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In vitro Antioxidant and Antidiabetic Activities of the Bark Extracts of *Kandelia candel* (L.) Druce

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In present study, various extracts using different solvents such as petroleum ether, ethyl acetate and ethanol were subjected to *in vitro* antioxidant and antidiabetic activities. Ethanolic extract showed the highest activities in all antioxidant studies such as DPPH scavenging, ABTS scavenging and nitric oxide methods but it is comparatively weaker than standard drug ascorbic acid. *In vitro* antidiabetic activities were performed by using α-amylase and α-glucosidase inhibition in both method ethanolic extract showed highest activity, but the standard drug acarbose showed more potent than all other extracts. The highest antioxidant and antidiabetic activity of ethanolic extract may be due to the highest content phenols and flavanoids. Therefore, the ethanolic extracts contains potent antioxidant and antidiabetic compounds their isolation will help the invention of a new potent drug for the treatment diabetes and oxidative stress.

Keywords: Antidiabetic, Antioxidant, Mangroves, *Kandelia candel*.

INTRODUCTION

Medicinal and aromatic plants are important sources of natural drugs which are mainly grown in the forests and many are cultivated. They are evolved alongside human civilizations since the beginning of life on universe. From the time immoral, medicinal plants were used for the treatment of human ailments and diseases because they contain the therapeutically important constituents. Increasing the complexity of various diseases the need for new drugs is of utmost importance, plants and other natural sources are a vast treasure of bio molecules which have potential to cure the most ailments and diseases of human [1]. Mangroves usually grow under stressful and extreme environmental conditions such as high temperature and radiation, salinity and anaerobic conditions that may not be a favourable condition for the normal plants to thrive. Hence, mangroves have developed special adaptation to remain survive in these conditions [2]. Mangroves have important role in protecting the nature it acts as sieve between sea and river and thereby supplying fresh water to the coastal regions, it acts as a green wall and protecting the sea cost by resisting the winds and from other natural calamities. It also provides food and shelter to marine organism and also for other terrestrial animals. Traditionally, the mangroves have been used as firewood and charcoal and for making boats, fishing gears, furniture, *etc*. Many mangroves are rich source of tannins and have been used for the extraction of tannins and leather production [3].

To survive in adverse environmental conditions, there is a possible alterations in their physiological processes which results in the synthesis of new chemical compounds; these compounds help them to protect from various biotic and a biotic conditions. Many of these compound possessing significant biological and medicinal properties and that can be cater the needs for the novel drugs for the treatment complex diseases in human [4]. Mangroves are the rich sources of secondary metabolites such as alkaloids, phenols, flavonoids, tannins, saponins, glycosides, terpenoids, *etc*. and medicinal value of mangroves are still not fully utilized in modern medicine [5].

Kandelia candel (L.) Druce is the only true mangrove species of the genus Kandelia belongs to the family Rhizopho-

raceae, distributed in South-East Asian mangrove communities. It grows on the slops of the river and ridge forests [6,7]. The bark is useful for tanning leather and for dyeing. Bark and leaves are used in the treatment of diabetes, bark mixed with dried ginger or long pepper and rose water is found to be useful for the cure of diabetes. Bark also showing antioxidant activity [8,9].

Oxidative stress is one of the reasons for the development and progression of cancer, diabetes mellitus, cardiovascular diseases, neurodegenerative diseases and inflammatory diseases among other syndromes [10]. Free oxygen and nitrogen species are unstable molecules, which are present in the nature and also generated by the body during the normal metabolic activities [11]. Human body having a antioxidant defence system, consisting of enzymatic and non-enzymatic pathways, which in the normal biological condition maintain an equilibrium between pro oxidants and antioxidants and thereby preventing damage to human body, enzymatic antioxidants constitutes the glutathione, catalase and superoxide dismutase and non-enzymatic antioxidants used by the body comprises the bilirubin, uric acid, lactoferrin, *etc*. In diseased conditions, the endogenous antioxidant systems are not effective in neutralizing the various oxidative species, leading to accumulation of excessive free radicals, which further leads oxidative stress-induced damages to the cellular system and leading to the progression various diseases including diabetes [12]. Various synthetic compounds are used for the management of oxidative stress like butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT) and propyl gallate (PG). These synthetic agents usually have some undesirable effects [13]. Many of them are hepatotoxic and some them have reported to be carcinogenic. Medicinal plants contain various secondary metabolites, which can effectively provide a safer and affordable therapies for the management of oxidative stress related disorders of human [14]. Major plant constituents that show antioxidant activity of plants include polyphenols and vitamins mainly A, C and E. Phenolic compound include phenols, flavonoids, coumarins, tannins and anthocyanidins [15].

Diabetes is an endocrine disease adversely affecting the metabolism of carbohydrate, lipid and protein. The abnormal metabolism is characterized by hyperglycaemia occurs while either the pancreatic cells do not secrete adequate insulin or while body cells are unable to successfully use the insulin formed. Type-II diabetes mellitus is the metabolic disorder commonly found in most of the people characterized by abnormally high blood sugar level due to abnormality in the secretion of insulin or due to the defects in the action or both [16]. Hydrolysis of the carbohydrates produces different sugars mainly glucose, which contributes the glucose in the blood. α-Glucosidase and pancreatic amylase mainly involved in the digestion of carbohydrate and glycoprotein, inhibitors of these enzymes may be found effective for the treatment of diabetes, Gaucher's disease, cancers and Alzheimer's disease [17,18]. Acarbose, miglitol and voglibose are the some of the commonly used inhibitors frequently used in combination with diet to regulate the blood glucose level of patients [19,20]. To reduce the side effects of synthetic drugs and also to introduce new candidates of drugs, it essential to investigate on plants which are the major source of complex phytochemicals. Recently, many efforts are made to develop a new α-glucodse inhibitor from natural sources [21].

In this work, preliminary phytochemical analysis and the quantitative estimation of phenolics and flavonoids on various extracts prepared from the coarsely powdered bark of the mangrove *Kandelia candel* were performed and *in vitro* antioxidant and antidiabetic activities of the different extracts is compared with standard drugs. Antioxidant activities of ethyl acetate, petroleum ether and ethanol extracts of bark of plants were conducted by the DPPH radical scavenging, ABTS radical scavenging and nitric oxide scavenging assay methods. The *in vitro* antidiabetic studies of the different extracts were performed by the estimating the inhibitory activity of different extracts on enzymes α-amylase and α-glucosidase.

EXPERIMENTAL

The plant material was procured from Valapattanam, Kannur district in Kerala state, India. Healthy plant with normal organs was selected for the study and identified by Botanical Survey of India, Coimbatore, India. The few dried pieces of the barks were preserved for the microscopical and powder analysis in shade and pulverized for further studies

Preparation of extract: The shade dried barks were finely grounded to coarse powder. The coarsely powdered bark then extracted by successive solvent extraction methods using solvents such as petroleum ether, ethyl acetate and ethanol with increasing polarity of the solvent by using Soxhlet apparatus. After completion of extraction each extracts were filtered by Whatman filter paper and the extracts were concentrated by using rotary vacuum evaporator at low pressure and temperature and measured the extractive values of each extract [22,23]. The residue obtained was stored in refrigerator for further study.

Preliminary phytochemical screening: The bark extracts of *Kandelia candel* were subjected to different chemical tests for the detection of phytoconstituents [24] such as alkaloids, carbohydrates, glycosides, phenolics, tannins flavonoids, steroids, triterpenoids, *etc.*

Tannins analysis: A small quantity of the extract was mixed with 20 mL of distilled water and boiled in a test tube. There wass an appearance of bluish black colour on the addition of few drops of 0.1% ferric chloride solution.

Saponins analysis: The prepared extract (1 mL) was mixed with 20 mL of distilled water and shake thoroughly. Formation of a stable foam indicate the presence of saponins.

Flavonoids analysis: Few mL of extract was added to conc. HCl and a small quantity of magnesium ribbon. An appearance of pink or magenta-red colour indicates the presence of flavonoids.

Phenolic analysis: Ferric chloride solution (2 mL) was added to a small quantity of the extract. The formation of a green or blue colour shows the presence of tannins.

Alkaloids analysis: Mayer's reagent (1 mL) added to 2 mL of extract, which was previously treated with 0.2 mL of 1% HCl. The formation of precipitate shows the presence of alkaloids.

Steroid analysis: Sulphuric acid (2 mL) was added to the sides of the test tubes containing a small portion of the extract. A formation of bluish-green or violet colour indicates the presence of steroids.

Terpenoid analysis: A small quantity of extract was added to 2 mL of chloroform and thoroughly shaken the mixture followed by addition of 3 mL of sulphuric acid. The formation of reddish brown or pinkish brown colour indicates the presence of terpenoids.

Glycoside analysis: A little quantity of extract was added to 2 mL glacial acetic acid and few drops of ferric chloride solution. The mixture was then added into another test tube containing 2 mL of conc. H_2SO_4. A formation of brown ring shows the presence of glycosides.

Carbohydrate analysis: Taken about 2 mL of extract in a test tube, then added 2 mL of Molisch's reagent, thoroughly mixed the solution followed by the addition of conc. H_2SO_4 along the sides of the test tube. A violet ring formation at the junction of two liquids indicates the presence of carbohydrate.

Determination of total phenolics: Total phenol contents in the plant extracts were determined by the modified Folin-Ciocalteu method [25]. A known concentration of extracts was mixed with 5 mL Folin-Ciocalteu reagent (previously diluted with water 1:10 v/v) and 4 mL (75 g/L) of sodium carbonate. The contents of the tube were mixed thoroughly for 15 s and kept for 0.5 h at 40 ºC for colour development. Absorbance was measured using spectrophotometer at 765 nm. Total phenolic content was expressed as mg/g gallic acid equivalent using the equation obtained from a calibration curve of gallic acid.

Determination of total flavonoid contents: Total flavonoid content of the extracts was evaluated through a method as described by Park *et al.* [26]. About 0.3 mL of extracts, 3.4 mL of 30% methanol, 0.15 mL of $NaNO_2$ (0.5 M) and 0.15 mL of $AlCl_3 \cdot 6H_2O$ (0.3 M) were mixed in a 10 mL test tube then 1 mL NaOH (1 M) was added after 5 min and mixed well. The absorbance was measured using spectrophotometer at 510 nm. The standard curve was prepared using quercetin standard solution (0-100 mg/L). The total flavonoid present in the samples were measured from the calibration curve prepared from the standard quercetin solution and is expressed as milligrams of quercetin equivalents per g of sample.

DPPH radical scavenging assay: The stock solution of 2,2′-diphenyl-1-picrylhydrazyl (DPPH) (0.1 mM) was made by adding 4 mg of DPPH in 100 mL methanol and kept at 20 ºC for further use. Further working solution was prepared from stock solution by diluting the DPPH solution with methanol to get an absorbance of about 1.2 ± 0.09 at 517 nm by using the spectrophotometer. A 3 mL portion of this solution was mixed with 100 µL of different concentrations of various extracts (100-500 µg/cm^3). The reaction mixture was then mixed thoroughly and incubated in for 0.5 h in dark at room temperature. Then measure the absorbance at 517 nm [27]. L-Ascorbic acid was used as standard drug. The same concentrations were also used for L-ascorbic acid. Control solution was prepared by the same procedure by using methanol instead of sample. The percentage scavenging activity can be measured by using the following equation:

$$\text{DPPH scavenging activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

ABTS radical scavenging assay: The working solution of ABTS scavenging assay were prepared by mixing two stock solution in equal quantities one of the stock solution contains 7 mmol/L ABTS solution and the other one consists of 2.4 mmol/L potassium persulfate solution. The prepared working solution then placed in dark for 12 h for the reactions to complete. Further the solution was mixed with 1 mL of freshly prepared ABTS solution to get an absorbance of 0.706 ± 0.001 units at 734 nm using the spectrophotometer [28]. Different concentration of various plant extracts (1 mL) and the standard drug gallic acid were mixed with 2.5 mL of ABTS solution and measured the absorbance at 734 nm after 7 min using the spectrophotometer. The ABTS scavenging capacity of the extract was compared with that of gallic acid percentage inhibition was calculated by the following equation:

$$\text{ABTS radical scavenging activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Nitric oxide scavenging activity: Nitric oxide scavenging activity was measured using Griess reagent [29]. Different extracts of the plant material and the standard drug in various concentrations were allowed to react with sodium nitroprusside (10 mmol/L) in phosphate buffer saline and the resultant mixture was then incubated at 25 ºC for 150 min. The samples were added to Griess reagent (2% H_3PO_4 1 %, sulphanilamide and 0.1 % napthylethylenediamine dihydrochloride). A chromophore was formed by the coupling of naphthylethylenediamine. The absorbance of chromophore formed was measured at 546 nm. L-Ascorbic acid was used as standard drug and the same concentrations of ascorbic acid were used to measure the absorbance of positive control. The percentage of inhibition were estimated by the following equation:

$$\text{Nitric oxide scavenging activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Inhibition of α-amylase enzyme: Test and standard drugs at the concentration of 100-500 µg/mL were added to 500 µL of 0.20 mM phosphate buffer (pH 6.9), which is previously mixed with α-amylase (0.5 mg/mL) solution and the whole solution were incubated at 25 ºC for 10 min. After the incubation, 500 µL quantity of 1% starch solution containing 0.02 M sodium phosphate buffer (pH 6.9) was added to each tube. The mixture was further incubated at 25 ºC for 10 min. Then colour reagent 3,5-dinitrosalicylic acid were added to the mixture to cease the reaction, the above solution was further incubated for 5 min in a boiling water bath and cooled to room temperature. Which was further diluted using 10 mL distilled water and the absorbance was measured at 540 nm. Standard drugs acarbose were prepared by the same concentration and measured the absorbance by the same procedure. A control sample is prepared by replacing extract with vehicle and measured the absorbance of the control [30]. The percentage inhibition of different extracts were measured by the following equation:

$$\alpha\text{-Amylase inhibition (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Inhibition of α-glucosidases enzyme: α-Glucosidase inhibition activity of the extracts were measured by incubating the 1 mL of 2% w/v maltose with 0.2 M Tris buffer of pH 8.0, then it was mixed with different concentrations of various extracts and incubated for 5 min at 37 ºC. The reaction was started by further addition of 1mL of α-glucosidase enzyme (1 U/mL) followed by incubation for 10 min at 37 ºC. Further reactions were stopped by heating the mixture for 2 min in boiling water. The amount of liberated glucose is estimated by glucose oxidase peroxidase method [31]. Percentage inhibition of three different extracts were measured by the following equation:

$$\alpha\text{-Glucosidase inhibition (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Statistical analysis: Statistical analysis was performed using GRAPHPAD PRISM® (version 8.00; GraphPad Software Inc., San Diego, CA, USA),

RESULTS AND DISCUSSION

In present study, the bark of true mangrove *Kandelia candel* was screened for their antioxidant and antidiabetic activity. Coarsely powdered dried bark of the plant was extracted by successive solvent extraction method using the solvents such as petroleum ether, ethyl acetate and ethanol according to their increasing polarity.

Preliminary phytochemical screening: The coarsely powdered bark was extracted with different solvents of varying polarity the ethanol extract had higher extractive value (13.5% w/w) followed by ethyl acetate (7% w/w) and petroleum ether (3.8% w/w), which indicated that the bark of the plant consists of more amount of polar compounds. The results of the preliminary phytochemical screening of the bark extract (Table-1) showed the presence of flavonoids, phenols, glycosides, steroids and terpenoids and the phenolics and flavanoids were estimated quantitatively (Table-2) and the results indicated the significantly higher concentration of phenolics and flavanoids in ethanol extract compare to ethyl acetate extract.

The phytochemical analysis of different extracts showed the presence of flavonoids, phenols, glycosides, steroids and terpenoids but in varying compositions. Terpenoids were found in petroleum ether and ethyl acetate fractions while the phenols, flavanoids and glycosides, steroids were found in ethyl acetate and ethanol extracts.

TABLE-1
PRELIMINARY PHYTOCHEMICAL SCREENING OF VARIOUS EXTRACTS OF THE BARK OF *Kandelia candel*

	Petroleum ether	Ethyl acetate	Ethanol
Alkaloids	Absent	Absent	Absent
Glycosides	Absent	Present	Present
Steroids	Present	Present	Present
Phenolics	Absent	Present	Present
Tannins	Absent	Absent	Present
Flavanoids	Absent	Present	Present
Triterpenes	Present	Present	Absent
Caebohydrates	Absent	Absent	Absent

TABLE-2
TOTAL PHENOLIC AND FLAVANOID CONTENT OF VARIOUS EXTRACTS OF THE BARK OF *Kandelia candel*

	Total phenols (mg of gallic acid/g)	Total flavanoids (mg of quercetin/g)
Petroleum ether	06.13 ± 0.11	03.40 ± 0.11
Ethyl acetate	46.32 ± 0.13	34.43 ± 0.06
Ethanol	65.50 ± 0.06	41.12 ± 0.03

***in vitro* antioxidant activity:** The *in vitro* antioxidant activity of the different extracts and the standard drugs were measured by three methods s such as DPPH radical scavenging (Figs. 1 and 2) ABTS radical scavenging (Figs. 3 and 4) and nitric oxide scavenging methods (Figs. 5 and 6) all the results

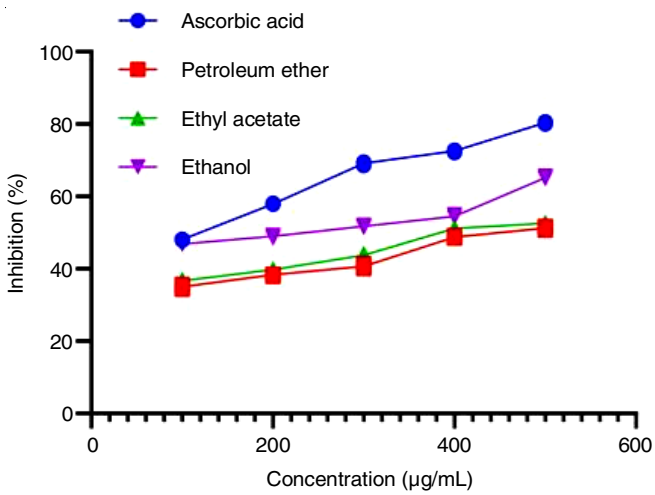

Fig. 1. DPPH Inhibition of various extracts of the bark of *Kandelia candel*

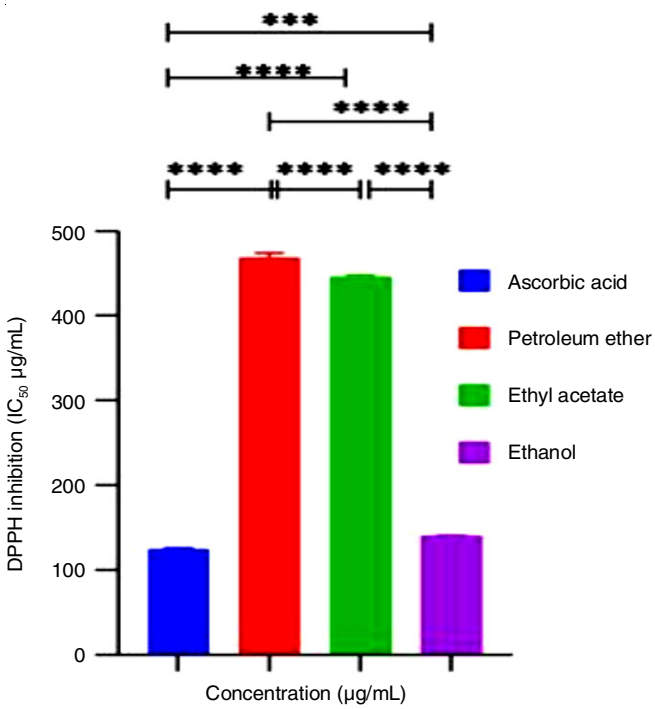

Fig. 2. DPPH inhibition, IC_{50} of the various extracts of *Kandelia candel* and standard drug ascorbic acid

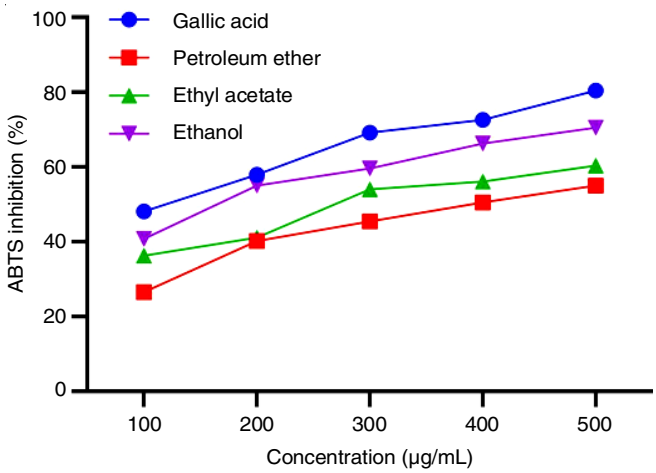

Fig. 3. ABTS Inhibition of various extracts of the bark of *Kandelia candel*

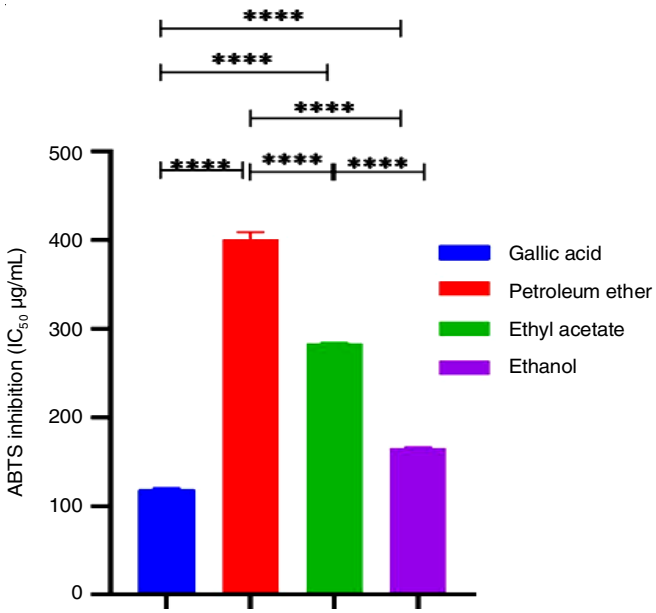

Fig. 4. ABTS inhibition, IC_{50} of the various extracts of *Kandelia candel* and standard drug gallic acid

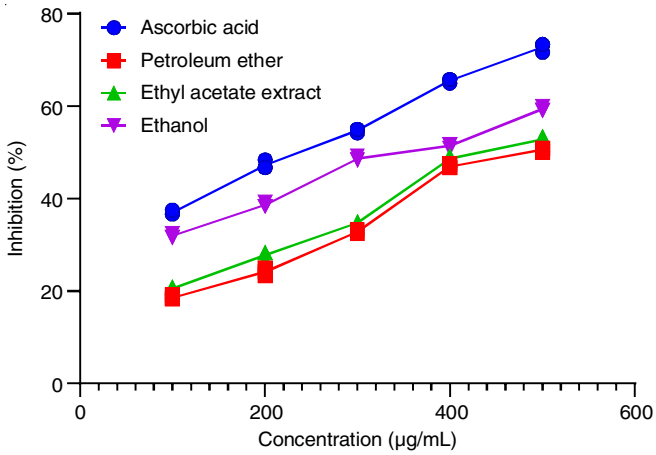

Fig. 5. Nitric oxide inhibition of various extracts of the bark of *Kandelia candel*

showed that standard drugs ascorbic acid and gallic acid showed the highest antioxidant activity followed by ethanol extract

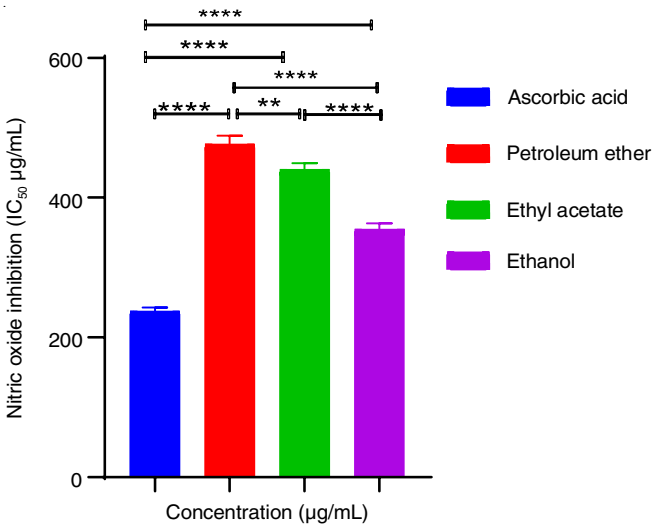

Fig. 6. Nitric oxide inhibition IC_{50} of the various extracts of *Kandelia candel* and standard drug ascorbic

and ethyl acetate extract, petroleum ether fraction showed the least antioxidant activity in all three methods. The IC_{50} values were determined from the dose response curve and is performed using Graph pad prism 8 software.

In this study, the extracts prepared from different solvents were studied for their antioxidant activities by different methods such as DPPH radical scavenging. ABTS radical scavenging and nitric oxide methods in all three methods IC_{50} values were determined the ethanol extracts showed the highest activity in comparison with ethyl acetate and petroleum ether. It could be due to high amount of flavanoids and phenolic compounds in ethanol extract. Those compounds had strong antioxidant properties and help to protect the human bodies against oxidative damage by scavenging the different reactive oxygen species.

***in vitro* antidiabetic activity:** The result of α-amylase (Figs. 7 and 8) and α-glucosidase inhibitory activity (Figs. 9 and 10) of the various extracts of the bark of *Kandelia candel*. Standard drug acarbose showed the highest activity in both α-amylase and α-glucosidase inhibition. Ethanol extract showed the comparatively higher activity than ethyl acetate extract and among the different extracts petroleum ether extract showed poor activity.

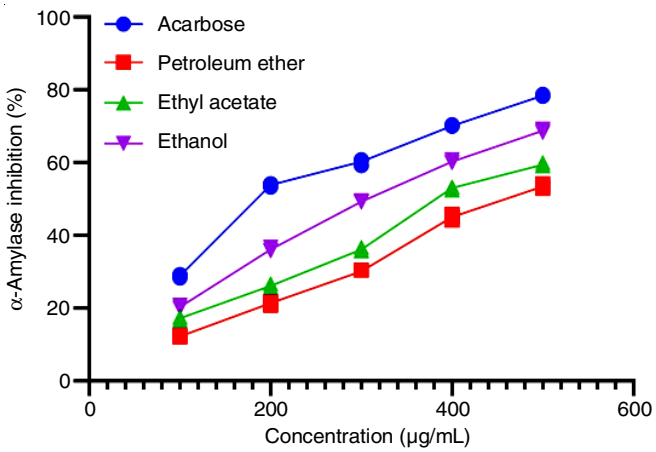

Fig. 7. α-Amylase inhibition of various extracts of the bark of *Kandelia candel*

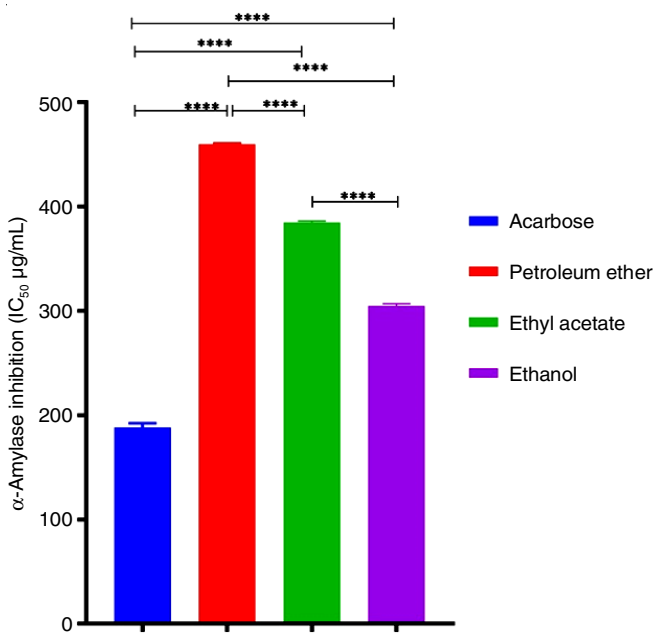

Fig. 8. α-Amylase inhibition IC_{50} of the various extracts of *Kandelia candel* and standard drug acarbose

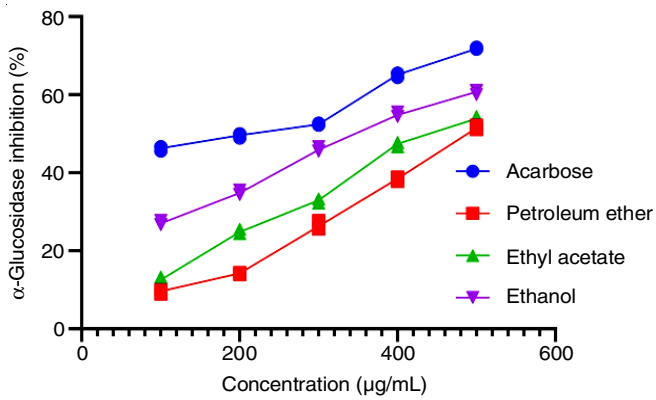

Fig. 9. α-Glucosidase inhibition of various extracts of the bark of *Kandelia candel*

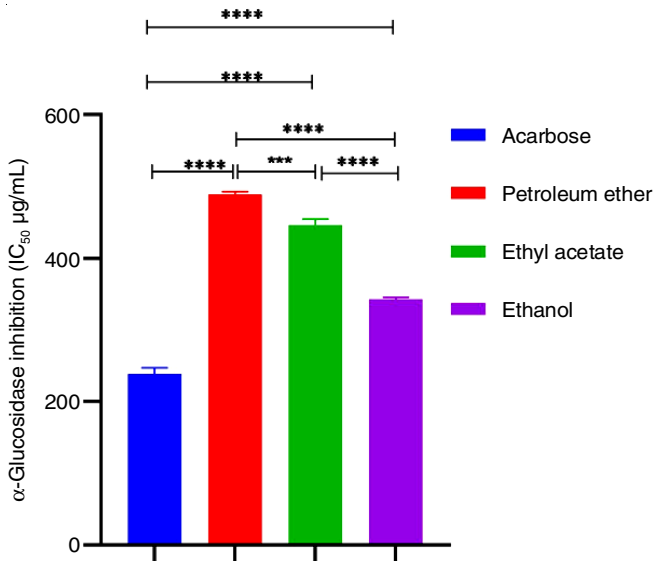

Fig. 10. α-Glucosidase inhibition IC_{50} of the various extracts of *Kandelia candel* and standard drug acarbose

In present study, the different bark extracts of the plant *K. candel* showed less activity in comparison with standard drug acarbose. Among the different extracts ethanol showed the highest inhibition against α-amylase and α-glucosidase it may be due highest concentration phenolic and flavanoid content of the extract. The phenolic compounds are known for their ability to inhibit actions of carbohydrate hydrolyzing enzymes and the favonoids was observed to possess high inhibitory activity against α-glucosidase in both *in vitro* and *in vivo* studies and may protect the pancreatic beta cell due to oxidative stress and thereby delaying the onset of type 2 diabetes.

Statistical analysis: All the studies were repeated thrice and the results were expressed as the mean ± SD. IC_{50} values of all the studies were determined from the dose response curve using The GraphPad Prism Software. Statistical significance of variance was determined by One way ANOVA statistical programs and followed Turkeys' tests for the comparison and separation of means. In all determination $p < 0.05$ was considered statistically significant.

Conclusion

The ethanolic extract of bark of mangrove *Kandelia candel* showed the highest antioxidant and antidiabetic activity in comparison with petroleum ether and ethyl acetate extracts, it may be due to high content phenolics and flavanoids in ethanol extract. So novel medicinal agents for the treatment for diabetes and oxidation stress can be developed from this mangrove, which is still under explored for their medicinal values. Further, isolation and *in vivo* evaluation can be done on these extracts which may lead to the discovery of new phytoconstituents useful for the treatment of diabetes mellitus.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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