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ORIGINAL ARTICLE

Exploration of natural enzyme inhibitors with hypoglycemic potentials amongst *Eucalyptus* Spp. by *in vitro* assays

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Abstract

AIM: To investigate the presence and potency of natural enzyme inhibitors with hypoglycemic potentials amongst *Eucalyptus* Spp. by *in vitro* assays.

METHODS: The leaf extracts of the three different *Eucalyptus* species [*E. globulus* (EG), *E. citriodora* (EC), *E. camaldulensis* (ECA)] were subjected to *in vitro* assay procedures to explore the prevalence of natural enzyme inhibitors (NEIs) after preliminary qualitative and quantitative phytochemical evaluations, to study their inhibitory actions against the enzymes like α-amylase, α-glucosidase, aldose reductase, angiotensin converting enzyme and dipeptidyl peptidase 4 playing pathogenic roles in type 2 diabetes. The antioxidant potential and total antioxidant capacity of the species were also evaluated.

RESULTS: Major bioactive compounds like polyphenols (341.75 ± 3.63 to 496.85 ± 3.98) and flavonoids (4.89 ± 0.01 to 7.15 ± 0.02) were found in appreciable quantity in three species. Based on the IC_{50} values of the extracts under investigation, in all assays the effectivity was in the order of EG > ECA > EC. The results of the ferric reducing antioxidant power assay showed that the reducing ability of the species was also in the order of EG > ECA > EC. A strong correlation (R^2 = 0.81-0.99) was found between the phenolic contents and the inhibitory potentials of the extracts against the targeted enzymes.

CONCLUSION: These results show immense hypoglycemic potentiality of the *Eucalyptus* Spp. and a remarkable source of NEIs for a future phytotherapeutic approach in Type 2 diabetes.

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Key words: Natural enzyme inhibitors; Hypoglycemic; *Eucalyptus*; *In vitro* assays; Pathogenic; Polyphenols; Flavonoids

Core tip: Enzymes play an essential role in mediating important biochemical processes of life but hyper or hypo activity of such enzymes leads to malfunctions of the processes. Etiopathogenesis of diseases at molecular level has shown that enzyme inhibitors can serve as effective therapeutic bullets for several diseases. The plant kingdom is a giant hub of phytomolecules with variant pharmacology, largely unexplored. Volatile and non-volatile fractions of *Eucalyptus* include bioactive compounds like terpenes, triterpenoids, flavonoids, polyphenols, *etc.* The exploration of enzyme inhibitors amongst *Eucalyptus* species by *in vitro* assays will help in bioactivity guided isolations of such inhibitors to be targeted as natural hypoglycemics.

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INTRODUCTION

Diabetes mellitus (DM) is fast becoming the epidemic of the 21st century, becoming one of the major killers of the health of mankind after Acquired Immuno Deficiency Syndrome, cancer and cerebrovascular diseases[1]. The statistics of the global diabetic population is expected to show a steady growth to 366 million by 2030. The international diabetes federation has estimated the number of diabetics in India to be 40.9 million, which is expected to grow to 60.9 million by 2025[1,2]. Diabetes is a common metabolic disorder with abnormal elevations in the blood glucose lipid profile, leading to major complications like diabetic neuropathy, nephropathy leading to end stage renal disease, retinopathy leading to blindness and diabetic foot ulcers necessitating limb amputations[1,2]. But despite tremendous strides in modern medicines, the availability of insulin therapy and synthetic hypoglycemics, their failure to restore normoglycemia without adverse effects calls for phytotherapy and alternative medicine[3,4]. Enzymes play a vital role in mediating essential biochemical life processes like metabolism, cell cycling, signal transduction, *etc.* However, hyper or hypo activity of such enzymes leads to malfunctions of the respective biochemical processes which in many cases are the underlying causes of diseases like diabetes, Alzheimer's disease, myasthenia gravis and Parkinson's disease, as depicted by their etiopathogenesis at the molecular level. It is anticipated that enzyme inhibitors serve as important therapeutic targets for these diseases[5]. It has been found that enzymes like α-amylase, α-glucosidase, dipeptidyl peptidase 4 (DPP4), aldose reductase (AR), angiotensin converting enzyme (ACE) and peroxisome proliferator activated receptor-γ (PPAR-γ) contribute significantly to the pathogenesis of type 2DM. Reactive oxygen species (ROS) also play a pathogenic role in type 2DM.

Phytomolecules, as natural enzyme inhibitors (NEIs), can serve as successful therapeutic bullets in the control of this chronic disease[2,5-8]. The World Health Organization has recommended phytotherapy for diabetes due to safety, effectivity, availability and affordability. The NAPRALERT database (NAtural PRoducts ALERT) and the ethnobotanical literature have reported more than 800 anti-diabetic plant species[4,7-9].

Eucalyptus, also known as "gum tree", is taxonomically from the family Myrtaceae, indigenous to Tasmania, Australia and cultivated mostly in subtropical and warm temperate regions of the world. The bark and leaves of *Eucalyptus* Spp. have been used in folk medicine for the treatment of ailments such as colds, fever, toothache, diarrhea and snake bites. Uses of *Eucalyptus* leaf hot decoctions as "herbal tea" have been recorded in Aboriginal, European and British Pharmacopeias for the traditional remedy of type DM[10-21]. A rich literature exists, reporting over 500 *Eucalyptus* species with different pharmacological actions[11-22]. Hypoglycemic potentials of *Eucalyptuses* are documented, but the mechanistic actions need to be explored further [11-21].

Table 1 List of phytochemicals of *Eucalyptus* Spp. inhibiting the enzymes

Phytochemicals	Enzymes inhibited ↓
Flavonoids, like quercetin, kaempferol, myricetin; Phenolics-tannins, ellagic acid, and gallic acid; terpinoids-ursolic acid, oleanolic acid, p-cymene, and 1,8-cineole, 1-(S)-α-pinene	α-amylase
Polyphenols, proanthocyanidins, anthocyanins	α-glucosidase
Flavonoids, flavonols, terpenoids, mono-terpenes	Aldose reductase
Flavonoids, flavonols, terpenoids, mono-terpenes	Angiotensin converting enzyme
Terpenoids	Peroxisome proliferator activated receptor
Triterpenoids, phenolic compounds	Di-peptidyl peptidase 4

Inhibiting the actions of carbohydrate hydrolyzing enzymes like α-amylase and α-glucosidase helps to reduce post-prandial (PP) hyperglycemia. Inhibition of other enzymes like AR, DPP-4, ACE and PPAR-γ also presents an effective strategy to combat type 2 DM naturally[5,6,8,11]. Extensive literature surveys and our previous works have reported that *Eucalyptus* shows the presence of terpenoids, triterpenoids, flavonoids, polyphenols and tannins in its various volatile and non-volatile fractions[8,21,22]. Major phytomolecules isolated from the Eucalyptus *Spp* and their inhibitory activity against the enzymes are depicted in Table 1.

AR, a member of the aldo-keto reductase super family, is the first and rate-limiting enzyme in the polyol pathway and reduces glucose to sorbitol, utilizing reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor. In type 2DM, due to increased availability of glucose in sensitive tissues like lens, nerves and retina, there is an increased formation of sorbitol through the polyol pathway. Intracellular accumulation of sorbitol leads to cataract, retinopathy and neuropathy. AR-inhibitors prevent the conversion of glucose to sorbitol and are capable of controlling diabetic complications[8,23-32]. Limited literature data and molecular docking analysis have shown that natural biomolecules with potent aldose reductase inhibitory actions include flavonoids like quercetin, quercitrin, myricitrin, coumarins, monoterpenes, stilbenes, *etc.* Flavonoids with binding energy (BE) ranging between -9.33 to -7.23 kcal/mol exhibited AR inhibitory properties, as evidenced by *in-silico* docking studies[8,32,33]. Five bioactive compounds, namely macrocarpals A-E detected in the ethanol extracts of the leaves of *E. globulus,* showed antibacterial actions, HIV-RTase (HIV-reverse transcriptase) inhibitory activity and also inhibited AR[8,32,33].

Attenuation in ROS level may be due to increased

production or diminished depletion of enzymes, like catalase, glutathione peroxidase and superoxide dismutase. Natural antioxidants which scavenge free radicals can provide a synergistic action to the overall antidiabetic potential of a plant[12,13]. Osawa and Namiki (1981, 1985) reported the presence of a powerful antioxidant, 4-hydroxytritriacontane-16,18 dione, in the leaf wax of different *Eucalyptus* species[24,25].

The enzyme ACE is associated with hypertension, a long term complication of diabetes. ACE activates histidyl leucine dipeptide called angiotensin-I into a potent vasoconstrictor called angiotensin-Ⅱ. Angiotensin-II influences aldosterone release which increases blood pressure by promoting sodium retention in distal tubules. Biomolecules like flavonoids, flavonols, anthocyanins and triterpenes are potent ACE inhibitors[8,34,35]. Molecular docking studies also recommend the use of herbal ACE inhibitors in the management of type 2 DM[8,34,35].

PPAR-γ is a key receptor in lipid and glucose homeostasis because of its ability to reduce the plasma free fatty acids and phytomolecules can exert their insulin sensitizing actions with their high affinity for the receptor PPAR-γ. Terpenoids act as PPAR modulators regulating carbohydrate and lipid metabolism. Several terpenoids have been isolated from the *Eucalyptus* species and PPAR antagonism is amongst the suggested modes of hypoglycemic action of *Eucalyptus*[8,36].

Glucagon-like peptide-1 (GLP-1) is a remarkable antidiabetic gut hormone with combinatorial actions of stimulating insulin secretion, inhibiting glucagon secretion, increasing beta cell mass, reducing the rate of gastric emptying and inducing satiety. DPP4 rapidly deactivates GLP-1. Phytomolecules, mostly triterpenoids, steroids and phenolic constituents with DPP4 inhibitory activity, help to increase the levels of endogenous active GLP-1 and act as an important therapeutic compound against type 2 DM, the fact being further supported by molecular docking studies[8,37].

The present report documents our studies aiming to explore the major phytochemicals amongst three *Eucalyptus* species, *E. globulus* (EG, blue gum or Tasmanian blue gum), *E. citriodora* (EC, lemon scented gum) and *E. camaldulensis* (ECA, river red gum or Murray red gum), along with the existence of NEIs of enzymes like α-amylase, α-glucosidase, AR, DPP4, ACE and antioxidant enzymes by *in vitro* assays, with the perspective to evaluate the potentiality of these three species to combat type 2 DM and its complications. Furthermore, such research will help in bioactivity guided isolation of potent NEIs.

MATERIALS AND METHODS

Plant materials

Fresh leaves of EG, EC and ECA were collected from natural and man-made forest areas of IIT Kharaghpur and adjoining areas, like Balarampur, Gopali and Arabari forest areas, and authenticated by Dr Shanta AK, Biotechnologist, Nirmala College of Pharmacy, Guntur, India.

Reagents and chemicals

Yeast α-glucosidase, bovine serum albumin, sodium azide, para-nitrophenyl α-D-glucopyranoside solution (pNPG), ACE (from rabbit lung, 3.5 units/mg of protein), starch azure, porcine pancreatic amylase, Tris-HCl buffer, hippuryl-L-histidyl-L-leucine (HHL) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Sigma Chemicals, United States. Other chemicals like diagnostic reagents, surfactants, polyphosphate, dextran sulphate, *etc*., were purchased from Merck Co, India. Acarbose was a kind gift sample from Zota Pharmaceuticals Pvt. Ltd, Chennai, India.

Preparation of eucalyptus leaf extracts

A uniform methodology was followed for preparing the leaf extracts of the three different species of *Eucalyptus*. Typically, the leaves were washed first with tap water and then with distilled water to remove all dust, subjected to shade drying at 25 ± 3 ℃ temperature, and then finely powdered in an electrical grinder (Bajaj GX 11, India). The leaf powder was stored at room temperature in an airtight container until use and labeled separately as EG, EC and ECA. Extraction was carried out as described by Sugimoto *et al*[20,21] with few modifications. Briefly, 500 g of leaf powder of each species was extracted separately with ethanol-water (1:2 v/v) under reflux for 2 h, filtered through a Whatman filter paper no. 1, concentrated using a rotary evaporator (Buchi, Flawil, Switzerland) and dried in a vacuum oven. The percentage yield of extracts was calculated with regard to the initial weight of dry powders and final weight of the extracts. These extracts were then subjected to preliminary and quantitative phytochemical evaluations and *in vitro* assay procedures.

Phytochemical investigations of the eucalyptus leaf extract

Phytochemical analysis of the major bioactive compounds of interest of the three different extracts (EG, EC and ECA) was performed using the methods of Harbone (1984), Trease and Evans (1989) and other literature methods[22,38]. The three extracts were analyzed for glycosides (Keller Killiani and Borntrager's tests), alkaloids (Mayer's, Dragendorff's reagents), saponins (Foam test), triterpenes (Salkowski and Libermann Burchard tests) and 1,8-cineole (Marquis reagent, Gallic acid reagent, conc. H_2SO_4 and phloroglucinol).

The total polyphenol content of the extracts was determined by ultra violet (UV) spectrophotometry (Perkin Elmer Lambda 25 UV-vis) at 760 nm using Folin-Ciocalteu reagent by the method of Othman *et al*[39] and Modnicki *et al*[40] (2009)[41,42]. The concentrations of the total polyphenols were determined in Gallic equivalents (GAE) per gram of the extract. The polyphenol content was calculated by the formula: $X = (5.6450 \times A)/m$. Where X is total phenolic compounds (%), A is absorbance of investigated extract and m is mass (g) of the investigated sample.

The total flavonoid content of the extracts was determined by the method of Djeridane *et al*[43], 2006, which

is based on the formation of a complex of flavonoid-aluminium, and the concentration of the flavonoids was expressed in terms of QE per gram extract.

The flavonol content of the extracts was determined according to Abdel-Hameed, 2009, which is based on the formation of a complex between the extract with $AlCl_3$ and sodium acetate and the total flavonol content was expressed in terms of quercetin equivalent (QE) per gram extract[42].

Tannins were measured according to the protocol of Hagerman and Butler, 1978, which is based on the obtention of a colored complex Fe^{2+}-phenol whose absorbance was measured spectrophotometrically at 510 nm. The tannin content was obtained in mg of tannic acid equivalent (TAE) per gram extract[44].

The three extracts were subjected to color reactions with Marquis Reagent, gallic acid reagent, concentrated H_2SO_4 and phloroglucinol reagent. Standard 1,8-cineole gives orange color with Marquis reagent, yellow color with gallic acid, dark yellow color with concentrated H_2SO_4, and no coloration with phloroglucinol reagent[22,38].

Gas chromatographic analysis of 1,8-cineole

Fresh leaves of the three *Eucalyptus* spp. (EG, EC and ECA) were air dried and 100 g leaves of each variety were subjected to hydrodistillation for 3-4 h to extract the essential oil, employing a Clevenger type apparatus[45]. Extracted oils were decanted from the water layer and dried over anhydrous sodium sulfate. The extracted oils of the three species were subjected to gas chromatographic (GC) analysis (perkin elmer clarus 500, with Flame Ionization Detector) as described by Quereshi *et al*[45]. The operating conditions were: nitrogen as carrier gas, injector and detector temperature of -250 ℃; column of 30 m (length) × 0.25 mm (inner diameter) and film thickness of 0.25 μm. The temperature was gradually increased at a rate of 15 ℃/min to 240 ℃ for 4 min.

In vitro assay procedures

After phytochemical investigations of the leaf extracts of EG, EC and ECA, *in vitro*-inhibitory assays of α-amylase, α-glucosidase, aldose reductase, ACE, DPP4, antioxidant assays like DPPH free radical scavenging activity, scavenging of hydrogen peroxide and total antioxidant activity in the ferric reducing antioxidant power (FRAP) assay were carried out following standard methods[46-57].

The enzymes mentioned above contribute to the pathogenesis of type 2 DM in one way or another. Inhibition of such enzymes helps to combat type 2 DM naturally. There are ample research works highlighting the hypoglycemic potentials of *Eucalyptus*. We explored such NEIs by the above mentioned *in vitro* assays and once again evaluated the hypoglycemic potentiality of *Eucalyptus*.

α-Amylase inhibitory assay: The study was carried out following standard literature methodologies with slight modifications[12,46,47]. Briefly, 2 mg of starch azure was suspended in a tube containing 0.2 mL of 0.5 mol tris-phosphate buffer (pH = 6.9) containing 0.01 mol calcium chloride as the substrate. After boiling the tube for 5 min, it was preincubated for 5 min at 37 ℃. Different concentrations (10, 20, 40, 60, 80 and 100 μg/mL) of the extracts of EC, EG and ECA were prepared by dissolving in 1 mL of 0.1% dimethyl sulfoxide. Then 0.2 mL of the extract of particular concentrations was put in the tube containing the substrate solution. Next, 0.1 mL of porcine pancreatic amylase in tris-HCL buffer (2 units/mL) was added to the tube containing extracts and substrate, at 37 ℃. After 10 min, the reaction was stopped by adding 0.5 mL of 50% acetic acid in each tube and the reaction mixture was centrifuged (Eppendorf-5804R) at 3000 *g* for 5 min at 4 ℃. The absorbance of the resulting supernatant was measured at 595 nm. Acarbose (A_{car}) in the concentration range of 1.25, 2.5, 5 and 10 μg/mL in distilled water was used to create the calibration curve. The assay was performed in triplicate. The concentration of the *Eucalyptus* extracts of three species (EG, EC and ECA) required to inhibit 50% of α amylase activity under the assay conditions is referred to as IC_{50} values. Absorbance was calculated using the formula: a amylase activity = [(Ac+) - (Ac-) - (As-Ab)]/[(Ac+) - (Ac-)] × 100.

α-glucosidase inhibitory assay: The assay procedure was developed as described by Basak *et al*[12] and Subramanian *et al*[47], with slight modifications. An aqueous ethanol extract of the three species (EG, EC and ECA) was used for the study. The yeast α-glucosidase enzyme solution was prepared by dissolving at a concentration of 0.1 U/mL in 100 mmol phosphate buffer, pH = 7.0, containing bovine serum albumin and sodium azide which was used as enzyme source. This enzyme solution was added to the aqueous-ethanolic extracts of EG, EC and ECA in increasing concentrations (1, 1.5, 2, 2.5, 3, 3.5 μL/mL). The reaction was initiated by adding 0.20 mL of para-nitrophenyl α-D-glucopyranoside solution (pNPG); 2 mmol pNPG in 50 mmol sodium phosphate buffer pH = 6.9) which acted as the substrate. The reaction was terminated by adding 1 mL 0.1 mol/L Na_2HPO_4. The test tubes were cooled under tap water and α-glucosidase inhibitory activity was determined at 405 nm by measuring the quantity of *P*-nitrophenol released from pNPG. The assay was performed in triplicate for each extract and the data presented as mean ± SD. The concentration of the *Eucalyptus* extracts (EG, EC and ECA) required inhibiting 50% of α-glucosidase activity under experimental conditions is defined as the IC_{50} value. Acarbose (A_{car}) was dissolved in distilled water to prepare a series of dilutions (1.25, 2.5, 5, 10 mg/mL) and was used as the positive control. The percentage inhibition was calculated according to the formula: %inhibition = (Abs400 control - Abs400 extarct)/Abs400 control.

IC_{50} values were determined from the plots of percentage inhibition *vs* log inhibitor concentration and were calculated by nonlinear regression analysis from the mean inhibitory values.

Aldose reductase inhibitory assay: The assay was carried out following reported literature methods and the experimental protocol approved by the Institutional Ethical Committee[48-50]. Two to three mo old healthy adult Wistar albino rats weighing about 150-200 g were acclimatized to laboratory conditions (12 h light and 12 h dark cycle, 25 ± 5 ℃, 30%-60% relative humidity) with free access to pelleted food and water *ad libitum*. Immediately after sacrifice, lenses were removed from the eyes, washed with saline and the fresh weights of a lens were measured. Next, a 10% homogenate was prepared from the rat lens in 0.1 mol/L phosphate-buffered saline (PBS) at pH 7.4, centrifuged at 5000 *g* for 10 min in the cold and the supernatant collected. The protein content of the supernatant was determined by literature methods[48-50].

For the determination of the aldose reductase (AR) inhibitory activity, 0.7 mL of phosphate buffer (0.067 mol), 0.1 mL of NADPH (25×10^{-5} mol), 0.1 mL of DL-glyceraldehyde (substrate, 5×10^{-4} mol) and 0.1 mL of lens supernatant were mixed in the sample cuvette. Absorbance was taken against a reference cuvette containing all other components except the substrate, DL-glyceraldehyde. The final pH of the reaction mixture was adjusted to pH = 6.2. On adding substrate to the solution mixture, the enzymatic reaction starts and absorbance (OD) was recorded at 340 nm for 3 min at 30 s intervals. AR activity was calculated and expressed as OD/min per milligram protein.

For the determination of the AR inhibitory activity of the *Eucalyptus* extracts, a stock solution was prepared by dissolving the *Eucalyptus* extracts (EG, EC and ECA) in PBS and different concentrations prepared from stock solutions were added to both the reference and standard cuvettes. The reaction was initiated by the addition of 0.1 mL DL-glyceraldehyde and the reaction rate measured as mentioned above. Percentage inhibitions of AR activity of the extracts were calculated with reference to normal rat lens to have 100% activity. The concentrations of the extracts required to inhibit 50% of AR activity under assay conditions is defined as the IC_{50} values which were calculated for each sample by plotting a graph between log dose concentrations *vs* percentage inhibition. Quercetin, a known AR inhibitor, was used as the positive control.

ACE inhibitory assay: The assay method was based on the liberation of hippuric acid from hippuryl-L-histidyl-L-leucine (HHL) catalyzed by the ACE. The assay procedure was carried as described [51,52] and other methods with slight modifications. Briefly, 50 μL of sample solutions (extracts of EC, EG and ECA) in the concentration range of 0.1-2.5 mg/mL were preincubated with 50 μL of ACE (25 mU/mL) at 37 ℃ for 10 min. Next, 150 μL of substrate solution (8.3 mmol HHL in 50mmol sodium borate buffer containing 0.5 mol NaCl at pH 8.3) was added and incubated for 30 min at 37 ℃. The reaction was terminated by addition of 250 μL 1.0 mol HCl. To the resulting solution, 0.5 mL of ethyl acetate was added and centrifuged (Eppendorf-5804R) for 15 min. Then, 0.2 mL of the upper layer was transferred to a test tube, evaporated under room temperature in vacuum and the liberated hippuric acid was dissolved in 1 mL distilled water and the absorbance was measured at 228 nm. Experiments were performed in triplicates. Captopril was used as standard (3.5 μg/mL) in the assay. The percentage of inhibition (ACEI) was calculated using the formula: %inhibition = (A-B)/(A - C) × 100. Where A is the OD at 228 nm with ACE but without inhibitor, B is the OD in presence of both ACE and inhibitor, C is the OD without ACE and inhibitor.

DPP4 inhibitory assay: The assay was carried out following reported literature methods using GPN-Tos (Gly-Pro p-nitroanilide toluenesulfonate salt) as the substrate[53-55]. Briefly, 0.5 mL of the assay mixture contained 40 mmol K-Na-phosphate buffer, pH 7.5, an enzyme sample. The reaction was initiated by adding a substrate to a concentration of 0.24 mmol and stopped by adding 0.2 mol acetic buffer at pH 5.5. The differential absorption at 390 nm was recorded against an identical mixture without the enzyme and the amount of p-nitroaniline depleted was evaluated from its extinction coefficient at the wavelength of 9.9 mmol/L/cm^{-1}.

Evaluation of antioxidant activity

Dpph free radical scavenging activity: The antioxidant activity of the *Eucalyptus* extracts (EC, EG and ECA) was determined on the basis of the scavenging effect on the stable DPPH free radical activity[12,39,51,56]. A stock solution of DPPH in methanol (33 mg in 1 L) was freshly prepared and kept in the dark at 4 ℃; after checking its initial absorbance, 5 mL of this stock solution was added to 1 mL of the solution of the extracts prepared in concentrations of 50-500 μg/mL. Next, 2.8 mL of 95% methanol was added and the mixture was shaken vigorously and after 30 min the absorption was measured at 517 nm. Ascorbic acid was used as the standard. The radical scavenging capacities of the test samples were expressed as percentage inhibition and calculated according to the equation: % inhibition of DPPH activity = (Absorbance control - Absorbance)/(Absorbance control) × 100.

Plotting was done of percentage inhibition *vs* concentration, and the concentration of sample required for 50% inhibition is regarded as IC_{50} value for each of the test samples.

Total antioxidant activity (FRAP assay): Total antioxidant activity was determined by the FRAP assay as described by Pracheta *et al*[56] and Shahwar *et al*[57]. It is a direct test of antioxidant capacity. The assay of reducing activity is based on the reduction of ferric to ferrous form in the presence of antioxidants in the tested samples (extracts of *Eucalyptus* species). The stock solutions included 10 mmol/L 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mmol HCl and 20 mmol $FeCl_3$, and 300 mmol acetate buffer (pH 3.6). The working solutions were freshly prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ and 2.5 mL of $FeCl_3$. The temperature of the solution

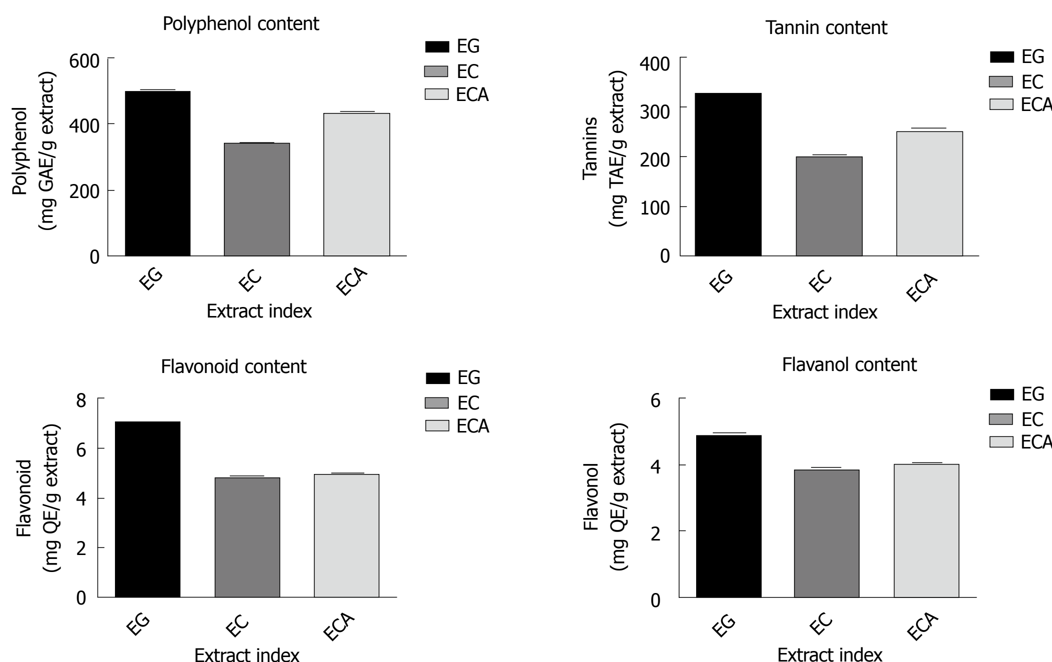

Figure 1 Graphical presentations of the presence of phytochemicals in Eucalyptus extracts. Data are presented as the mean ± SD of each triplicate test. EG: *E. globulus*; EC: *E. citriodora*; ECA: *E. camaldulensis*.

Table 2 Total polyphenol, flavonoid, flavonol and tannin contents of *E. globulus*, *E. citriodora* and *E. camaldulensis*

Extract[1]	Polyphenol (mg/g extract[2])	Tannins (mg/g extract[2])	Flavonoid (mg/g extract[2])	Flavonol (mg/g extract[2])
EG	496.85 ± 3.98	329.06 ± 6.25	7.15 ± 0.02	4.98 ± 0.01
EC	341.75 ± 3.63	199.75 ± 5.49	4.89 ± 0.01	3.87 ± 0.05
ECA	429.91 ± 4.03	253.15 ± 4.96	5.01 ± 0.02	4.09 ± 0.01

[1]Content expressed per gram of relevant extracts (EG, EC and ECA); [2]Values are expressed as mean ± SD from triplicate determination. EG: *E. globulus*; EC: *E. citriodora*; ECA: *E. camaldulensis*.

Table 3 Color test results for the presence of 1,8-cineole in *E. globulus*, *E. citriodora* and *E. camaldulensis* extracts

Extracts	Marquis test	Gallic acid test	Concentrated H_2SO_4	Phloroglucinol
EG	Orange	Yellow	Dark yellow	No color
EC	Orange	Dark Yellow	Dark yellow	No color
ECA	Orange	Yellow	Bright orange-yellow	Pink

EG: *E. globulus*; EC: *E. citriodora*; ECA: *E camaldulensis*.

was raised to 37 ℃ prior to use. *Eucalyptus* extracts (200 μL) were allowed to react with FRAP solution (2900-3000 μL) for 30 min in the dark. Absorbance of the colored product formed (ferrous tripyridyl triazine complex) was recorded at 595 nm. Results were expressed in μM equivalent to $FeSO_4$ by extrapolation from the calibration curve.

Statistical analysis

The experimental results were expressed as mean ± SD of three replicates. The data were subjected to one way analysis of variance (ANOVA) using commercially available software (Prism version 5.0; Graph Pad Software, San Diego, CA, United States). Results were analyzed by Student's *t* test (paired or unpaired, as appropriate) or Tukey's multiple comparison test. Statistical analysis was performed by using GraphPad Prism where $P < 0.05$ was considered statistically significant.

RESULTS

The yield of the *Eucalyptus* leaf extracts (extractions carried out in triplicates) were 49% ± 3.3% for EG, 46.5% ± 4.2% for EC, and 45.8 ± 3.9% for ECA. The details of phytochemicals amongst *Eucalyptus* Spp. and the enzymes inhibited by them are presented in Tables 1 and 2 and Figure 1. The color test results for the presence of 1,8-cineole in the extracts of EG, EC and ECA are presented in Table 3. GC analysis of the oils extracted from three species (EG, EC and ECA) showed the highest 1,8-cineole content in EG (about 50%). ECA also showed the presence of 1,8-cineole in addition to several other peaks indicating the presence of other compounds. In EC citronellal was found to be the major component.

All three extracts (EG, EC and ECA) showed promising inhibitory potentials for enzymes, including α-amylase, α-glucosidase, AR, ACE and DPP4. The antioxidative potential of the extracts were determined by DPPH radical scavenging and the total antioxidative capacity by the FRAP assay. The results of all such inhibitory assays are presented in Figure 2 and the summary of the IC_{50} values of tested samples in Table 4.

The correlation coefficient (R^2) between polyphenol and flavonoid content and IC_{50} inhibitory values of the enzymes ranged between 0.81-0.99 and 0.57-0.99 respec-

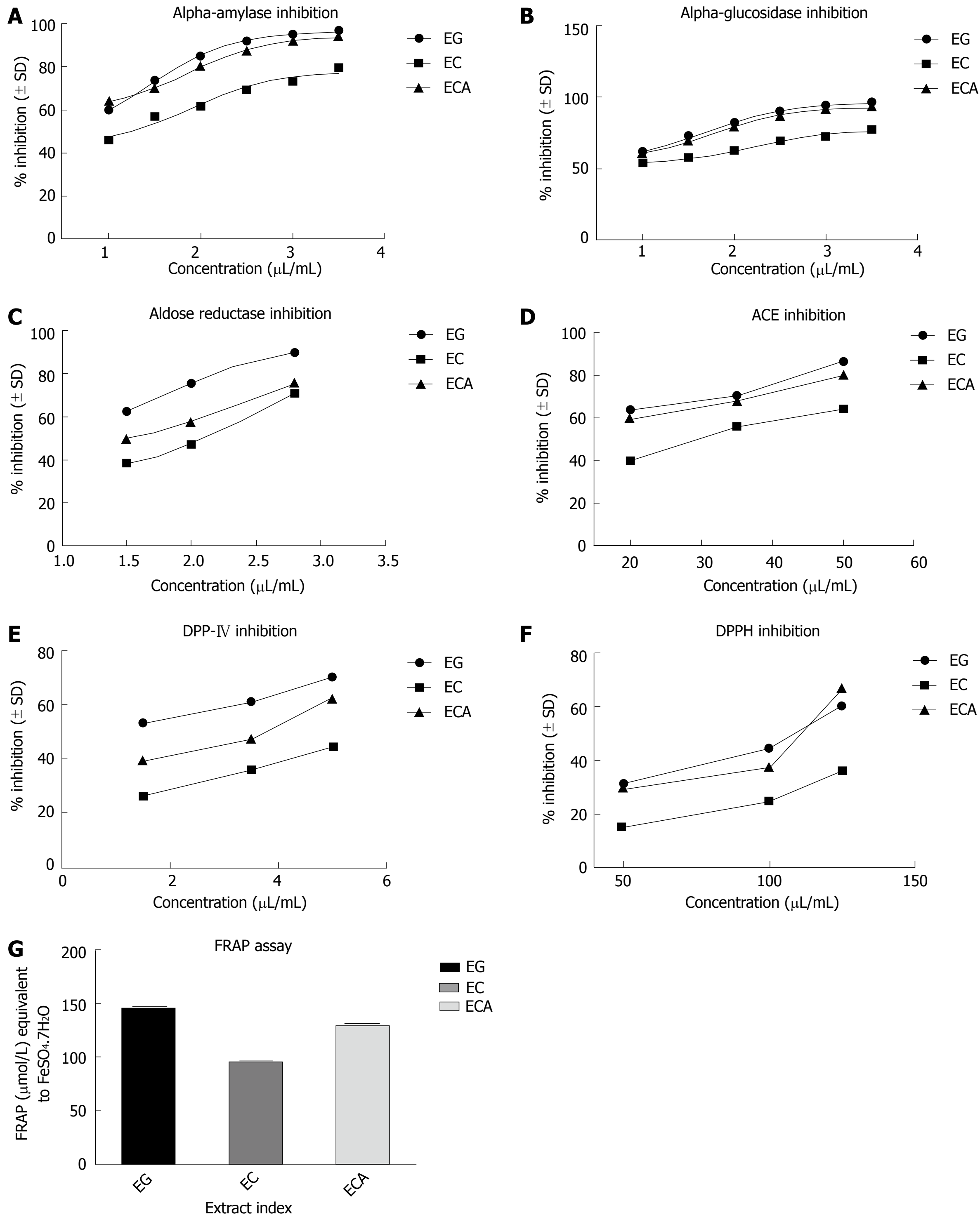

Figure 2 Eucalyptus extracts. A: Alpha-amylase; B: Alpha-glucosidase; C: Aldose-reductase; D: Angiotensin converting enzyme; E: Dipeptidyl peptidase 4; F: 1,1-Diphenyl-2-picrylhydrazyl; G: FRAP assay. Data are presented as the mean ± SD of each triplicate test. EG: *E. globulus*; EC: *E. citriodora*; ECA: *E. camaldulensis;* FRAP: Ferric reducing antioxidant power.

tively.

The polyphenol content of three *Eucalyptus* Spp. (EG, EC and ECA) was compared with the IC_{50} values of different inhibitory assays using Tukey's multiple comparison test (one-way ANOVA), considering $P < 0.05$ as significant. All P values were found to be < 0.05. The results suggested that the inhibitory potentials of the extracts are largely dependent upon the polyphenol content in *Eucalyptus* Spp.

DISCUSSION

Qualitative and quantitative phytochemical investigations of the *Eucalyptus* leaf extracts EG, ECA and EC showed appreciable levels of bioactive components like polyphenols and flavonoids. From the IC_{50} values of *Eucalyptus*

Table 4 IC_{50} inhibitory values of Eucalyptus extracts *E. globulus*, *E. citriodora* and *E. camaldulensis* in different assays

Assays	EG	EC	ECA
α-amylase	3.01 ± 0.01	4.13 ± 0.09	3.65 ± 1.04
α-glucosidase	2.08 ± 0.01	2.68 ± 0.11	2.11 ± 0.19
Aldose reductase	2.06 ± 0.03	6.72 ± 0.65	2.56 ± 0.84
Angiotensin converting enzyme	4.31 ± 0.09	30.83 ± 0.45	6.85 ± 0.98
Dipeptidyl peptidase	3.098 ± 0.09	6.138 ± 0.68	3.99 ± 0.91
1,1-diphenyl-2-picrylhydrazyl (DPPH)	12.32 ± 0.91	68.42 ± 0.05	14.44 ± 1.91

EG: *E. globulus*; EC: *E. citriodora*; ECA: *E. camaldulensis*.

extracts in different assays (Table 4), it appears that all three extracts showed significant inhibitory potentials against the six enzymes assayed, in the order EG > ECA > EC. Based on the results of FRAP assay, the reducing ability of EG was highest and that of EC lowest (Figure 2). 1,8-cineole is the major constituent of the volatile fractions in EG and ECA, whereas in EC the major constituent is citronellal with citronellol and spathulenol. According to the literature, compounds with highest reducing ability have delocalized chemical bonds[56-60]. Prior research suggested a strong positive correlation (R^2 = 0.99) between phenolic content and antioxidative potential[12,18,58,59]. Polyphenols received wide attention because of their antioxidant properties which refers to their ability to prevent damage from ROS through radical scavenging or prevent the generation of these species by iron chelation[61]. Polyphenols also bind and inhibit the enzymes α-amylase and α-glucosidase[61]. Polyphenols have also been shown to facilitate insulin response and attenuate secretion of glucose dependent insulinotropic polypeptide and glucagon like GLP-1. Other suggested mechanisms for the hypoglycemic actions of polyphenols were down regulation of the expression of liver glucokinase, upregulation of phosphoenolpyruvate carboxykinase (PEPCK), induction of the AMP-activated protein kinase (AMPK) pathway, enhancing peripheral glucose utilization by stimulating glucose transporter subtype 4 (GLUT-4), *etc.*[62]. In this context, it is to be mentioned that green tea extract (GTE) contains polyphenols like catechin, epicatechin, *etc.* Epigallocatechin gallate (EGCG), an abundant form of catechin, is the major attributable factor for the beneficial effects of green tea. EGCG inhibits adipocyte proliferation, increases fat oxidation and enhances the expression of GLUT-4, as shown in animal studies[63,64].

Literature surveys have shown that flavonoids and its subfamilies significantly inhibit the ACE enzyme by generating chelate complexes within the active center of ACE[65]. Flavonoids were found to attenuate hepatic gluconeogenesis by decreasing the activity of glucose-6-phosphate and PEPCK, subsequently improving glycemic control[65]. Our research data are in accordance with this phenomenon. A strong correlation was found between polyphenol (R^2 = 0.81-0.99) and flavonoid contents (R^2 = 0.57-0.99) with the antioxidative and enzyme inhibitory potentials of the extracts.

NEIs can serve as an important therapeutic tool against type 2 DM. The current research aims to provide the state-of-the-art search of NEIs amongst *Eucalyptus* Spp. by *in vitro* assays which can be further utilized for bioactivity-guided isolations of such enzyme inhibitors. Our research results show the hypoglycemic potential of the *Eucalyptus* Spp. (extracts) for future exploitations in phytotherapy of type 2 DM. However, further extensive pharmacology and toxicological studies in animal and human models are warranted.

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COMMENTS

Background

The current research aims to explore the presence of biomolecules by *in vitro* assays amongst three eucalyptus species acting as natural enzyme inhibitors for enzymes with significant pathogenic roles in type 2 diabetes.

Research frontiers

Enzymes like α-amylase, α-glucosidase, aldose reductase, angiotensin converting enzyme and dipeptidyl peptidase 4 play important pathogenic roles in type 2 diabetes. Phytomolecules acting as inhibitors of such enzymes can act as effective therapeutic targets in type 2 diabetes. Volatile and non-volatile fractions of *Eucalyptus* Spp. include biomolecules like terpenes, triterpenoids, flavonoids, polyphenols, *etc.* The exploration of enzyme inhibitors amongst *Eucalyptus* Spp. by *in vitro* assays will help in bioactivity guided isolation of such inhibitors to be targeted as natural hypoglycemics.

Innovations and breakthroughs

Enzymes play a vital role in mediating essential biochemical life processes. However, hyper or hypo activity of such enzymes leads to malfunctions of the respective biochemical processes, which in many cases are the underlying causes of diseases like diabetes. The current research aims to provide the state-of-the-art search of natural enzyme inhibitors amongst *Eucalyptus* Spp. by *in vitro* assays which can be further utilized for bioactivity-guided isolations of such enzyme inhibitors. Those research findings have shown that the *Eucalyptus* Spp. under study have immense hypoglycemic potentials with high IC_{50} values against the targeted enzymes. Moreover, the inhibitory potentials of the species are also well correlated with the polyphenol-flavonoid contents of the species.

Applications

The *Eucalyptus* Spp. (extracts) under study showed significant hypoglycemic potentialities for future exploitations in phytotherapy of type 2 DM.

Terminology

Natural Enzyme Inhibitors: Malfunctions of certain enzymes are the root causes of many diseases. Effective enzyme inhibitors have great clinical significance and a substantial role in the drug delivery process. Such enzyme inhibitors of natural origin are more acceptable due to safety and lower incidences of side effects on short and long term treatment modalities.

Peer review

Dey *et al* investigated the potential hypoglycemic actions of Eucalyptus extracts *in vitro*. The extracts were found to significantly inhibit a number of enzymes related to T2DM, such as amylase, glucosidase, dipeptidyl peptidase 4, *etc.* The rationale of this study and methodology were adequately described. The selection of enzymes and antioxidant activity is based on the hypothesis that these activities are involved in the pathogenesis of type 2 diabetes. The three extracts show broad enzyme inhibitory activity and antioxidant activity, which differs in

magnitude between the three extracts. The authors conclude that the extracts might serve as starting material for new therapeutic modalities for type 2 diabetes and that their data fit with the idea that leaves from trees could provide a base material for drug discovery and development programs.

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