Original article

Chemical characterization, antidiabetic and anticancer activities of Santolina chamaecyparissus

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A B S T R A C T

Santolina chamaecyparissus is an important medicinal plant growing in the Mediterranean region and has been reported as a potent anti-inflammatory, antibacterial, antioxidant, and antifungal agent. The purpose of the current research is to identify the chemical constituents in ethyl acetate extract (EAE) from the leaves of S. chamaecyparissus, and to evaluate antidiabetic, and anticancer activity. Chemical constituents of EAE were identified by GC-MS, and the anticancer activity was evaluated by α-glucosidase inhibition assay. The anticancer activity was assessed by Epidermal Growth Factor Receptor (EGFR) expression in human breast cancer cell line (MCF7) by using quantitative RT-PCR method. GC-MS analysis of EAE of S. chamaecyparissus yielded 44 compounds. Tetrapentacontane (27.15%), eicosyl acetate (8.40%), 2-methylhexacosane (6.87%), and n-pentadecanol (5.44%) were found as major chemical constituents. The EAE of S. chamaecyparissus showed concentration dependent inhibition of α-glucosidase enzyme and the IC50 value (IC50 110 ± 4.25 μg/mL) was found comparable with standard acarbose (IC50 105 ± 3.74 μg/mL). The real-time qRT-PCR results showed that the EGFR protein (bcl-2) in human breast cancer cell line (MCF7) was negatively expressed with a value of –0.69297105 after treatment with EAE (100 μg/mL). The study results are suggesting the possible use of S. chamaecyparissus in the management of diabetes, and human breast cancer.

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1. Introduction

Medicinal plants are the major source of remedies for the treatment of chronic human ailments including diabetes, cancer, cardiovascular complications, etc. In recent years, natural products play a very important role in drug discovery for life-threatening diseases (Verpoorte, 2000). Natural drugs derived from medicinal plants are considered safe and effective compared to synthetic drugs from natural sources (Newman et al., 2003). Cur- rent therapy to alleviate cancer, and metabolic disorders such as diabetes mellitus, cardiovascular complications are not optimal and thus efforts have been made to develop effective and better drugs from natural sources (Newman et al., 2003).

Santolina (Asteraceae) species are found throughout the Mediterranean and European region. The most common species of the Santolina genus are S. viridis (South of France, and North of Spain), S. africana (Iberian Peninsula) and S. chamaecyparissus L. (Synonym: Ormenis fricana). Santolina chamaecyparissus has been reported as anti-inflammatory (Cuellar et al., 1998; Sala et al., 2000); antioxidant and antimicrobial (Nouasri et al., 2015; Djeddi et al., 2012); anticandidal (Suresh et al., 1997); antibacte- rial, and antifungal (Salah-Fatnassi et al., 2017); CNS depressant, and anti-cholinergic (Giner et al., 1988). Phytochemical investigations of essential oil of S. chamaecyparissus by GC-MS identified as vincristine, vinblastine, topotecan, irinotecan, docetaxel, pacli-taxel, etc. (Farnsworth et al., 1985). Even the discovery of approved and widely used anti-diabetic drug, metformin came from the traditional approach of using Galega officinalis (Grover et al., 2002). Current therapy to alleviate cancer, and metabolic disorders such as diabetes mellitus, cardiovascular complications are not optimal and thus efforts have been made to develop effective and better drugs from natural sources (Newman et al., 2003).

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several phytochemicals, the most abundant ones belong to mono and sesqui-terpenes, the major constituents include camphor, cubenol, \( \beta \)-cymene, sabine, 1,8-cineole, \( \alpha \)-phellandrene, \( \beta \)-eudesmol and terpinene-4-ol (Garg et al., 2001; Perez-Alonso and Velasco-Negueruela, 1992; Derbey et al., 1989). In the present research work, the chemical composition of ethyl acetate extract from leaves of \( S. \) chamaecyparissus was determined by GC-MS. The present study was also designed to evaluate \( S. \) chamaecyparissus extract efficacy in the treatment of diabetes mellitus via \( \alpha \)-glucosidase enzyme inhibition. Further, the anticancer activity was assessed by evaluation of EGFR protein expression using quantitative RT-PCR technique.

### 2. Materials and methods

#### 2.1. Plant materials and chemicals

The fresh \( S. \) chamaecyparissus L. leaves (500 g) were collected from the Northern region of Saudi Arabia. The authenticity of \( S. \) chamaecyparissus leaves (Voucher number: PL/2020-21/008) was ascertained by Pharmacognostic Dr. Abuzer Ali, College of Pharmacy, Taif University. Anticancer cell line study was carried out by Trichy Research Institute of Biotechnology Pvt. Ltd., Trichy, Tamil Nadu. The MCF-7 (human breast cancer) cell line was purchased from NCCS Pune, India. SUPER-SCRIPT\textsuperscript{TM}II RNase H- Reverse Transcriptase was received from Gibco (USA). Human EGFR and \( \beta \)-actin forward and reverse primers were purchased from Xcelris Pvt. Ltd. India (Table 1). The \( \alpha \)-glucosidase enzyme was brought from Subra Scientific Company Chennai, India.

#### 2.2. Preparation of ethyl acetate extract (EAE) of \( S. \) chamaecyparissus

The leaves of the \( S. \) chamaecyparissus were dried for one week in the shade. \( S. \) chamaecyparissus, leaves were pulverized into a coarse powder. Coarse powder (50 g) was extracted at 200 W ultrasonic power with ethyl acetate (250 mL) in an ultrasonic flask (Elma, Germany) at 35 °C for 20 min. The extract was filtered and concentrated using a rotary evaporator (Buchi, Switzerland). Further, EAE was freeze-dried and stored at 2–4 °C.

#### 2.3. GC-MS analysis and identification of chemical constituents of \( S. \) chamaecyparissus

The chemical composition of \( S. \) chamaecyparissus EAE was determined by GC-MS. The sample was run on Agilent Bench Top GC-MS (Agilent Technologies, Wilmington, DE, USA) equipment with ethyl acetate (250 mL) in an ultrasonic flask (Elma, Germany) at 35 °C for 20 min. The extract was filtered and concentrated using a rotary evaporator (Buchi, Switzerland). Further, EAE was freeze-dried and stored at 2–4 °C.

#### 2.5. Anticancer activity: EGFR expression by quantitative RT-PCR

### 2.5.1. Cell culture and cell treatment

The cell line of MCF-7 was cultured in liquid Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 u/mL penicillin, and 100 \( \mu \)g/mL streptomycin and maintained at 37 °C under a 5% CO\(_2\) atmosphere. The \( S. \) chamaecyparissus EAE sample was tested for EGFR and \( \beta \)-actin gene expression using MCF-7 cells according to the method reported earlier (Eimani et al., 2014). In brief, trypsinization was used to harvest the MCF-7 cells, pooled in a 15 mL tube. The cells were then inserted into a 6-well tissue culture plate in a DMEM medium containing 10% FBS and 1% antibiotic solution for 24 h at 37 °C at a density of 1 to 106 cells/well (1 mL). The wells were washed with sterile PBS, and 100 \( \mu \)g/mL of \( S. \) chamaecyparissus EAE was treated in a serum free DMEM medium and incubated at 37 °C in a humidified 5% CO\(_2\) incubator for 24 h. The complete RNA from the MCF-7 cell line was isolated using the Trizol method after the incubation time (Neah and Ujjwala, 2011).

### 2.5.2. RNA isolation

Total RNA isolation was performed using the Trizol method (Chomczynski and Mackey, 1995). The samples were centrifuged, using diethylpyrocarbonate treated centrifuge tubes at 5000 rpm for 10 min to get the cell pellet. To cell pellet (1 \( \times \) 10\(^6\) cells), Trizol (700 \( \mu \)L) was added to cell lysis. The lysate was collected into 1.5 mL tubes and vigorously pipetted. Then 300 \( \mu \)L of chloroform

### Table 1

| Genes       | Directions | Sequence (5’ – 3’) | TM   | Product size |
|-------------|------------|--------------------|------|--------------|
| Human       | Forward    | 5’-GGCAGCCTTGAAGAGATCATTTCCTC-3’ | 7.06 | 163          |
| EGFR        | Reverse    | 5’-CTGTGTGAGGCCCACTGAG-3’         | 61.5 | 179          |
| Human       | Forward    | 5’-ATCGTGGCTGACTTAAGGAGAAC-3’    |      |              |
| \( \beta \)-actin | Reverse | 5’-AGGAAGAAGGCTCTGAAGACTG-3’     |      |              |
was added and mixed vigorously for 5 min at room temperature. The aqueous layer was separated by centrifugation at 12000 rpm for 20 min at 4 °C. The aqueous layer was collected in a fresh 1.5 mL tube. RNA was precipitated by adding 700 μL of isopropanol. Precipitated RNA was pelleted by centrifugation at 12000 rpm for 20 min at 4 °C. The pellet was washed with 70% ethanol. Finally, air-dried RNA pellet was mixed into 30 μL double distilled autoclaved water and stored at –80 °C till the next use. The quantity and quality of the isolated RNA were estimated by Labman UV–visible spectrometer and resolved in 1.5% agarose gel, respectively.

2.5.3. DNase treatment

DNA contamination was removed by the DNase treatment. The reaction volume was set up to 20 μL containing 1U of DNase. It was incubated at 37 °C for 45 min, then 20 μM of 2 μL EGTA was added and further incubated at 66 °C for another 10 min. Sodium acetate (1/10 V) and absolute ethanol (2 V) were added and incubated at –20 °C for 60 min. Then the mixture was centrifuged at 12000 rpm for 20 min at 4 °C, the supernatant was discarded, and the pellet was washed with 500 μL of 75% ethanol. The sample was air-dried and dissolved in 20 μL of sterile water and stored till further process.

2.5.4. Gene level detection of micrometastases

Total RNA (1.5 μg) was converted to cDNA using a reaction mixture containing Reverse transcriptase (MMLV). The cDNA synthesis was carried out at 25 °C for 10 min followed by 37 °C for 2 h. Denaturation of cDNA and RNA hybrid along with inactivation of reverse transcriptase was carried out at 85 °C for 2 min. The prepared cDNA was used as a template to detect metastasis. The expression levels of the selected genes (Primer sequence, Table 1) were assessed by qRT-PCR in ABI StepOne Plus (Applied Biosystems, CA, USA) using the relative quantification (2^−ΔΔCT) method. Expression was normalized using the endogenous control (β-actin), and control cells were used as the calibrator.

2.5.5. RT-PCR condition

The initial melting temperature was set at 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 sec. Annealing at 57 °C for 15 sec, and the extension temperature was at 72 °C for 20 sec (Table 2). The real-time data was captured at the end of each extension stage.

3. Results

3.1. GC-MS analysis of EAE of S. chamaecyparissus

The chemical composition of EAE from S. chamaecyparissus leaves was determined by GC-MS method (Fig. 1) and results are presented in Table 3. A total of 44 components representing 97.62% were identified. The major constituents in EAE from S. chamaecyparissus included tetrapentacontane (27.15%), eicosyl acetate (8.40%), and 2-methylhexacosane (6.87%). However, phytol (0.85%), 2-methylhexacosane (6.87%). However, phytol (0.85%), N-acetyl-tyrosine (0.72%), β-camphor (0.5%), and α-curcumene (0.35%) were detected in trace. This is the first GC-MS study on EAE of S. chamaecyparissus. However, the previous studies on essential oil composition of S. chamaecyparissus had shown the presence of camphor, arometis ketone, α-phellandrene, aromadendrene, cubenol, p-cymene, α-terpinol, carophyllene oxide, and 1,8-cineole as major chemical constituents (El-Sharkawy, 2014; Giner et al., 1988; Garg et al., 2001; Perez-Alonso and Velasco-Negueruela, 1992; Derbesy et al., 1989). In the current study, Phytol detected in EAE, is an aromatic diterpene alcohol, reported to have immunostimulant, antioxidant, antiallergic, anti-inflammatory, antinociceptive, and antimicrobial potentials (Moraes et al., 2014). N-acetyl-L-tyrosine found in EAE improves cognitive function, as it acts as a precursor for dopamine.

3.2. α-Glucosidase inhibition activity of EAE from S. chamaecyparissus

The EAE from leaves of S. chamaecyparissus showed concentration dependant α-glucosidase enzyme inhibition that varies from 17.36 ± ± 3.32 to 64.51 ± 1.37 μg/mL for concentration ranging from 31.25 to 1000 μg/mL (Table 4 and Fig. 2). Acarbose was used as a positive standard and it also showed concentration dependant inhibition of α-glucosidase enzyme ranging from 21.94 ± 2.45 to 81.26 ± 3.21 μg/mL for the same concentration as the test sample. The IC50 values for S. chamaecyparissus and acarbose were found as 110 ± 4.25 and 105 ± 3.74 μg/mL, respectively against the α-glucosidase enzyme.

3.3. Anticancer activity: Expression of EGFR in human breast cancer cell line (MCF-7)

An investigation was performed to assess the EGFR expression using human breast cancer cell line (MCF7) and the results are presented in Table 5 and Figs. 3a-3b. The results showed negative expression of EGFR protein compared to the control group. β-Actin protein was used as an endogenous control for normalisation of expression EGFR (Fig. 3a). Table 5 also represents the EGFR (bcl-2) protein levels from multiple quantitative real-time RT-PCR runs relative to normalized levels of β-actin. The results showed that the downregulation of EGFR protein with a value of –0.69297105 after treatment with EAE from leaves of S. chamaecyparissus (100 μg/mL).

4. Discussion

Essential oils obtained from plants emerged as the major source of fragrance and are used as medicines; an important component in aromatherapy, and used as flavouring agents in foods, medicinal products, and cosmetics (Ali, 2001). S. chamaecyparissus is an important medicinal plant of the Mediterranean region and has been reported as anti-inflammatory, antioxidant, antimicrobial, anticandidal, antibacterial, antifungal, CNS depressant, and anti-cholinergic (Tundis and Loizzo, 2018). GC-MS analysis of S. chamaecyparissus yielded 44 chemical constituents in EAE and the major constituents were tetrapentacontane (27.15%), eicosyl acetate (8.40%), and 2-methylhexacosane (6.87%). However, phytol (0.85%), N-acetyl-tyrosine (0.72%), β-camphor (0.5%), and α-curcumene (0.35%) were detected in trace. This is the first GC-MS study on EAE of S. chamaecyparissus. However, the previous studies on essential oil composition of S. chamaecyparissus had shown the presence of camphor, arometis ketone, α-phellandrene, aromadendrene, cubenol, p-cymene, α-terpinol, carophyllene oxide, and 1,8-cineole as major chemical constituents (El-Sharkawy, 2014; Giner et al., 1988; Garg et al., 2001; Perez-Alonso and Velasco-Negueruela, 1992; Derbesy et al., 1989). In the current study, Phytol detected in EAE, is an aromatic diterpene alcohol, reported to have immunostimulant, antioxidant, antiallergic, anti-inflammatory, antinociceptive, and antimicrobial potentials (Moraes et al., 2014). N-acetyl-L-tyrosine found in EAE improves cognitive function, as it acts as a precursor for dopamine.

| Fragments | Initial denaturation | Number of cycles | Denaturation | Annealing | Extension |
|-----------|---------------------|------------------|-------------|-----------|-----------|
| EGFR      | 95 °C-15 min        | 40               | 95 °C for 10 sec | 57 °C-15 s | 72 °C-20 s |
| β-actin   | 95 °C-15 min        | 40               | 95 °C for 10 sec | 61 °C-15 s | 72 °C-20 s |
Oral N-acetyl-L-tyrosine is also reported increasing L-tyrosine levels in the brain (Jongkees et al., 2015; Topall and Laborit, 1989). α-Curcumene identified in EAE is reported as antibacterial and antifungal against selected microorganisms (Silva et al., 2015). Camphor detected in EAE, is used as a skin penetration enhancer, and reported to possess counterirritant, rubefacient, mild analgesic, antimicrobial, insecticidal, anticancer, and antitus-sive activities (Zuccarini and Soldani, 2009; Chen et al., 2013). Inhibition of carbohydrate metabolizing enzymes such as α-amylase and α-glucosidase is an important strategy to control postprandial hyperglycaemia in diabetic patients (Subramanian et al., 2009).
Expression of EGFR in breast cancer cells (MCF-7) following treatment with *S. chamaecyparissus*. The results of the current enzyme inhibition study indicate that the *S. chamaecyparissus* EAE moderately inhibit \( \alpha \)-glucosidase enzyme with IC\(_{50}\) value of 110 ± 4.25 \( \mu \)g/mL. \( \alpha \)-Amylase and \( \alpha \)-glucosidase enzymes are present in brush border of the gastrointestinal tract and responsible for the breakdown of polysaccharides and disaccharides, respectively. Inhibition of these enzymes leads reduction of monosaccharides available for absorption in blood and ultimately it controls the sudden rise of blood glucose level after meal. The increased blood sugar level after meal may be a potential candidate for \( \beta \)-amylase \( \alpha \)-glucosidase. Hence, the present study provides preliminary evidence that *S. chamaecyparissus* may be a potential candidate for the development of antidiabetic drugs through the inhibition of carbohydrate metabolizing enzyme i.e. \( \alpha \)-glucosidase.

![Image](Image61x530 to 265x637)

**Fig. 3a.** 1: Control group; 2: treated with *S. chamaecyparissus* (100 \( \mu \)g/mL).

![Image](Image265x530 to 61x637)

**Fig. 3b.** Real-time quantitative measurement of EGFR in breast cancer cells (MCF-7) cell lines in response to 48 h treatment with *S. chamaecyparissus* (130.4 \( \mu \)g/ml).

5. Conclusion

The ethyl acetate extract (EAE) from leaves of *S. chamaecyparissus* was determined by the GC-MS method, and tetrapentacontane, eicosyl acetate, 2-methylhexacosane, \( n \)-pentadecanol were found as major chemical constituents. The in-vitro \( \alpha \)-glucosidase enzyme inhibition activity of EAE from leaves of *S. chamaecyparissus* showed concentration dependant enzyme inhibition, and IC\(_{50}\) value was found comparable with standard acarbose. The study findings also indicate the possible anticancer effects of *S. chamaecyparissus*, as it showed the negative expression of EGFR protein in human breast cancer cell line (MCF7). The current findings support the possible use of *S. chamaecyparissus* in the management of diabetes and in human breast cancer. Further research studies are required to isolate the chemical compounds from *S. chamaecyparissus* and to evaluate their possible antidiabetic and anticancer effects.

**Declaration of Competing Interest**

The authors declare that there is no conflict of interest.

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