase and α-glucosidase inhibition assay

Extracts	IC_{50} (µg/ml)	
	α-amylase inhibition assay	α-glucosidase inhibition assay
Ethylacetate	84.12	78.43
Ethanol	69.92	65.48
Aqueous	99.17	89.38
Acarbose	49.67	51.62

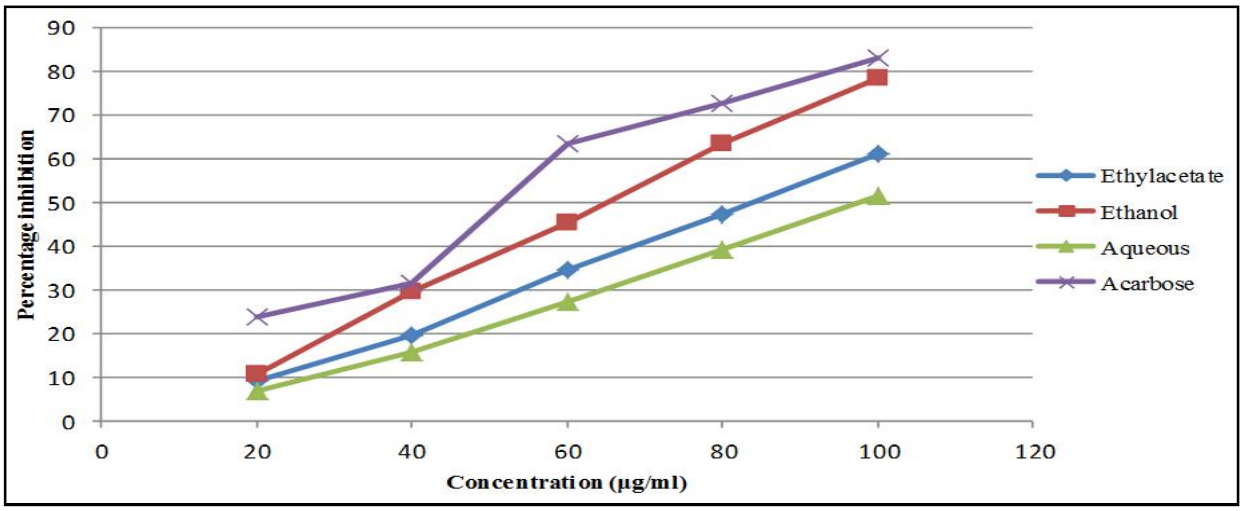

Figure 4: Percentage inhibition of α-amylase activity in ethylacetate, ethanol, aqueous extracts of *S. zeylanicum*.

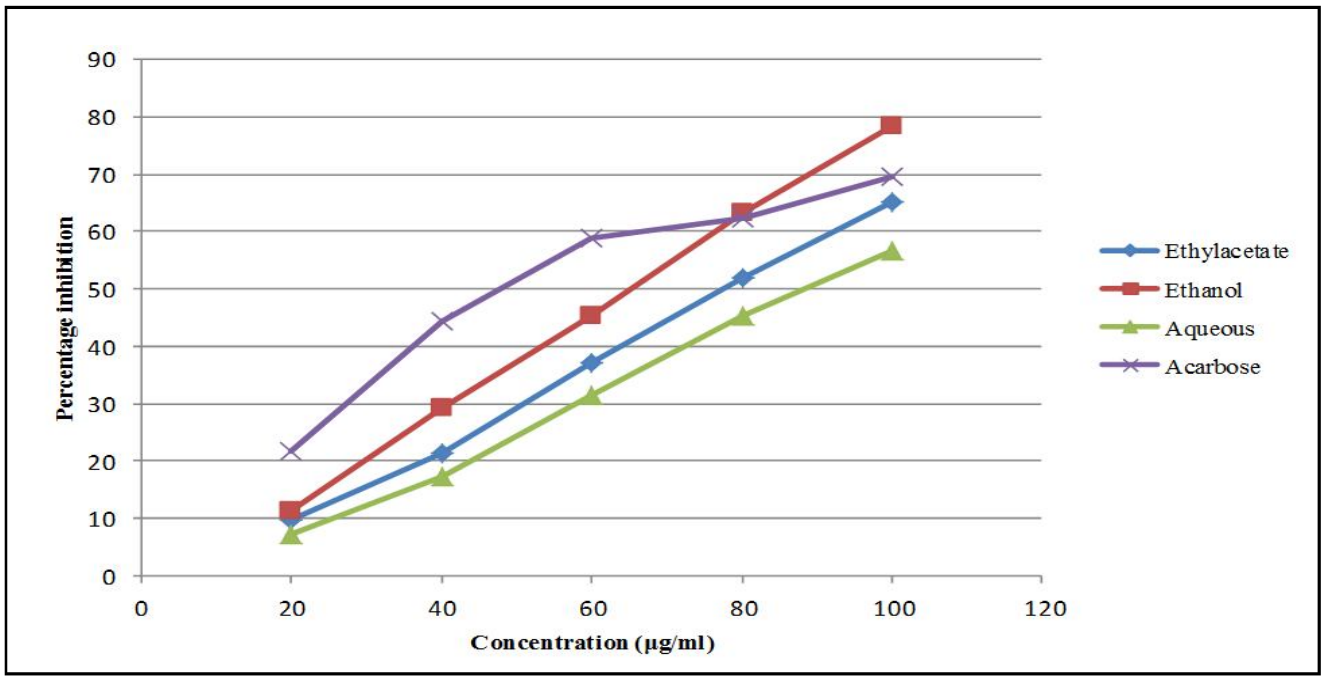

Figure 5: Percentage inhibition of α-glucosidase activity in ethylacetate, ethanol, aqueous extracts of *S. zeylanicum*.

4. Discussion

Numerous studies have demonstrated the critical role free radicals play in the emergence of a number of communicable diseases, including cancer, heart disease, ageing, cataracts, and others. Antioxidants can reduce the pace of oxidation by removing these erratic free radicals (Asimi *et al*., 2013). The expensive cost and side effects of antioxidant medicines, however, drive many individuals to turn to herbal therapies, which have fewer negative effects. Other than the numerous approaches available for evaluating antioxidant activity *in vitro* and *in vivo*, only a limited number of reliable methodologies can be employed to assess plant extract (Miller *et al.*, 1993). Additionally, the potential of the plant extracts to scavenge the stable free radical DPPH, the cation ABTS, FRAP as well as total antioxidant capacity (TAC) were assessed to investigate their antioxidant activity.

The persistent free radical DPPH, which has a nitrogen centre, is frequently employed to evaluate the scavenging ability of plant extracts and antioxidant standards. It has a unique absorbance at 517 nm when free radical scavengers are present. The DPPH solution turns yellow diphenylpicryl hydrazine by absorbing hydrogen from a matching donor, losing its distinctive dark purple hue (Conforti *et al*., 2008). Using the DPPH assay, several researche published recently documented the antioxidant characteristics of medicinal plant compounds (Tirzitis and Barlosz, 2010). The scavenging action was seen in all *S. zeylanicum* extracts and increased with sample concentration. The DPPH test was performed on the ethyl acetate, ethanol, and aqueous extracts at different concentrations ranging between 20 µg/ml to 100 µg/ml, and the results were compared to the standard (ascorbic acid). The ethanol extract had the best scavenging effectiveness (IC_{50}=56.12 µg/ml) in comparison to the other ethyl acetate extract (IC_{50}=65.63 µg/ml) and the aqueous fraction (IC_{50}=113.5 µg/ml). When compared to an aqueous extract, the ethanol and ethyl acetate extracts statistically demonstrated a considerable increase in DPPH radical scavenging. It was also noted that the IC_{50} value was quite similar to the ascorbic acid (13.85 µg/ml).

The free form of 2, 2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical cation is stable. This radical's concentration can be found by measuring the absorbance at 734 nm. When an antioxidant is added to a solution containing this radical, the radical is reduced and its absorbance is decreased. This decline is dependent not only on the test compound's antioxidant activity but also on concentration and duration. An enhanced ABTS decolorization test was used for the research (Tachakittirungrod *et al*., 2007). The scavenging action grew as the test compound's concentration increased. For ethyl acetate, ethanol, and aqueous extracts, as well as the standard ascorbic acid, the percentage inhibition values at 100 µg/ml were 73.43, 83.62, 39.95, and 88.12%, respectively. The ascorbic acid fraction has the highest scavenging effectiveness against ABTS radicals (IC_{50} = 14.34 µg/ml) out of all the combinations. When compared to ethyl acetate and aqueous extract, the antioxidant activity of ABTS showed much greater activity in the ethanol fraction.

Free radical scavenging in the sample caused the Fe3+/ferricyanide complex to reduce to the blue ferrous form in the FRAP method. This reduction can be used to measure the antioxidant capacity, sometimes referred to as the reducing power (Benzie *et al*, 1996). The reducing ability of the three extracts is measured in mg of ascorbic acid equivalents (AAE)/g of extract. Of the three extracts, ethanolic extract was determined to have the greatest decreasing ability. Since the total antioxidant activity is expressed as ascorbic acid equivalent, the phosphomolybdate method is quantitative. As the Phosphomolybdate ions get reduced in the presence of an antioxidant, the total antioxidant capacity (TAC) assay measures the green phosphate/MoV complex using spectrophotometric analysis (Kumaran, 2007). The ethanolic extract had the highest overall antioxidant activity, measuring 370.23 mg equivalents of ascorbic acid. It was followed by ethyl acetate with 300.67 mg equivalents and aqueous extracts with 180.92 mg equivalents.

α-amylase and α-glucosidase are two examples of carbohydrate metabolising enzymes that are blocked, which decreases postprandial blood glucose levels by slowing down the breakdown of carbohydrates and subsequent glucose absorption. It has been shown that inhibiting these enzymes is one of the most effective ways to manage hyperglycemia in type 2 diabetes (Kim *et al*., 2005; David *et al*., 2017). The percentage inhibition of α-amylase at greater concentrations of each fraction was found to be 60.86, 78.42, and 51.46% for ethyl acetate, ethanol, and aqueous extract, respectively. These values were compared to the standard acarbose (82.92%). The IC_{50} values for ethyl acetate, ethanol, and aqueous extract were found to be 84.12, 69.92, and 99.17 µg/ml, respectively. Acarbose, on the other hand, has α-amylase inhibitory action, with an IC_{50} value of 49.67 µg/ml. When compared to the standard, the ethanolic extract exhibits the highest inhibitory impact on α-amylase out of all of them.

While α-glucosidase inhibitory activity was seen at 20, 40, 60, 80, and 100 µg/ml concentrations for all extracts of *S. zeylanicum* leaves, the ethanolic extract showed the highest percentage of inhibition (65.46%) across all fractions. The ethanol fraction had the highest inhibitory activity, as shown by the sequence of ethanol extract (65.43 µg/ml) > ethyl acetate extract (78.48 µg/ml) > aqueous extract (89.38 µg/ml). The IC_{50} values of the three fractions varied from 20 to 100 µg/ml. Therefore, in comparison to ethylacetate and aqueous extracts, the ethanolic fraction exhibited a notable decrease in IC_{50}. In the current research, the *in vitro* studies were performed to choose the best extract which will be used in further studies such as *in vivo* antidiabetic, isolation and characterization.

5. Conclusion

The study indicates that ethanolic extract showed the highest antioxidant and *in vitro* antidiabetic activity. The antidiabetic potential of *S. zeylanicum* leaf extracts was assessed in this work utilizing a variety of *in vitro* test techniques. These methods were chosen based on the extracts' ability to inhibit enzymes that metabolise carbohydrates, such as α-amylase and α-glucosidase. A research of DPPH, ABTS scavenging activities, FRAP and total antioxidant capacity was conducted to assess the antioxidant potential. The extracts from *S. zeylanicum* leaves demonstrated a strong antidiabetic and radical scavenging action, as shown by the results. As a result, the ethanolic extract may provide a promise in the treatment of diabetes problems. Increased activity in the ethanolic extract may be due to the presence of secondary metabolites such as flavonoids and phenols.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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