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Original Article

EVALUATION OF α-GLUCOSIDASE INHIBITORY POTENTIAL OF METHANOLIC LEAF EXTRACT OF *OCIMUM CANUM*

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ABSTRACT

Objective: The present investigation was designed to study the inhibitory effects of methanolic leaf extract of *Ocimum canum (O. canum)* on α-glucosidase using *in vitro* model followed by an assessment of bioactive compounds.

Methods: The methanolic leaf extract was prepared by Soxhlet extraction method and partially purified by thin layer chromatography (TLC). Each band was subjected to α-glucosidase inhibition study. The positive bands were further characterized by high-performance liquid chromatography (HPLC) and quadrupole time of flight (Q-TOF) micro mass spectrometer.

Results: Out of the several combinations of solvent systems, toluene, ethyl acetate and formic acid combination in the ratio of 7:2:1 revealed 5 bands on the TLC sheet. Among all the TLC bands, 2 bands (band A and B) showed the significant inhibitory effect on α-glucosidase activity. HPLC analysis of band A and B revealed the presence of two important polyphenolic compounds, namely rosmarinic acid (RA) and ursolic acid (UA). Q-TOF micromass spectrometer analysis revealed the percentage availability of RA, caffeic acid, tartaric acid, quercetin and other polyphenolic components in the bioactive bands.

Conclusion: The study revealed that methanolic leaf extract of *O. canum* exhibits potent inhibition of α-glucosidase activity. Inhibition of α-glucosidase activity might be attributed to the presence of the polyphenolic compounds like RA and UA. Therefore, this finding can lead to the development of natural α-glucosidase inhibitors by the *O. canum* leaf extract.

Keywords: *Ocimum canum,* α-glucosidase, Hyperglycemia, Rosmarinic acid, and Ursolic acid

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INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia, which results from improper regulation of carbohydrate and lipid metabolism by insulin. The disorder causes considerable long-term complications and is also a major risk factor for cardiovascular diseases. Type-II diabetes is more prevalent than type I and is caused by an imbalance in insulin secretion. Postprandial hyperglycemia is an important symptom of type II diabetes and is one of the primary anti-diabetic targets. Treatment of postprandial hyperglycemia can be achieved by inhibiting key carbohydrate hydrolyzing enzymes like α-glucosidase and α-amylase [1, 2]. Alpha glucosidase is a membrane-bound enzyme which facilitates absorption of glucose by the small intestine. The inhibition of α-glucosidase in the small intestine delays the rate of hydrolytic cleavage of oligosaccharides. The process of digestion spreads to the lower part of small intestine, which delays the overall absorption rate of glucose into the blood and decreases the postprandial rise of blood sugar [1].

Herbal drugs are widely used in the treatment of diabetes with lesser side effects [3, 4] due to their availability and cost-effectiveness. Hence, screening of medicinal plants for inhibition of carbohydrate-hydrolyzing enzymes holds an enormous potential for developing new drugs for the treatment of diabetes.

Among different medicinal plants, the genus *ocimum* has a strong therapeutic potential [5]. They are distributed throughout the Indian subcontinent and most of them are traditionally used for the treatment of ailments such as cardiovascular disorders, diabetes, fungal infections, bronchitis, bronchial asthma, malaria, diarrhoea and arthritis [6]. *O. canum* belongs to the family Lamiaceae and is traditionally used in West Africa for the treatment of hyperglycemia [7]. Aqueous leaf extract of this plant was reported as having anti-hyperglycemic property [8]. The aqueous extract of both fresh and dried leaves of *O. canum* lowers fasting blood glucose levels as well as the body weight of genetically diabetic mice [8].

Earlier reports suggest that the presence of phenolic acids like rosmarinic acid might be responsible for the antihyperglycemic property of this plant [9]. Scarpatti and G Oriente (1958) have shown that RA is a polyphenol which is ubiquitously present in species of the Boraginaceae, the subfamily Nepetoideae of the family of Lamiaceae [10].

RA is also reported to be present in *O. canum* and several other Lamiaceae members [9]. UA is another important polyphenol reported from genus *Ocimum* exhibiting a wide range of pharmaceutical properties [11].

In the past few decades, substantial research work has been devoted to understanding the biological activities of phytochemicals. None of the research, however, fully demonstrates the mechanism which can be utilized to manage diabetes and associated complications. Moreover, not much focus was given to identifying exactly which constituents present in this plant aids in treating hyperglycemia. There are very few reports which concentrated on the effects of RA and UA on the α-glucosidase activity. Therefore, the present study aims to analyze the inhibitory effect of the leaf extracts of *O. canum* on the carbohydrate metabolizing enzyme and examines its correlation with the presence of polyphenolic compounds.

MATERIALS AND METHODS

Collection of plant material

Seeds of *O. canum* were collected, identified and authenticated from the Institute of Integrated Ayurveda and Medicine, Yelhanka (13.1005 °N and 77.5940 °E), Bangalore. The authenticated voucher specimen of *O. canum* (voucher number: BUB/MB-BT/KM/*O. c*/01) was deposited in the Department of Microbiology and Biotechnology, Bangalore University, Bangalore.

Plants were maintained for three months and leaves were harvested at full bloom. Leaves were weighed and air dried in paper bags at room temperature for 15 d.

Preparation of leaf extract

The dried leaf powder of *O. canum* 50g was extracted with 200 ml of methanol 95% v/v by a Soxhlet apparatus at 50 °C temperature for 36 h. The process was repeated twice and the extract obtained was filtered and evaporated to dryness by a rotary evaporator (Superfit, R/150/22, India) at 40 °C [12]. The dried extract was weighed and kept at -20°C for further analysis.

Chemicals used

Dipotassium hydrogen phosphate and potassium dihydrogen phosphate for preparing phosphate buffer were purchased from Merck Millipore, Germany. Alpha-glucosidase and alpha-D gluco-pyranoside (PNPG) was purchased from SRL, Mumbai. Sodium carbonate, methanol, toluene, ethyl acetate, formic acid, tetra-hydrofuran, orthophosphoric acid and dimethyl sulphoxide (DMSO) were purchased from Himedia, Bengaluru. Silica Gel (GF-254 8x5) was purchased from Merck. Standard rosmaranic acid and ursolic acid were purchased from Sigma-Aldrich.

Thin layer chromatography

A hundred microlitre of the crude methanolic extract was applied on silica gel GF-254(Merck, 8x5 cm) plates. Toluene: ethyl acetate: formic acid (7:2:1v/v) was used as a developing solvent system. TLC bands were visualized under visible and UV light and retention factor (Rf) values were calculated. Each TLC band (1 mg) was collected and dissolved in 10% of dimethyl sulphoxide (HPLC grade), filtered and the samples were stored separately. Different concentrations of extract such as 50, 100, 150 and 200 µg/ml were prepared for the assessment of the inhibitory effect on α-glucosidase activity.

Alpha-glucosidase inhibitory assay

The α-glucosidase inhibitory activity was assayed as per the procedure of Kim *et al.* (2005) [13]. Alpha-D glucopyranoside (PNPG) in 0.1 M potassium phosphate buffer (pH 6.9) was used as a substrate. The enzyme solution (0.1 U/ml) was prepared in 0.01 M phosphate buffer (pH 6.0). The reaction mixture consisted of phosphate buffer, 0.25 ml of 0.5 mmol 4-nitrophenyl α-D-glucopyranoside (PNPG), 0.1 ml α-glucosidase, and 100 µl of the sample. The reaction was carried out at 37 °C for 20 min and was stopped by adding 0.1 M sodium carbonate solution. The enzyme activity was monitored using spectrophotometer (UV-1800 Shimadzu) at 400 nm by the estimation of p-nitrophenol released. All experiments were carried out in triplicate and the inhibitory effect of the extract on α-glucosidase was assessed and calculated as percent inhibition.

HPLC of the bioactive bands

HPLC was performed on bioactive bands and the system used was SPD-10Avp/10vvP (Shimadzu, Japan). It was carried out using a C18 column (250x4.6 mm, 5 µm) as the stationary phase and methanol: tetrahydrofuran: 0.1% orthophosphoric acid (55:5:40 v/v) as the mobile phase at a flow rate 0.7 ml/min and a pressure of 139 kgF/cm at a temperature of 40 °C. The TLC bands were sonicated and filtered through syringe filter before use. RA (≥ 98% HPLC Sigma-Aldrich) and UA (≥ 90% HPLC Sigma-Aldrich) used as a standard during HPLC analysis of polyphenolic compounds. The injection volume was 20 µl (1 mg/ml in methanol) and elutions were detected at 210 and 330 nm for RA and UA respectively [14]. Compounds were identified by comparing the retention time and absorption spectra with their standards.

Mass spectrometer analysis of the bioactive bands

Mass spectrometry was performed using a micromass Q-Tof-micro ™(Waters) operating in positive ion electrospray mode. The general conditions were the source temperature of 373 K, capillary voltage 2.5 kV, cone voltage of 45V, syringe rate was 50 ul and the sample volume was 20 µl. Mass spectra were acquired and accumulated over 60 s and the spectra were scanned in the range of 100 to 1200 m/z. The ions of interest (on the basis of m/z) were selected and deposited for collision with argon in the collision quadrupole. Collision gas pressure was tuned to generate extensive fragmentation of the ions under observation.

RESULTS

The inhibitory effect of the methanolic leaf extract of *O. canum* on α-glucosidase activity and its relation to the polyphenol compounds present in the extract was analysed and the results are presented in fig. and tables.

Thin layer chromatography

Fig. 1 shows the TLC plate of the methanolic leaf extract. Among the different combinations of nonpolar solvents used, toluene: ethyl acetate: formic acid (7:3:1) combination showed the best separation of compounds. Five clearly separated bands were obtained. The Rf values of band A, B, C, D and E were found to be 0.5, 0.68, 0.73, 0.78 and 0.85 respectively.

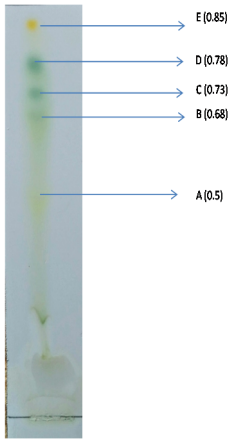

Fig. 1: TLC of methanolic extract of *Ocimum canum*, with the solvent system, toluene: ethyl acetate and formic acid (7:2:1)

Effect of *Ocimum canum* extract on α-glucosidase activity

The α-glucosidase activity was found to be inhibited by the leaf extracts on a dose-dependent manner with a correlation of r^2= 0.929, (fig. 2). TLC band B displayed the maximum inhibition of α-glucosidase activity followed by bands A, C and D, whereas band E did not show any inhibitory effect on the α-glucosidase activity (fig. 2). The IC_{50} of band B was found to be 159.21 µg, followed by bands A, C and D which were found to be 191.53 µg, 430.03 µg and 498.00 µg respectively. The IC_{50} values of different TLC bands are presented in table 1.

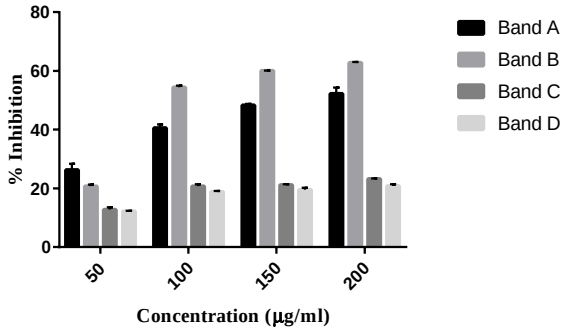

Fig. 2: Inhibitory activity of *Ocimum canum* leaf extract (TLC bands A, B, C and D) against the α-glucosidase activity. Results are mean±SD-standard deviation, (n=3)

Table 1: IC_{50} value of different TLC bands of *Ocimum canum* leaf extract

S. No.	Leaf extract	IC_{50}
1	Band A	191.53 µg
2	Band B	159.21 µg
3	Band C	430.03 µg
4	Band D	498.00 µg

HPLC analysis of bioactive TLC bands

Bioactive compounds of TLC bands A and B were confirmed by HPLC and results were presented in fig. 3, fig. 4, fig. 5, fig. 6, fig. 7 and fig. 8. HPLC analysis of the TLC bands showed the presence of RA and UA together with several other compounds. The RA was detected at 330 nm after 5.45 min (fig. 3-5) which has an absorbance maximum at 330 nm and a minimum at 265 nm. The percent yield of RA in the TLC bands A and B were found to be 0.067 and 0.079 respectively (table 2). The UA was detected at 210 nm after 12.55 min as shown in (fig. 6-8). The percent yield of UA in the TLC bands A and B were found to be 0.122 and 0.160 respectively (table 3).

Mass spectrometer analysis of bioactive TLC bands

TLC bands A and B were further characterized by mass spectrometer and results (mass spectrum) were presented in fig. 9 and fig. 10. Most of the compounds were common in both the bands but percentage availability of these compounds was different. In TLC band A predominant organic acids and phenolic compounds were observed as tartaric acid of m/z 149, the caffeic acid of m/z 179, quercetin of m/z 305 and RA of m/z 360 (fig. 9). In TLC band B the compounds were found to be the tartaric acid of m/z 149, the caffeic acid of m/z 179, quercetin of m/z 305, RA of m/z 360 RA of m/z 361 (fig. 10). The percentage availability of quercetin (69 %) and RA (55%) in band B was found to be significantly higher than band A.

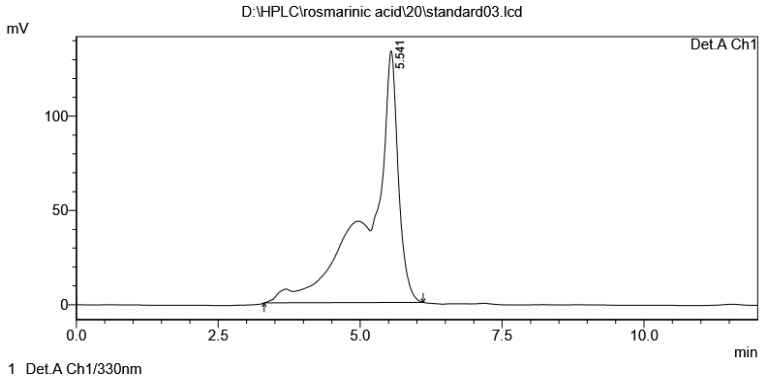

Fig. 3: HPLC chromatogram of rosmarinic acid (RA) standard solution

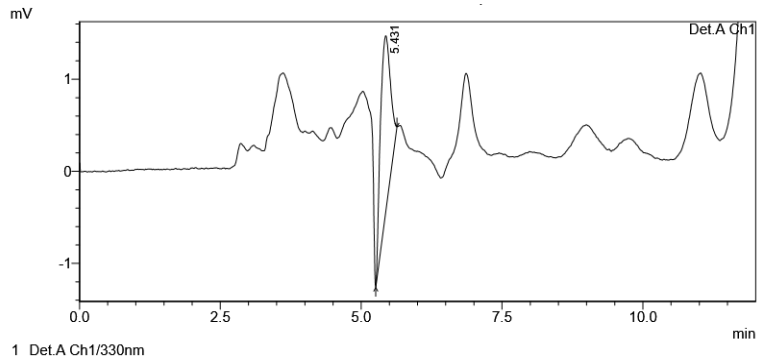

Fig. 4: HPLC chromatogram of rosmarinic acid (RA) present in the TLC band A of methanolic extract of *Ocimum canum*

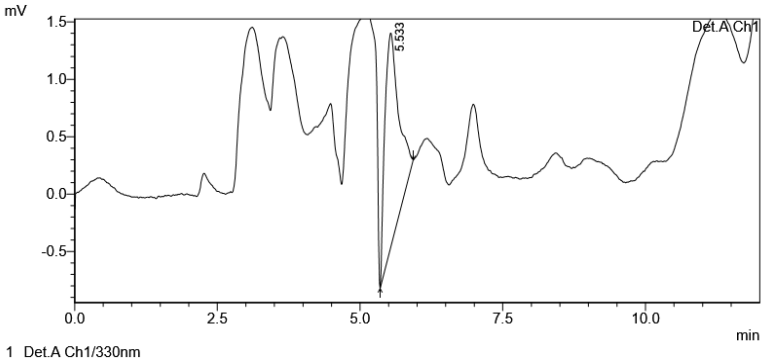

Fig. 5: HPLC chromatogram of rosmarinic acid (RA) present in the TLC band B of methanolic extract of *Ocimum canum*

Table 2: Quantification of rosmarinic acid (RA) present in the TLC bands of methanolic extract of *Ocimum canum*

S. No.	Compounds	Conc (mg/ml)	Ret. time (min)	% yield
1.	RA (std.)	0.10	5.541	98
2.	Band A	10.00	5.431	0.067
3.	Band B	10.00	5.531	0.079

Conc-concentration, Ret. time-retention time, Std-standard

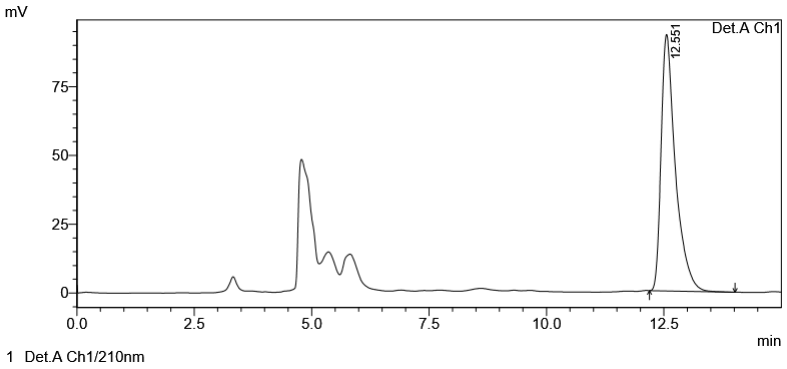

Fig. 6: HPLC chromatogram of ursolic acid (UA) standard solution

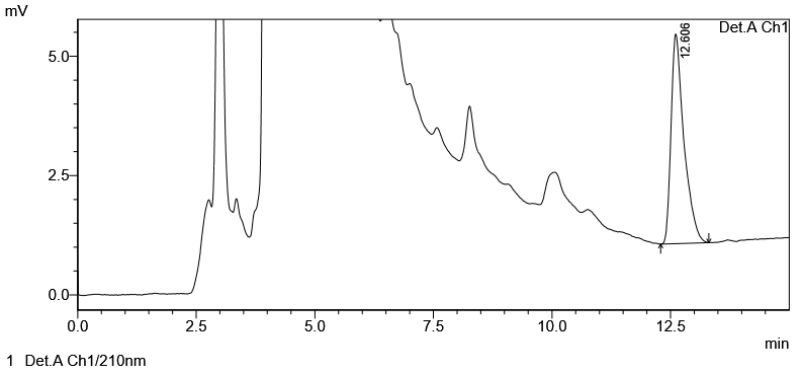

Fig. 7: HPLC chromatogram of ursolic acid (UA) present in the TLC band A of methanolic extract of *Ocimum canum*

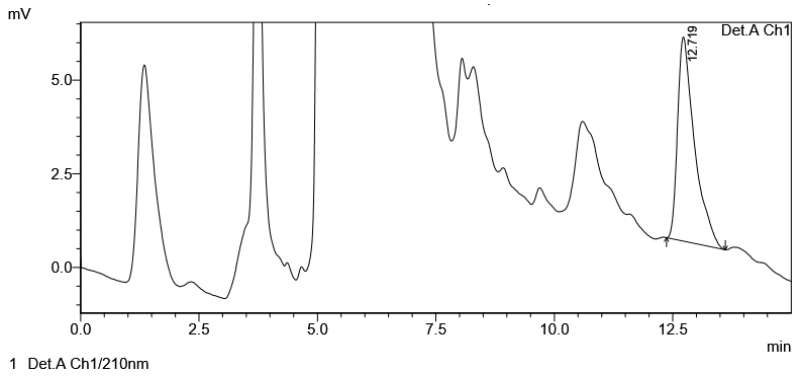

Fig. 8: HPLC chromatogram of ursolic acid (UA) present in the TLC band B of methanolic extract of *Ocimum canum*

Table 3: Quantification of ursolic acid (UA) present in the TLC bands of methanolic extract of *Ocimum canum*

S. No.	Compounds	Conc (mg/ml)	Ret time (min)	% yield
1.	UA (std.)	0.10	12.55	95
2.	Band A	10.00	12.60	0.122
3.	Band B	10.00	12.71	0.160

Conc-concentration, Ret. time-retention time, Std-standard

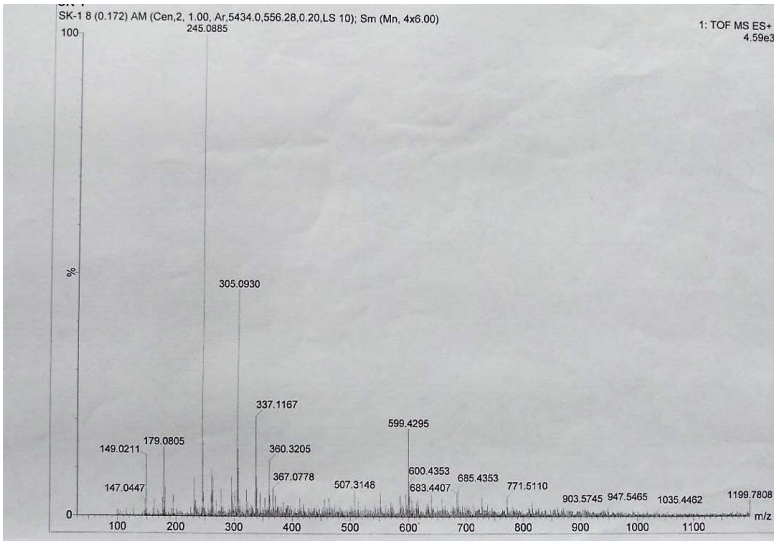

Fig. 9: Mass spectrum of TLC band A of methanolic extract of *Ocimum canum*

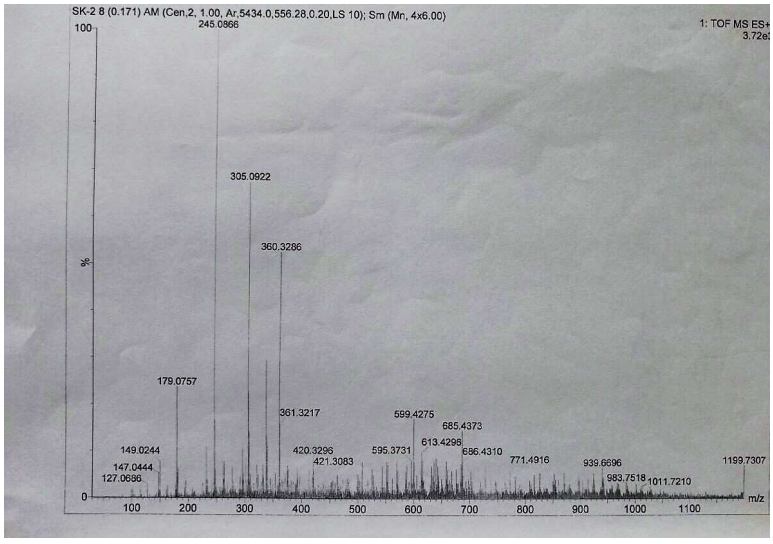

Fig. 10: Mass spectrum of TLC band B of methanolic extract of *Ocimum canum*

DISCUSSION

Several plants have been shown to impart antihyperglycemic effect through the inhibition of carbohydrate-hydrolyzing enzymes in the small intestine [15-18]. It is evident from the present results that the methanolic leaf extract of *O. canum* inhibited α-glucosidase activity *in vitro* which is in agreement with the earlier reports [15-18]. As illustrated in the results the activity of α-glucosidase decreased with increased concentrations of the extract. TLC bands A and B of the leaf extract showed maximum enzyme inhibition when compared to bands C and D. Therefore, A and B bands were further subjected to HPLC to analyze the bioactive compounds. The yield percentage of RA obtained by HPLC method, and the availability percentage obtained by mass spectrometry in TLC band B was found to be higher with maximum inhibitory activity on α–glucosidase when compared to band A. This may be due to the fact that RA might be the lead compound that produces the enzyme inhibitory action. Further, the plant polyphenol reduces oxidative stress as well as inhibits carbohydrate hydrolyzing enzyme to control hyperglycemia [19, 20]. The RA being a derivative of polyphenol might also act as a strong antioxidant [21]. Previous studies revealed the therapeutic properties of RA, which mainly attributed to its potent antioxidative effects [22]. Initial studies have shown that RA controls plasma glucose by modulating SGLT1 trafficking to the intestinal brush border membrane in diabetic animals [23]. Antioxidant supplementation has been shown to improve the treatment of chronic diabetes. Consistent hyperglycemia and elevated levels of free fatty acids contribute to oxidative stress and cause progression of diabetes [24]. Therefore, antioxidants are frequently included in complementary therapeutics. The UA was detected as another bioactive compound in the leaf extract of *O. canum,* which is in concordance with the earlier reports [25, 26]. Silva *et al.,*(2008) [27] have shown that UA is detectably present in *Ocimum basilicum,* which is a triterpenoid and has been described as an anti-inflammatory, hepatoprotective, antitumoral and antidiabetic agent [28]. Further, UA has been reported as an uncompetitive inhibitor of α-glucosidase [29]. It also stimulates glucose uptake through a cross-talk between different signalling pathways, linking the P13K and MAPK pathways with Ca++-CaMKII network in translocation and GLUT4 expression in skeletal muscle [30].

The mass spectrometer method is a sensitive and selective way to identify polar organic compounds with acidic sites such as phenolic, organic acids and flavonoids [31]. Therefore, this method has been used to identify some of the important polyphenolic compounds present in *O. canum* methanolic extract. Hence, the inhibition of α-glucosidase by *O. canum* extract may be presumed as a synergistic effect of RA, UA and other polyphenolic compounds. In addition, the

antioxidant property of RA and UA along with their α-glucosidase inhibitory property, these bioactive compounds might serve as potential candidates for the treatment of hyperglycemia.

CONCLUSION

Current studies provide an experimental justification to the traditional use of *O. canum* for the management of hyperglycemia and provide a preliminary understanding of the possible mechanism through which this plant may aid to overcome diabetes and associated complications. TLC bands A and B of the *O. canum* leaf extract showed significant inhibition of α-glucosidase and two important phenolic acids RA and UA were identified in these bioactive bands. Although the same compounds have been detected in both the bands but yield percentage and the availability percentage of RA in TLC band B was found to be higher, with maximum inhibitory activity on α–glucosidase. Therefore, band B could be the better choice to develop a therapeutic strategy for the treatment of hyperglycemia.

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AUTHORS CONTRIBUTIONS

The study concept was designed by Dr. K. Manjunath. Data acquisition, data analysis, and manuscript preparation were done by Kumari Smita. Final approval and overall checking were done by Dr. S. K. Sarangi.

CONFLICT OF INTERESTS

Declared none

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