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Essential Oil from Clove Bud (*Eugenia aromatica* Kuntze) Inhibit Key Enzymes Relevant to the Management of Type-2 Diabetes and Some Pro-oxidant Induced Lipid Peroxidation in Rats Pancreas *in vitro*

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Abstract: The inhibition of enzymes involved in the breakdown of carbohydrates is considered a therapeutic approach to the management of type-2 diabetes. This study sought to investigate the effects of essential oil from clove bud on α -amylase and α -glucosidase activities. Essential oil from clove bud was extracted by hydrodistillation, dried with anhydrous Na_2SO_4 and characterized using gas chromatography (GC-MS). The effects of the essential oil on α -amylase and α -glucosidase activities were investigated. The antioxidant properties of the oil and the inhibition of Fe^{2+} and sodium nitroprusside-induced malondialdehyde (MDA) production in rats pancreas homogenate were also carried out. The essential oil inhibited α -amylase ($\text{EC}_{50}=88.9 \mu\text{L/L}$) and α -glucosidase ($\text{EC}_{50}=71.94 \mu\text{L/L}$) activities in a dose-dependent manner. Furthermore, the essential oil inhibited Fe^{2+} and SNP-induced MDA production and exhibited antioxidant activities through their NO^* , OH^* , scavenging and Fe^{2+} -chelating abilities. The total phenolic and flavonoid contents of the essential oil were 12.95 mg/g and 6.62 mg/g respectively. GC-MS analysis revealed the presence of α -pinene, β -pinene, neral, geranial, gamma terpinene, cis-ocimene, allo ocimene, 1,8-cineole, linalool, borneol, myrcene and pinene-2-ol in significant amounts. Furthermore, the essential oils exhibited antioxidant activities as typified by hydroxyl (OH) and nitric oxide (NO) radicals scavenging and Fe^{2+} -chelating abilities. The inhibition of α -amylase and α -glucosidase activities, inhibition of pro-oxidant induced lipid peroxidation in rat pancreas and antioxidant activities could be possible mechanisms for the use of the essential oil in the management and prevention of oxidative stress induced type-2 diabetes

Key words: essential oil, clove bud, diabetes, α -amylase, α -glucosidase

1 Introduction

Recent reports have revealed that hyperglycemia and oxidative stress both play a crucial role in the early onset and progression of diabetes via glycosylation of macromolecules and oxidative damage to pancreatic β -cells^{1,2}. The pancreas is thought to be susceptible to oxidative stress due to low antioxidant capacity³. However, an approach to prevent/manage type-2 diabetes is to decrease postprandial hyperglycemia and augment the body's antioxidant status through dietary means.

Diabetes mellitus is a chronic metabolic disease associat-

ed with hyperglycemia, resulting from insufficient or inefficient insulin secretion⁴. Recent trends in the management of diabetes involve regulation/control of postprandial blood glucose. This is because in diabetic conditions there are elevated levels of glucose in the blood due to the inability of the pancreas to secrete insulin. Carbohydrate hydrolyzing enzymes such as α -amylase and α -glucosidase helps to breakdown complex carbohydrates to facilitate the release of glucose into the blood. Inhibition of these enzymes thereby retards the absorption of glucose. Natural sources of α -amylase and α -glucosidase inhibitors are of great im-

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portance in folk medicine for the treatment and management of type-2 diabetes^{4,5}. Essential oils with strong antioxidant properties have been reported to be good inhibitors of these enzymes thereby acting on them to control hyperglycemia and prevent other diabetic complications which can be triggered by oxidative stress^{6,7}.

Clove is an evergreen tree which belongs to the family Myrtaceae. It produces a flower bud which has good fragrance and taste and commonly used as spice for cooking in different parts of the world. Clove bud has numerous medicinal properties. It has been reported that it has antioxidant, antimicrobials, anti-inflammatory, antiseptic, analgesic and anti-convulsant properties^{8,9}. Some bioactive compounds such as the terpenoids, monoterpenes, sesquiterpenes, and other compounds have been reported to be present in the clove bud essential oil^{10,11}. Moreover there is dearth of information on the inhibitory effects of essential oil from clove bud on α -amylase and α -glucosidase activities. This study investigated the anti-hyperglycemic and antioxidant properties of clove bud essential oil

2 Materials and Methods

2.1 Materials

2.1.1 Sample collection

Clove buds were collected from the Akure main market (South West of Nigeria) and ground to fine powder and using Warring Commercial heavy Duty Blender (Model 37BL18; 24 \varnothing CB6). Authentication of the samples was carried out at the Department of Crop, Soil and Pest management (CSP), Federal University of Technology, Akure, Nigeria.

2.1.2 Essential oil isolation

100 g of the ground clove bud powder was subjected to hydrodistillation for 3 h in an all glass Clevenger – type apparatus according to the method recommended by the European Pharmacopoeia¹². The extracted oil sample was passed over anhydrous sodium sulphate and stored in sealed vials at 4°C for further analysis¹³.

2.1.3 Chemicals and reagents

Chemicals and reagents used such as thiobarbituric acid TBAR, 1,10-phenanthroline, deoxyribose, gallic acid, Folin-Ciocalteus reagent were procured from Sigma-Aldrich, Inc., St Louis, MO, trichloroacetic acid TCA was sourced from Sigma-Aldrich, Chemie GmbH Steinheim, Germany, hydrogen peroxide, methanol, acetic acid, thiourea, Copper sulphate, sulfuric acid, sodium carbonate, aluminum chloride, potassium acetate, sodium dodecyl sulphate, Iron(II) sulfate, potassium ferricyanide and ferric chloride were sourced from BDH Chemicals Ltd., Poole, England, Porcine pancreatic α -amylase and rat intestinal α -glucosidase were purchased from Sigma Chemical Co. (St. Louis, MO). Except stated otherwise, all other chemicals and reagents

were of analytical grades and the water was glass distilled.

2.2 Methods

2.2.1 α -Amylase inhibition assay

The essential oil (500 μ L) and 500 μ L of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing Hog pancreatic α -amylase (EC 3.2.1.1) (0.5 mg/mL) were incubated at 25°C for 10 minutes. Then, 500 μ L of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added to each tube. The reaction mixture was incubated at 25°C for 10 minutes and stopped with 1.0 ml of dinitrosalicylic acid colour reagent. Thereafter, the mixture was incubated in a boiling water bath for 5 min, and cooled to room temperature. The reaction mixture was then diluted by adding 10 mL of distilled water, and absorbance measured at 540 nm¹⁴.

2.2.2 α -Glucosidase inhibition assay

The essential oil (50 μ L) and 100 μ L of α -glucosidase solution (1.0 U/mL) in 0.1 M phosphate buffer (pH 6.9) was incubated at 25°C for 10 min. Then, 50 μ L of 5 mM p-nitrophenyl- α -D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added. The mixtures were incubated at 25°C for 5 min, before reading the absorbance at 405 nm in the spectrophotometer. The α -glucosidase inhibitory activity was expressed as percentage inhibition¹⁵.

2.2.3 Determination of total phenol content

The total phenol content was determined according to the method of Singleton *et al.*¹⁶. Briefly, appropriate dilutions of the extract was oxidized with 2.5 mL 10% Folin-Ciocalteu's reagent (v/v) and neutralized by 2.0 mL of 7.5% sodium carbonate. The reaction mixture was incubated for 40 min at 45°C and the absorbance was measured at 765 nm in the spectrophotometer. The total phenol content was subsequently calculated as gallic acid equivalent.

2.2.4 Determination of total flavonoid content

The total flavonoid content of the oil extracts was determined using a slightly modified method reported by Meda *et al.*¹⁷. Briefly, 0.5 mL of appropriately diluted sample extract were mixed with 0.5 mL methanol, 50 μ L 10% AlCl₃, 50 μ L 1 M potassium acetate and 1.4 mL water and allowed to incubate at room temperature for 30 min. The absorbance of the reaction mixture was subsequently measured at 415 nm and the total flavonoid content calculated as quercetin equivalent.

2.2.5 Gas chromatography analysis

The analytical GC was carried out by Hewlett-Packard 5890 gas chromatograph Hewlett-Packard Corp., Palo Alto, CA) equipped with Flame Ionization Detectors (FID) with DB-5 column (30 m length, 0.25 mm column id., 0.25 μ m film thickness). The following conditions were applied: Injection temperature: 290°C. Injection volume: 1.0 μ L. Injection mode: Split (1:50). Temperature program: 50°C for 4 min, rising at 3°C /min to 240°C, then rising at 15°C/min to

300°C, held at 300°C for 3 min. FID (290°C): H₂ flow: 50 ml/min; air flow: 400 mL/min.

2.2.6 Lipid peroxidation assay

2.2.6.1 Preparation of tissue homogenates

The rats were decapitated under mild diethyl ether anaesthesia and the pancreas was rapidly isolated and placed on ice and weighed. This tissue was subsequently homogenized in cold saline (1/10 w/v) with about 10 up and down strokes at approximately 1200 rev/min in a Teflon glass homogenizer. The homogenate was centrifuged for 10 min at 3000 xg to yield a pellet that was discarded, and a low-speed supernatant (S1) was kept for lipid peroxidation assay¹⁸⁾.

2.2.6.2 Lipid peroxidation and thiobarbituric acid reactions

The lipid peroxidation assay was carried out using the modified method of Ohkawa *et al.*¹⁹⁾. Briefly, 100 µL S1 fraction was mixed with a reaction mixture containing 30 µL of 0.1 M pH 7.4 Tris-HCl buffer, extract (0 – 100 µL) and 30 µL of 250 µM freshly prepared FeSO₄ (the procedure was also carried out using 7 mM sodium nitroprusside). The volume was made up to 300 µL by water before incubation at 37°C for 2 h. The colour reaction was developed by adding 300 µL 8.1% SDS (Sodium dodecyl sulphate) to the reaction mixture containing S1, which was subsequently followed by the addition of 600 µL of acetic acid/HCl (pH 3.4) mixture and 600 µL 0.8% TBA (Thiobarbituric acid). This mixture was incubated at 100°C for 1 h. TBARS (Thiobarbituric acid reactive species) produced were measured at 532 nm and the absorbance was compared with that of standard curve using MDA (Malondialdehyde).

2.2.7 Nitric oxide radical scavenging assay

The scavenging effect of the extract on nitric oxide (NO[•]) radical was measured according to the method of Mercocci *et al.*²⁰⁾. 100 – 400 µL of the oil extract was added in the test tubes to 1 ml of sodium nitroprusside solution (25 mM) and tubes incubated at 37°C for 2 h. An aliquot (0.5 ml) of the incubation was removed and diluted with 0.3 mL Griess reagent (1% sulphanilamide in 5% H₃PO₄ and 0.1% naphthylethylene diaminedihydrochloride). The absorbance of the chromophore formed was immediately read at 570 nm against distilled water as blank.

2.2.8 Fe²⁺ chelation assay

The Fe²⁺ chelating ability of the volatile oil was determined using a modified method of Minotti and Aust²¹⁾ with a slight modification by Puntel *et al.*²²⁾. Freshly prepared 500 µM FeSO₄ (150 µL) was added to a reaction mixture containing 168 µL 0.1 M Tris-HCl (pH 7.4), 218 µL saline and the extracts (0 – 25 µL). The reaction mixture was incubated for 5 min, before the addition of 13 µL 0.25% 1, 10-phenanthroline (w/v). The absorbance was subsequently measured at 510 nm in a spectrophotometer. The Fe(II) chelating ability was subsequently calculated.

2.2.9 Fenton reaction (Degradation of deoxyribose)

The method of Halliwell and Gutteridge²³⁾ was used to determine the ability of the extract to prevent Fe²⁺/H₂O₂ induced decomposition of deoxyribose. The extract 0-100 µL was added to a reaction mixture containing 120 µL of 20 mM deoxyribose, 400 µL of 0.1 M phosphate buffer, 40 µL of 500 mM of FeSO₄, and the volume were made up to 800 µL with distilled water. The reaction mixture was incubated at 37°C for 30 minutes and the reaction was then stopped by the addition of 0.5 mL of 28% trichloroacetic acid. This was followed by addition of 0.4 mL of 0.6% thiobarbituric acid solution. The tubes were subsequently incubated in boiling water for 20 min. The absorbance was measured at 532 nm in a spectrophotometer.

3 Results and Discussion

3.1 α-Amylase and α-glucosidase activity

Increase in blood glucose has been linked to diabetes and its complications which gives a pointer to the control of postprandial glucose. However, inhibition of carbohydrate hydrolyzing enzymes (α-amylase and α-glucosidase) would slow down the absorption of glucose, reduce plasma glucose level and consequently decrease postprandial hyperglycemia^{24, 25)}. α-amylase and α-glucosidase are key enzymes responsible for the breakdown of dietary carbohydrates to glucose. While α-amylase hydrolyses the internal glycosidic linkages to produce oligosaccharides, α-glucosidase breaks down the oligosaccharides to monosaccharides especially glucose. The glucose released is absorbed across the intestinal enterocytes and thereby released into the blood.

The interaction of the clove bud oil with α-amylase and α-glucosidase is presented in Figs. 1 and 2. At the concentrations tested (40– 160 µL/L), the results revealed that the essential oil inhibited α-amylase activity in a dose-dependent fashion between 35-78%. Similarly, dose dependent inhibition of α-glucosidase activity ranged from 58–90% at the same concentrations of the sample. The EC₅₀ values for the dose inhibition of α-amylase and α-glucosidase activities by the clove bud oil are 88.89 µL/L and 71.94 µL/L respectively. The EC₅₀ values for acarbose inhibition of α-amylase and α-glucosidase activities are 18.63 µg/mL and 21.1 µg/mL respectively. Essential oils have been discovered to play a crucial role in carbohydrate catabolism via inhibition of carbohydrate hydrolyzing enzymes and can be possibly used as nutraceuticals for the prevention and/or management of diabetes²⁶⁻²⁸⁾. The inhibitory effects of the oil on these enzymes could therefore be the possible mechanism by which it can be used in the management/treatment of diabetes. Although, the positive control (acarbose) showed to have stronger inhibition of the carbohydrate hydrolyzing enzymes compared to the studied essen-

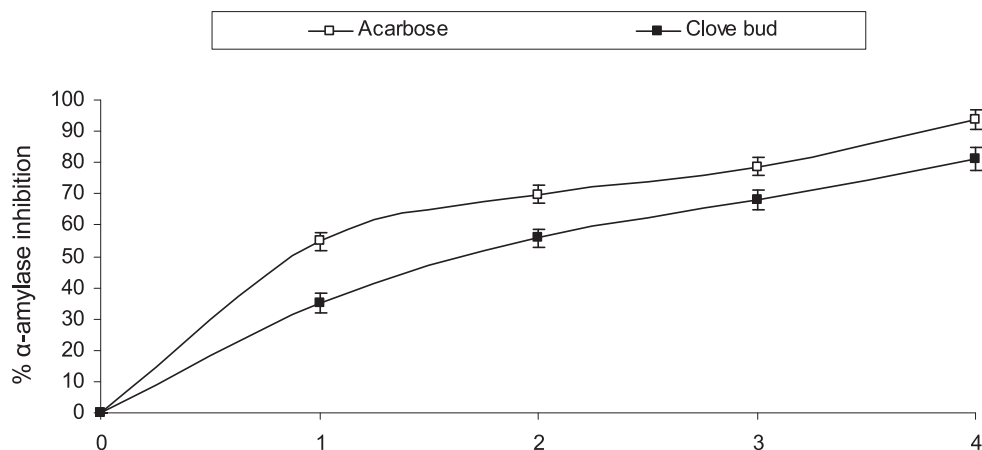


Fig. 1 α -Amylase inhibition by essential oil extracts from clove bud and acarbose. The essential oil extract concentrations for the plot are 0 $\mu\text{L/L}$, 40 $\mu\text{L/L}$, 80 $\mu\text{L/L}$, 120 $\mu\text{L/L}$, 160 $\mu\text{L/L}$. the concentrations of acarbose used in the plot are 0 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$, 20 $\mu\text{g/mL}$, 30 $\mu\text{g/mL}$ and 40 $\mu\text{g/mL}$. Values represent means \pm of standard deviation of triplicate readings.

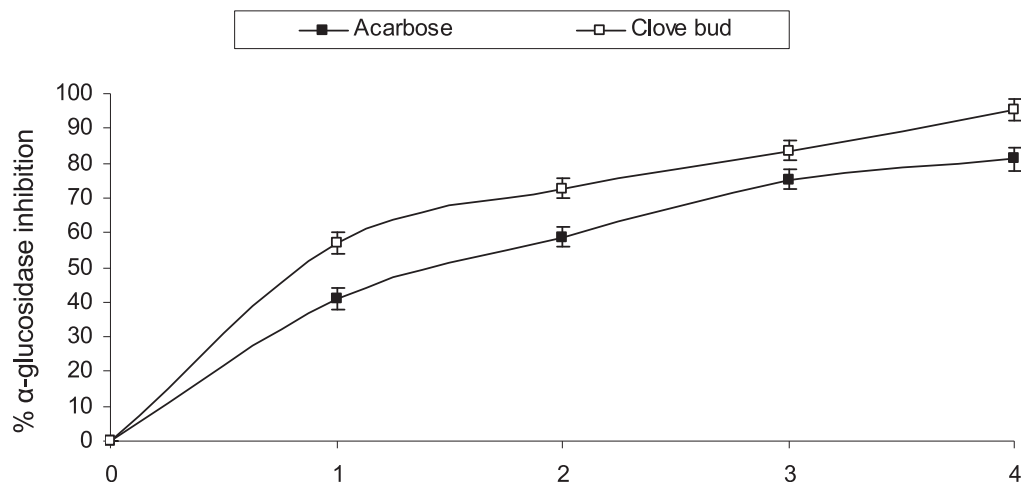


Fig. 2 α -Glucosidase inhibition by essential oil extracts from clove bud and acarbose. The essential oil extract concentrations for the plot are 0 $\mu\text{L/L}$, 40 $\mu\text{L/L}$, 80 $\mu\text{L/L}$, 120 $\mu\text{L/L}$, 160 $\mu\text{L/L}$. the concentrations of acarbose used in the plot are 0 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$, 20 $\mu\text{g/mL}$, 30 $\mu\text{g/mL}$ and 40 $\mu\text{g/mL}$. Values represent means \pm of standard deviation of triplicate readings.

tial oil, however the side effect of synthetic inhibitors such acarbose are not expected to be experience with the natural compounds like studied essential oil.

3.2 Phenolic distribution and chemical constituent

Furthermore, the antidiabetic activity of the essential oil could be linked to the phenolic content, as some phenolic rich plant extracts have been shown to inhibit starch hydrolyzing enzymes²⁹. The results of the total phenol and flavonoid distribution in the clove bud essential oil are presented in **Table 1**. The total phenolic content reported as gallic acid equivalent was 12.95 mg/L, while the total flavonoid content reported as quercetin equivalent was 6.6 mg/L. The percentage yield of the oil obtained from 100 g of

the powdered clove bud is shown in **Table 2**. In addition, the inhibitory effects of the oil on the carbohydrate hydrolyzing enzymes can also be linked to some bioactive components present in the oil. GC analysis as presented in **Table 3** revealed the presence of α -pinene (13.09%), β -pinene (45.44%), myrcene (5.13%), gamma terpinene (5.08%), pinene-2-ol (2.34%), 1,8-cineole (16.27%), linalool (0.71%), borneol (0.25%), α -terpineol (0.42%), terpinen-4-ol (0.35%), neral (0.2%), geranial (0.43%) and limonene (0.12%). α -pinene, 1,8-cineole and limonene have been reported to possess strong α -glucosidase activity²⁶. The inhibition of this enzyme is a good therapeutic approach for the control of hyperglycemia.

Table 1 The total phenolic content reported as gallic acid equivalent and total flavonoid content reported as quercetin equivalent.

Parameter (unit)	Value
Total phenol (mg/GAE/L)	12.95 ± 0.61
Total flavonoid (mg/QE/L)	6.62 ± 0.52

Values represent means of triplicate readings

Table 2 Percentage yield (w/v) of clove bud essential oil.

Sample	% Yield
Clove Bud	12.95

3.3 Inhibition of lipid peroxidation

The observed inhibition of MDA production in rat pancreas in the presence of Iron II (Fe^{2+}) (Fig. 3) revealed that the essential oil has a strong antioxidant activity. The essential oil inhibited MDA production in a dose-dependent manner with EC_{50} value of 88.13 $\mu\text{L/L}$ (Table 4). Fe^{2+} Participates in the Fenton's reaction by reacting with hydrogen peroxide (H_2O_2) to generate a highly reactive hydroxyl radical (OH^\cdot) which is capable of causing damage to membrane lipids and cell death³⁰. Increased level of Fe^{2+} in the pancreas leads to the damage of the β -cells and results in deficiency of insulin, decreased uptake of glucose and hyperglycemia. Similarly Incubation of the rat pancreas in the presence of sodium nitroprusside (SNP) caused a significant increase in the MDA content of the pancreas as presented in Fig. 4 and EC_{50} (92.94 $\mu\text{L/L}$) in Table 4. SNP releases NO which is capable of reacting with superoxide to form peroxynitrite a powerful oxidant that can induce lipid peroxidation thereby causing oxidative damage. The inhibition of SNP-induced lipid peroxidation in pancreas tissues by the clove bud essential oil (Fig. 4) could be attributed to the ability of the bioactive constituents to scavenge NO radical produced by SNP, thus protecting the alteration of the

Table 3 Chemical composition of clove bud essential oil.

Composant	RT	%
Camphene	4.780	0.23
limonene	7.796	0.12
α -pinene	9.688	13.09
β -pinene	11.366	45.44
benzyl alcohol	11.694	0.87
cis ocimene	12.521	2.32
myrcene	12.993	5.13
allo ocimene	13.190	2.78
pinene-2-ol	13.822	2.34
α -thujene	14.381	3.36
gamma terpinene	14.929	5.08
neral	15.292	0.20
geranial	15.388	0.43
isoartemisia	16.448	0.20
1,8-cineole	16.540	16.27
linalool	17.699	0.71
borneol	17.835	0.25
terpinen-4-ol	18.677	0.35
α -terpineol	18.773	0.42
thymyl methyl ether	19.725	0.17
α -copane	24.783	0.18

RT: retention time

structure and function of β -cells in the islet of Langerhans of the pancreas³¹. Moreover, the ability of the clove bud essential oil to inhibit Fe^{2+} and SNP- induced oxidative damage in the rat pancreas could be beneficial in the control and/or management of type-2 diabetes.

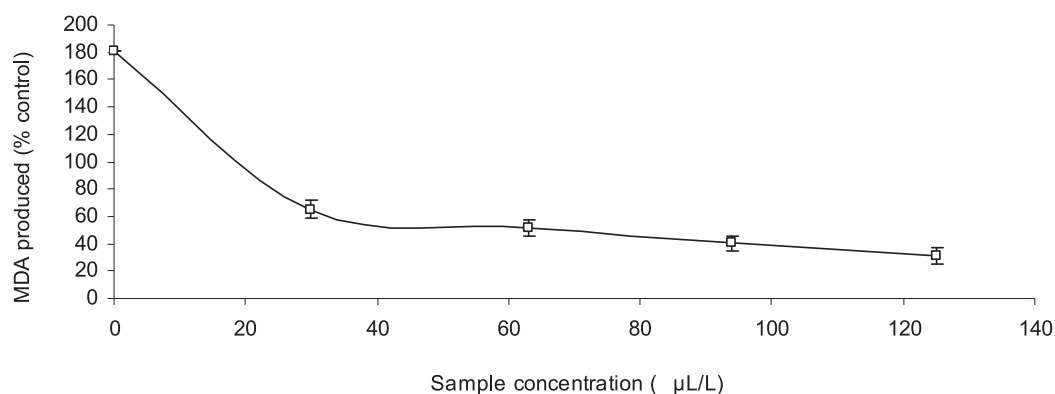
**Fig. 3** Inhibition of Fe^{2+} - induced lipid peroxidation in rats pancreas homogenates by essential oil from clove bud. Values represent means \pm of standard deviation of triplicate readings.

Table 4 EC₅₀ of inhibition of α -amylase and α -glucosidase activities, inhibition of Fe²⁺ and SNP - induced lipid peroxidation in rat's pancreas homogenates and radicals (NO, OH) scavenging and Fe²⁺ chelating abilities of clove bud essential oils (μ L/L).

α -Amylase	88.89 \pm 5.01
α -glucosidase	71.94 \pm 5.26
Lipid Peroxidation	
Fe ²⁺ - induced	88.13 \pm 0.14
SNP - induced	92.94 \pm 0.11
Radicals scavenging ability	
OH [•]	90.62 \pm 0.42
NO [•]	195.77 \pm 8.68
Fe ²⁺ - chelating ability	134.15 \pm 14.50

Values represent means of triplicate.

3.4 Antioxidant activity

Oxidative stress contributes to the pathogenesis and progression of diabetes and studies have reported that the pancreatic β -cells are susceptible to free radical induced-oxidative damage due to the fact that they have low antioxidant enzymes such as glutathione peroxidase, and superoxide dismutase³. In this study, the essential oil scavenged, OH[•] and NO[•] in a dose-dependent manner as shown in Figs. 5 and 6 respectively. The EC₅₀ values were 90.62 μ L/L (OH[•]) and 195.77 μ L/L (NO[•]) (Table 4). Figure 7 also revealed that the essential oil had a dose-dependent Fe²⁺ chelating ability with EC₅₀ value of 134.15 μ L/L (Table 4). OH[•] is a highly reactive radical capable of causing DNA damage in the β - cells via the breakdown of hydrogen per-

oxide in a reaction triggered by Fe²⁺. Although NO is needed for signal transmission, blood pressure and immune response, it has been implicated in diabetic complications⁶. Overproduction of NO[•] can lead to initiation of lipid peroxidation³². The observed radical scavenging and Fe²⁺ chelating ability of the clove bud essential oil could be ascribed to the presence of phenolic monoterpenes such as α -pinene, 1,8-cineole, linalool and borneol which has been revealed by Mastelić *et al.*³³ to be strong radical scavengers. Hence, the radical scavenging and chelating ability of the oil could be a practical approach through which oxidative stress-induced type-2 diabetes is controlled.

Conclusion

The inhibition of α -amylase and α -glucosidase and Fe²⁺ and SNP-induced MDA production as well as radicals (OH[•] and NO[•]) scavenging and Fe²⁺ abilities are possible mechanisms by which clove bud essential oil could be used in the management and/or prevent type-2 diabetes.

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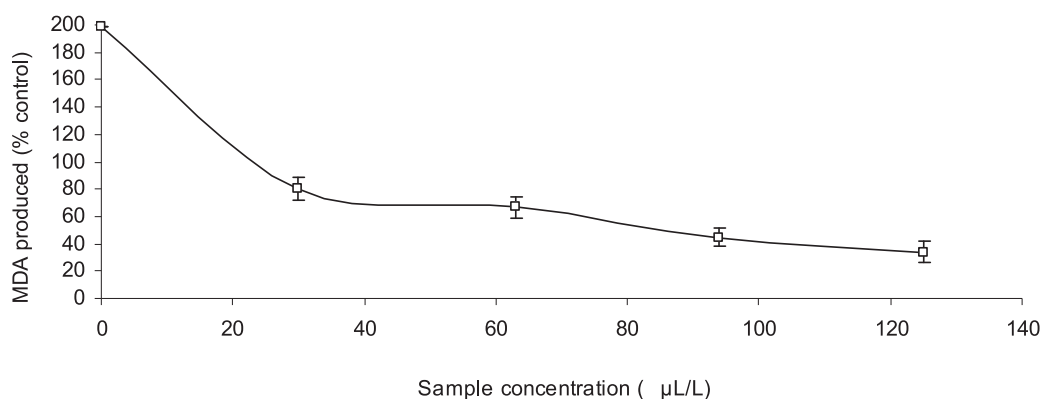


Fig. 4 Inhibition of sodium nitroprusside induced lipid peroxidation in rats pancreas homogenates by essential oil from clove bud.

Values represent means \pm of standard deviation of triplicate readings.

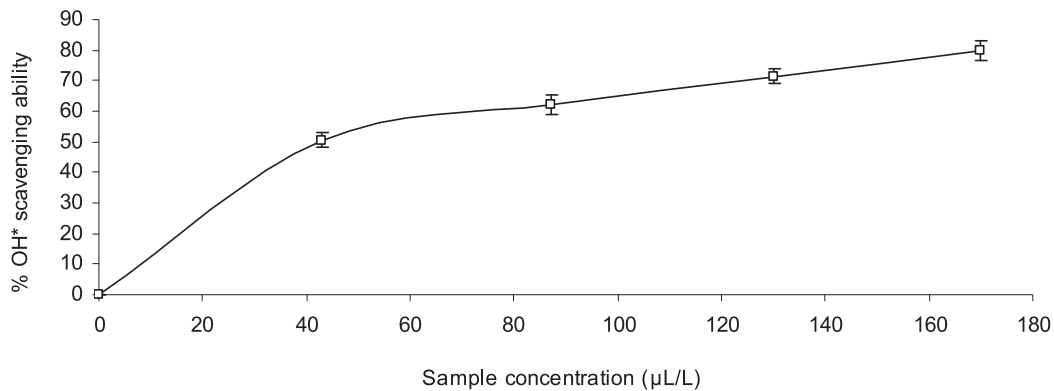


Fig. 5 OH^{*} Scavenging ability of essential oil clove bud. Values represent means ± of standard deviation of triplicate readings.

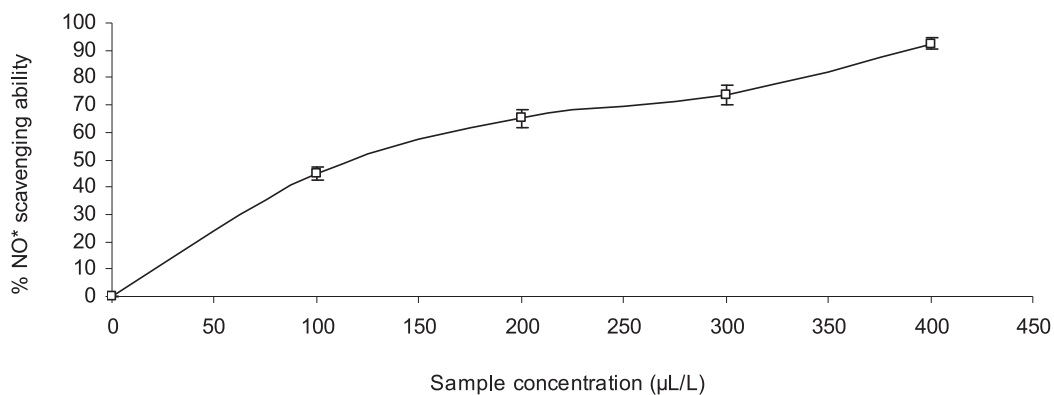


Fig. 6 NO^{*} Scavenging ability of essential oil clove bud. Values represent means ± of standard deviation of triplicate readings.

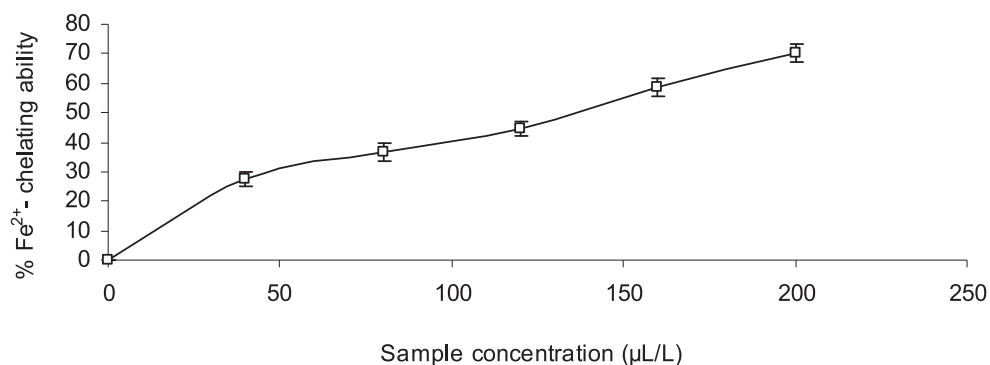


Fig. 7 Fe²⁺ - Chelating ability of essential oil clove bud. Values represent means ± of standard deviation of triplicate readings.

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