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Bioactive compounds from marigold processing waste: Extraction, isolation, and antidiabetic activity

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ABSTRACT

Diabetes is a chronic metabolic disorder characterized by excessive blood sugar levels. According to the International Diabetes Federation, 537 million adults are living with diabetes today and is projected to rise to 643 million by 2030 and 783 million by 2045. Marigold or *Tagetes erecta* Linn. is a common plant used in diverse cultures in India. Different parts of this plant are used in folk medicine to treat colic, diarrhea, vomiting, fever, skin diseases, and hepatic disorders. During marigold cultivation, the leaves are the most common agricultural waste. In the present study, we aim to evaluate the *in-vitro* antidiabetic activity of hydroalcoholic extract of leaves of marigold and the isolation and characterization of active constituents. The hydroalcoholic extract was fractionated by column chromatography using different stationary phases and solvents ranging from highly non-polar to polar as mobile phase by gradient elution method. *In-vitro* alpha-glucosidase inhibition of the hydroalcoholic extract and isolated compounds was performed and compared with the standard drug acarbose. The isolated compounds were tentatively characterized as kaempferol di-O-rhamnoside (compound 1), kaempferol O-rhamnoside-O-pentoside (compound 2), and kaempferol O-rhamnoside (compound 3). The isolated compounds were more active than the hydroalcoholic extract. Kaempferol O-rhamnoside was found to possess the highest antidiabetic activity with an IC_{50} value of 61 µg/ml.

INTRODUCTION

Tagetes erecta Linn. (African marigold) is a common ornamental plant (Madanan *et al*., 2021), belongs to the Asteraceae family native to Mexico, Southwestern United States, South America, Africa, and India (Tripathi *et al*., 2012). The plant is slightly coarse, erect, and branched up to 1-m in height. Flowers are large, globe-shaped, bright yellow, brownish yellow, or orange, and deeply incised and leaves are sharply toothed (Priyanka *et al*., 2013; Singh *et al*., 2020). Traditionally leaves are effective against piles, kidney troubles, muscular pain, ulcers, wounds, boils, and carbuncles (Kiranmai *et al*., 2011). Flowers are used to curing fever, epileptic fits, liver complaints, eye diseases, bleeding piles, rheumatism, cold, and bronchitis (Karwani and Sisodia, 2015; Nikkon *et al*., 2009). The plant was reported as having antibacterial, antimicrobial, antioxidant, hepatoprotective, insecticidal, mosquitocidal, nematicidal, wound healing, antioxidant, analgesic, and larvicidal properties (Gopi *et al*., 2012). They were found to be a rich source of flavonoids, xanthophylls, glycosides, and saponins. The major constituents reported from the plant are (Phrutivorapongkul *et al*., 2013), patulitrin (Rhama and Madhavan, 2011), β-sitosterol, erethrodiol-3-palmitate, α-tertheenyl, lupeol, β-daucosterol, erethrodiol, erethrodiol-3 palmitae, kaempferol, palmitin, quercetagetin, and

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quercetaetin-7-methyl ether (Xu *et al*., 2011). Marigold petals are the main industrial source of lutein, which is used to color foods (Kashyap *et al*., 2022).

Diabetes is a chronic metabolic disorder characterized by excessive blood sugar levels. According to the International Diabetes Federation, 537 million adults are living with diabetes today and is projected to rise to 643 million by 2030 and 783 million by 2045 (Elyamani *et al*., 2021; Sun *et al*., 2022). The α-glucosidase is an enzyme located in the brush border of the enterocytes of the jejunum, and its function is to cleave oligosaccharides and disaccharides into monosaccharides for better absorption (Akmal and Wadhwa, 2022). The inhibition of this enzyme results in the reduction in postprandial blood glucose levels (Bu *et al*., 2010). However, there is no systematic study reported on the fractionation and isolation of antidiabetic principles from the leaves of *T. erecta*. Hence, in the present study, it was proposed to evaluate leaves of *T. erecta* to isolate active constituents and compare the activity of the hydroalcoholic extract, and isolated compounds by *in-vitro* α-glucosidase enzyme inhibitory model.

MATERIALS AND METHODS

Identification and extraction

Leaves of *T. erecta* L. were obtained from Mysuru, Karnataka, authenticated, and the voucher specimen of the same was deposited at National Ayurveda Dietetics Research Institute, Bangalore (Voucher no: RRCBI-MUS-138). Extraction was carried out by the reflux method. 500 g of the dried, powdered leaves were extracted with hydro alcohol (1:8 drug to solvent ratio), the extract was concentrated in a rotary evaporator and dried using a vacuum tray drier to obtain powder and was stored in polythene bags until analysis.

Chromatographic separation and isolation

The hydroalcoholic extract was subjected to fractionation by silica gel (60–120 mesh) column chromatography (column 1), and elution was started with hexane, ethyl acetate (EA), and methanol. Eleven fractions were collected, based on the thin layer chromatography (TLC) pattern [n-Butanol: Glacial acetic acid: Water (16:4:20)], five fractions (5%, 25%, 50%, 75% methanol in EA, and 100% methanol) were combined, subjected to further column chromatography (column 2), and performed the same above-mentioned procedure. From column 2, 12 fractions were collected and 5% methanol in EA, 5 subfractions were collected. First and second subfractions yielded crude crystals with impurities, and TLC showed two bands, so these fractions were combined and subjected to further silica gel (300–400 mesh) column chromatography (column 3). Elution has started with hexane, chloroform, EA, and methanol. Twenty-one fractions were collected, from 20% EA in chloroform, four subfractions were collected, first and second fractions got purified spots in TLC, so these fractions were combined (compound 3).

From column 1, 25% of methanol in EA, the major part, was utilized for column 2, green to white specs were observed in the sides of the test tubes, and acetone was added to obtain precipitate (compound 1). From column 2, two subfractions 5% and 7.5% of methanol in EA were separately concentrated, acetone was added to each fraction, and a precipitate was obtained and showed a similar band pattern in TLC. Thus, these compounds were combined to obtain compound 2.

Characterization

The isolated compounds were characterized using ultra violet (UV), TLC, Fourier transform infrared (FTIR), and mass spectrophotometer. The MALDI—Mass spectrophotometer data were collected utilizing a 25 kV accelerating voltage, a 90 ns time delay, and a 50 Hz pulsed nitrogen laser (λ = 337 nm) on an Ultra-flex TOF/TOF spectrometer (Bruker Daltonics, Bremen, Germany, and Billerica, Massachusetts, USA). TLC of all three compounds showed a single spot (Fig. 14); therefore, all three compounds were subjected to the high-performance liquid chromatography (HPLC) analysis for assessing their purity. A new HPLC method was developed and standardized in the laboratory using a Shimadzu HPLC instrument (Scientific Instruments, Kyoto, Japan), with the following conditions:

- Column: Shim-pack C18 (250 × 4.6 mm, 5 microns),
- Mobile phase: Acetonitrile in phosphate buffer, pH:3.5,
- Elution: Gradient elution,
- Flow rate: 1.5 ml/minute,
- Detector: Photodiode array detector, and
- Injection volume: 20 µl.

Sample preparation

2 mg of each sample was dissolved in 5 ml of methanol with the help of sonication for 2 minutes. The mobile phase was prepared by mixing HPLC grade acetonitrile with phosphate buffer of pH 3.5 by adding 0.136 g of anhydrous potassium dihydrogen orthophosphate (KH_2PO_4) in 900 ml of HPLC grade water (obtained from Millipore, Milli-Q Water purification system) and 0.5 ml of orthophosphoric acid and volume was made up to 1,000 ml with water, filtered through 0.45 µ membrane and degassed in a sonicator for 3 minutes (Table 1).

*Compound **1** (From column 1)*

From column 1, 25% of methanol in EA, the major part, was utilized for column 2, green to white specs were observed in the sides of the test tubes, acetone was added, and a precipitate was obtained; light green, crystalline powder; the yield was 94.5 mg; melting point or mp 201°C–205°C; HPLC three peaks were observed. The retention time of one major peak was 11.432 minutes with 77% area and two minor peaks were 11.297 and 11.668 minutes with 13.3% and 8% areas, respectively. UV-Vis (λ_{max}, ethanol): 263, 344 nm; FTIR (KBr, cm^{-1}): 3,352.39 (–OH), 2,980.12 (–CH aromatic), 2,926.11 (–CH aliphatic), 1,602.90, 1,492.95 (C=C

Table 1. The gradient elution conditions.

Time (minute)	Buffer concentration (Solvent A)	Acetonitrile concentration (Solvent B)
0.01	95	5
18	55	45
25	20	80
28	20	80
35	55	45
40	95	5
45	95	5

aromatic), 1,656.91 (>C=O) 1,352.14 (C–O–C); MS m/z 577.27, two MS2 fragment ions at *m/z* 431 (M-146)-, *m/z* 283 (M-146-146-2H)-.

*Compound **2** (From column 2)*

From column 2, two subfractions 5% and 7.5% of methanol in EA were separately concentrated, acetone was added to each fraction, and a precipitate was obtained, which showed a similar band pattern in TLC and similar peaks in HPLC. Thus, these compounds were combined. Light green crystalline powder; the yield was 240 mg; mp 240°C–245°C; HPLC three peaks were observed. One major peak was seen at 16.109 minutes and two insignificant minor peaks. UV-Vis (λ_{max}, ethanol): 265, 347 nm; FTIR (KBr, cm^{-1}): 3,419.90 (–OH), 3,976.26 (–CH aromatic), 2,924.18 (–CH aliphatic), 1,597.11 (C=C aromatic), 1,658.91 (>C=C), 1,346.36 (C–O–C); MS *m/z* 563 two MS2 fragment ions at *m/z* 431 (M-132)-, *m/z* 285 (M-132-146)-.

*Compound **3** (From column 3)*

From column 3, 20% EA in chloroform, four subfractions were collected, first and second fractions got purified spots in TLC, so these fractions combined. Pale yellow semi-solid; the yield was 700 mg; HPLC chromatogram showed a single peak with 97% purity with a retention time of 16.559 minutes mp 150°C–155°C; UV-Vis (λ_{max}, ethanol): 264, 363 nm; FTIR (KBr, cm^{-1}): 3,473.91 (–OH), 3,093.92 (–CH aromatic), 2,929.97 (–CH aliphatic), 1,554.68 (C=C aromatic) 1,668.48 (>C=O), 1,176.62 (C–O–C). MS *m/z* 431, MS2 fragment at *m/z* 285 (M-146)-.

Alpha-glucosidase inhibitory assay

The inhibition of α-glucosidase activity was assessed using the spectrophotometric microplate reader. 10 µl of α-glucosidase enzyme (1 U/ml) was pre-incubated with different concentrations of the test material (100, 150, 200, and 400 µg/ml) at 37°C and allowed to react with PNPG (20 µl, 5 mM). The reaction was terminated by adding sodium carbonate solution (50 µl, 0.1 M), and p-nitrophenol released in the reaction mixture was monitored at 410 nm compared with the control, and acarbose was used as a standard. Acarbose is the most widely prescribed α-glucosidase inhibitor. The experiment was conducted in triplicates and percentage inhibition was calculated (Telagari and Hullatti, 2015).

RESULTS AND DISCUSSION

Isolation and characterization

The hydroalcoholic extract was fractionated by column chromatography using different stationary phases and solvents ranging from highly non-polar to polar as mobile phase by gradient elution method. The isolated kaempferol di-O-rhamnoside (**1**) was a light green, crystalline powder with mp 201°C–205°C. In HPLC, three peaks were observed. The retention time of one major peak was 11.432 minutes (77% area) and two minor peaks was 11.297 and 11.668 minutes (13.3% and 8% areas, respectively) (Fig. 2). From UV-Vis, major peaks at 263 and 344 nm (Fig. 5) indicate the **1** to be flavonol derivatives (Negri *et al*., 2013). TLC (n-butanol: glacial acetic acid: water-16:4:20) showed a single black to greenish-brown spot with ferric chloride reagent indicating a pure, single compound and polyphenolic in nature (Forsyth, 1955) (Fig. 14). FTIR showed prominent peaks at frequencies, 3,352.39 cm^{-1} for the –OH stretching. The –CH stretching of aromatic nature was interpreted by a peak at 2,980.12 and 2,926.11 cm^{-1} indicating the presence of –CH aliphatic stretching, 1,602.90 and 1,492.95 cm^{-1} representing the C=C aromatic stretching. The additional peaks at 1,656.91 cm^{-1} correspond to an ethylenic double bond and C–O–C stretching (Fig. 8). Negative electrospray ionization mass spectrometry (ESI-MS) spectra showed molecular ion peak at m/z 577.27 that generated two MS2 fragment ions at *m/z* 431 (M-146), loss of a rhamnose moiety; and at *m/z* 283 (M-146-146-2H)-, loss of two rhamnose moieties (Barros *et al*., 2011). The position of substitution of the sugar residues could not be concluded and the fragmentation pattern was similar to flavonoid di-O-glycosides (Fig. 11). The result indicates that **1** could be kaempferol di-O-rhamnoside (**1)** (Fig. 1). The isolated kaempferol O-rhamnoside-O-pentoside (**2**) was light green crystalline powder with mp 240°C–245°C. HPLC, one major peak was at 16.109 minutes and two insignificant minor peaks (Fig. 3). UV-Vis major peaks at 265 and 347 nm indicate **2** to be flavonol derivatives (Negri *et al*., 2013) (Fig. 6). TLC showed similar features to **1** and confirms it as polyphenolic (Forsyth, 1955) (Fig. 14). FTIR showed prominent peaks at frequency 3,419.90 cm^{-1} representing –OH stretching. The peak at 3,976.26 cm^{-1} accounted for –CH aromatic stretching and 2,924.18 cm^{-1} for –CH aliphatic stretching. 1,597.11 cm^{-1} corresponds to C=C aromatic stretching, 1,658.91 and 1,346.36 cm^{-1} indicated C=C and C–O–C groups, respectively (Fig. 9). The

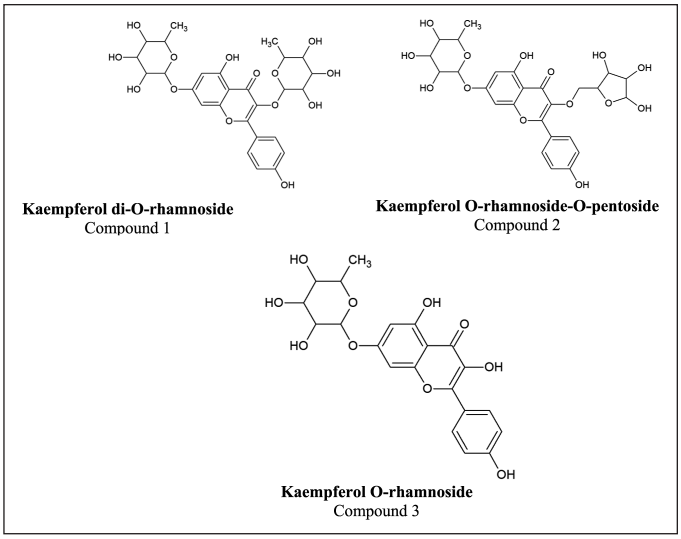

Figure 1. Chemical structure of isolated compounds.

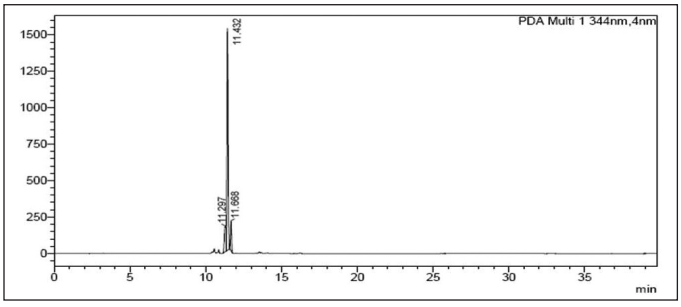

Figure 2. HPLC chromatogram of isolated kaempferol di-O-rhamnoside (1).

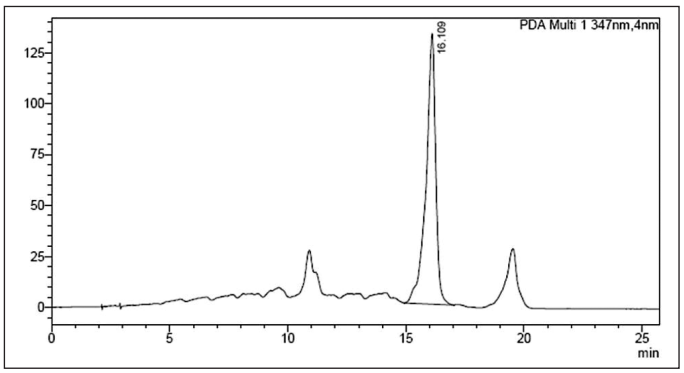

Figure 3. HPLC chromatogram of isolated kaempferol O-rhamnoside-O-pentoside (2).

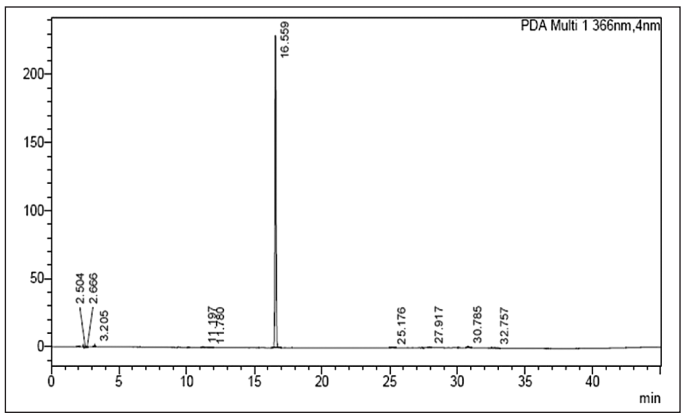

Figure 4. HPLC chromatogram of isolated kaempferol O-rhamnoside (3).

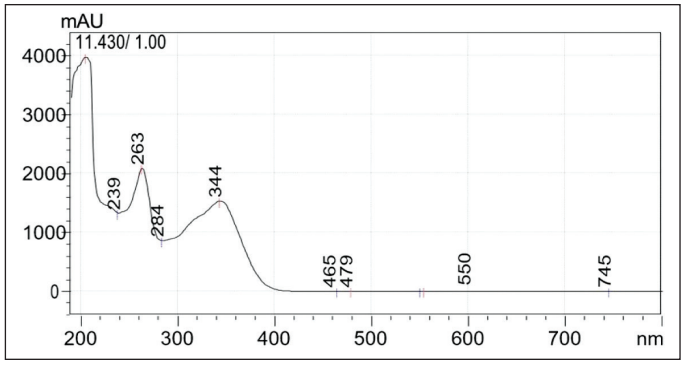

Figure 5. UV-Vis spectra of isolated kaempferol di-O-rhamnoside (1).

negative ESI-MS spectrum showed a molecular ion peak at (M–H)—at *m/z* 563. Its MS2 gave two fragment ions, a major fragment at *m/z* 431 (M-132)-, loss of a pentose moiety, a minor one at *m/z* 285 (M-132-146)-, loss of rhamnosyl-pentoside moiety (Barros *et al*., 2011) (Fig. 12), and relative abundances of the two fragment ions suggested that they were located at different positions on the aglycone, although it was also not possible to conclude about the precise position. The compound was tentatively assigned as kaempferol O-rhamnoside-O-pentoside (**2**) (Fig. 1). The isolated kaempferol O-rhamnoside **(3)** was a pale-yellow semi solid with mp 150°C–155°C. HPLC chromatogram showed a single

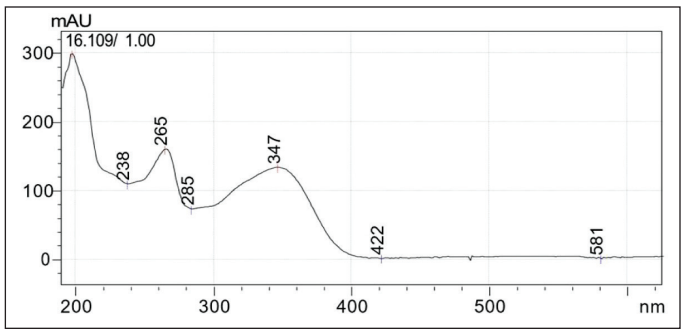

Figure 6. The UV-Vis spectra of isolated kaempferol O-rhamnoside-O-pentoside (2).

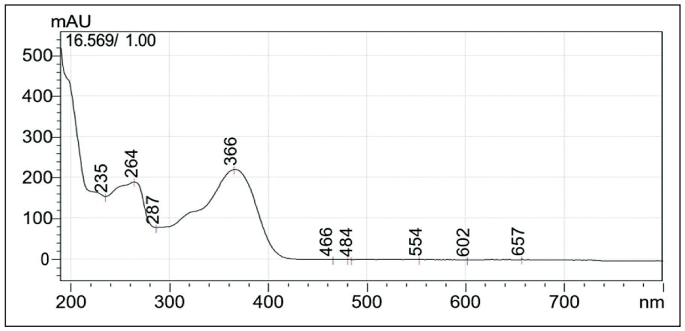

Figure 7. UV-Vis spectra of isolated kaempferol O-rhamnoside (3).

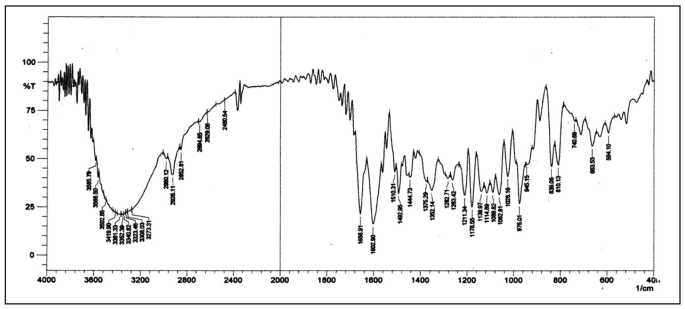

Figure 8. FTIR spectra of isolated kaempferol di-O-rhamnoside (1).

peak with 97% purity with a retention time of 16.559 minutes (Fig. 4). UV showed two peaks at 264 and 363 nm indicating the presence of flavonol derivatives (Negri *et al*., 2013) (Fig. 7). TLC showed similar features to **1** and confirms it as polyphenolic (Forsyth, 1955) (Fig. 14). FTIR showed prominent peaks at 3,473.91 cm^{-1} for –OH stretching and 3,093.92 cm^{-1} for –CH aromatic stretching. –CH aliphatic represents 2,929.97 cm^{-1} and C=C aromatic stretching at 1,554.68 cm^{-1}. Ethylenic double bond at 1,668.48 and 1,176.62 cm^{-1} for C–O–C stretching (Fig. 10). Negative ESI-MS of **3** showed a molecular ion peak (M–H) - at *m/z* 431. Its MS2 gave a major fragment at *m/z* 285 (M-146)-, loss of a rhamnose moiety (Zhao *et al*., 2014) (Fig. 13). Relative abundances of the two fragment ions suggested that they were located at different positions on the aglycone, although it was also not possible to conclude the precise position. Thus, the compound was tentatively assigned as kaempferol O-rhamnoside (**3**) (Fig. 1).

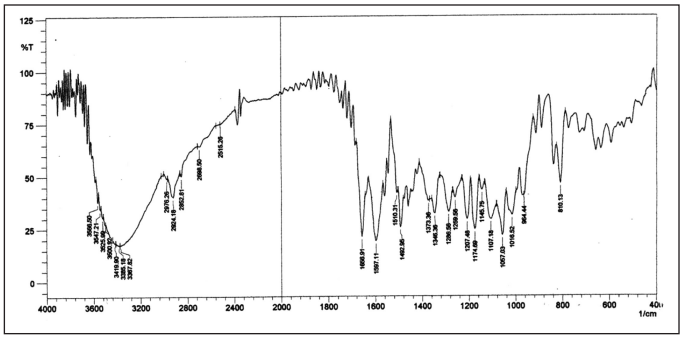

Figure 9. FTIR spectra of isolated kaempferol O-rhamnoside-O-pentoside (2).

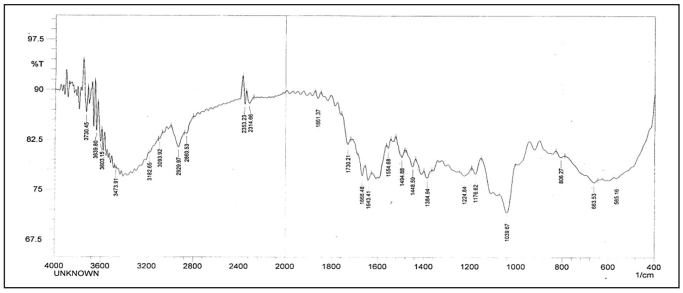

Figure 10. The FTIR spectra of isolated kaempferol O-rhamnoside (3).

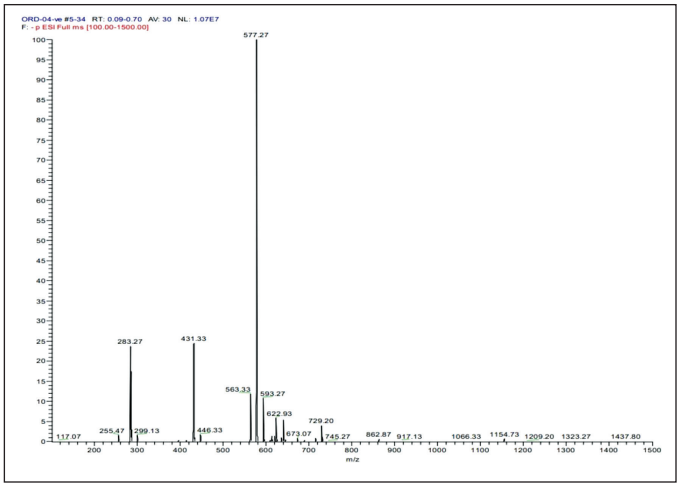

Figure 11. MS spectra of isolated kaempferol di-O-rhamnoside (1).

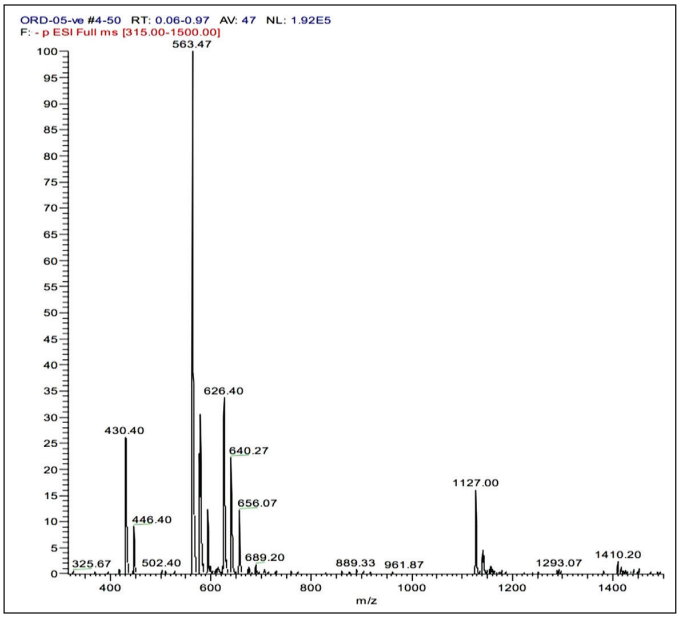

Figure 12. MS spectra of isolated kaempferol O-rhamnoside-O-pentoside (2).

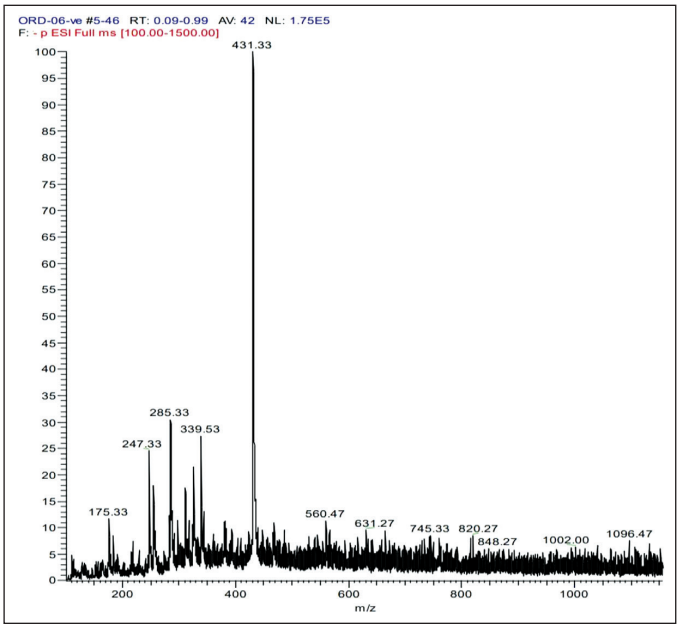

Figure 13. The MS spectra of isolated kaempferol O-rhamnoside (3).

Alpha-glucosidase enzyme inhibitory assay

The α-glucosidase enzyme inhibitory assay demonstrated that the hydroalcoholic extract is somewhat pharmacologically active and less powerful than isolated components in contrast to standard acarbose (Table 2). With complex combinations of natural products, less well-known phenomena termed antagonism; where the effects of active materials are hidden by those of other compounds. Removing these molecules is one of the key rationales stated for isolating active compounds in the conventional pharmaceutical development process (Caesar and Cech, 2019). The results showed α-glucosidase inhibitors from the leaves were kaempferol di-O-rhamnoside (**1**), kaempferol O-rhamnoside-O-pentoside (**2**), and kaempferol O-rhamnoside (**3**). Three compounds showed potent anti-α-glucosidase activity, kaempferol O-rhamnoside (IC_{50} value 61 µg/ml) was more active followed by kaempferol O-rhamnoside-O-pentoside (IC_{50} value 84.7 µg/ml) and kaempferol di-O-rhamnoside (IC_{50} value 118.81 µg/ml) (Table 2). Our present study revealed the leaves extract and isolated compounds along with the previously reported articles on the plant make the plant a good source of active antidiabetic agents.

Quercetagetin, the primary flavonoid in marigold inflorescence residue, was isolated and purified. Quercetagetin's *in-vitro* anti-diabetic efficacy was tested and compared to conventional quercetin and rutin. The IC_{50} values for quercetagetin on α-glucosidase and α-amylase were 180.11 ± 3.68 and

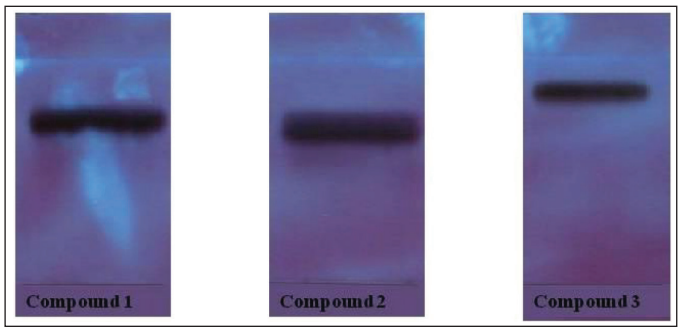

Figure 14. TLC of isolated compounds kaempferol di-O-rhamnoside (1), kaempferol O-rhamnoside-O-pentoside (2), and kaempferol O-rhamnoside (3).

Table 2. The α-glucosidase inhibitions by hydroalcoholic extract and isolated compounds.

Test agent	α-glucosidase inhibition (µg/ml)
Hydroalcoholic extract	240.2
Compound 1	118.81
Compound 2	84.71
Compound 3	61
Acarbose	50.90

137.71 ± 3.55 µmol/l, respectively (Wang *et al*., 2016). Lutein extract from marigold flowers can reduce blood glucose levels and works as an antioxidant in mice, as indicated by a drop in malondialdehyde levels (Kusmiati *et al*., 2019). Esterified lutein from *T. erecta* flower petals exhibits considerable antidiabetic action in alloxan-induced diabetic mice (Saigun and Sasidhar, 2019).

Kaempferol glycosides are reported as antidiabetic and radical scavengers (Adhikari-Devkota *et al*., 2019; Liu *et al*., 2018). Kaempferol di-O-rhamnoside (**2**) was reported as antioxidant and hypoglycemic (de Sousa *et al*., 2004). Kaempferol glycosides also reported a variety of biological activities both *in-vitro* and *in-vivo*. These include cytotoxic (Dimas *et al*., 2000), antinociceptive, anti-inflammatory (De Melo *et al*., 2009), antiviral (Mitrocotsa *et al*., 2000), antioxidant, antidiabetic, neuroprotective, anticancer, antimicrobial, cardioprotective, anti-osteoporotic, estrogenic/antiestrogenic, anxiolytic, analgesic, and antiallergic activities (Calderon-Montano *et al*., 2011).

CONCLUSION

The present study revealed that the hydroalcoholic extract of *T. erecta* leaves showed potent antidiabetic activity. On further fractionation of the hydroalcoholic extract, three active principal constituents were identified, namely kaempferol di-O-rhamnoside (**1**), kaempferol O-rhamnoside-O-pentoside (**2**), and kaempferol O-rhamnoside (**3**) which were not previously reported from this plant. All the compounds were tentatively characterized using UV, FTIR, and MS analyses. On the other hand, their purity was evaluated by HPLC and mp. This appears to be the first report on the comparative study of hydroalcoholic extract, and three isolated compounds using the *in-vitro* α-glucosidase inhibitory assay. The pharmacological activity of *T. erecta* leaf extract and column fractions could be due to the presence of kaempferol glycosides. Furthermore, based on the *in-vitro* results, the *in-vivo* evaluation will be carried out using the streptozotocin-induced diabetic rat model.

LIST OF ABBREVIATIONS

EA: Ethyl acetate; ESI-MS: electrospray ionization mass spectrometry; FTIR: fourier transform infrared; HPLC: High-performance liquid chromatography; TLC: Thin layer chromatography; UV: ultra violet.

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AUTHOR CONTRIBUTIONS

All authors agreed to submit the article to the current journal, gave final approval of the version to be published, made significant contributions to conception and design, data collection, analysis, and interpretation, participated in its writing or critically revised it for important intellectual content, and agreed to be responsible for all aspects of the work.

FUNDING

There is no funding to report.

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

There are no human or animal subjects in this study's experiments.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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