Antioxidant activity and -glucosidase inhibition by essential oils from *Hertia cheirifolia* L.

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Abstract: Essential oils from *Hertia cheirifolia* L. were evaluated for antioxidant activities by the 1,1-diphényl- 2-picrylhydrazyl (DPPH), reducing power and carotene/linoleic acid and inhibitory properties against α-glucosidase. The essential oils (EOs) have been analyzed by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS). Chemical analyses showed that the EOs were rich in terpenes. α-Pinene was identified as major component in *H. cheirifolia* essential oils. Studies on kinetic behavior of the EOs showed that the oils of this species were non-competitive inhibitors and the flowers oil exhibited a strong □-glucosidase inhibitory activity with IC_{50} value of 0.24 ± 0.01 mg/mL. These results show that *H. cheirifolia* could be a natural source of potent antioxidants and α-glucosidase inhibitors

1. Introduction

Diabetes mellitus (DM) is an endocrine disorder resulting in hyperglycemia due to insulin deficiency, insulin resistance or both (Lee et al., 2012). This heterogeneous disease which runs an insidious course may result from a complex interplay of metabolism, environmental and genetic factors (Kadan et al., 2013). Type 2 dia- betes is the most common form, accounting for more than 90% of patients, and is caused by an imbalance between blood sugar absorption and insulin secretion. In addition, oxidative stress is implicated as one of the main factors responsible for the induction of type 2 diabetes mellitus (Shinde et al., 2011). One therapeutic approach to treat diabetes is to retard the absorption of glucose via inhibition of α-glucosidase. In fact, this enzyme, located on the surface of the brush border of the intestinal cells, is crucial for the digestion of oligosaccharides to monosaccharides which are absorbed easily by the intestine (Kim, 2013). Many recent studies on the treatment of type 2 diabetes have focused on the potential use of plant extracts and natural components that could be safer than synthetic sources. Indeed, many oral hypoglycemic agents, such as biguanides and sulfonylureas, are available along with insulin for treatment of diabetes (Mannucci et al., 2004), but these synthetic agents are expensive and can produce adverse side effects. Hence, recently, many medicinal herb extracts have been used for the treatment of diabetes mellitus due to low side effects (Ping et al., 2010). As well, some studies suggested that essential oils may improve useful in the battle against insulin resistance and type 2 diabetes mellitus, and various oils have been used in the market as therapeutic agents for years without occurrence of significant adverse health effects (Hammer et al., 1999; Tomaino et al., 2005). Consequently, there has been a growing interest in herbal essential oils, due to their antioxidative activity (Su-Tze et al., 2012). The families extensively studied for essential oils were Lamiaceae, Apiaceae and Asteraceae (Bas¸er, 2002).

The genus *Hertia*, which belongs to the Asteraceae family, contains 12 species distributed all over South and North Africa and Southwest Asia (Akhgar et al., 2012). In Tunisia, we found only the species *Hertia cheirifolia* L.

H. cheirifolia is endemic to both Tunisia and Algeria. It grows in large clumps. This plant has fleshy

stems, branched and very leafy 20–40 cm. The leaves are alternate and fleshy. The heterogamous capitula have a big solitary of 2–3 cm in diameter. The flowers are lemon yellow, those peripheral and ligulate are fertile and the others tubular are sterile (Pottier-Alapetite, 1981).

Traditionally in Pakistan, the decoction of leaves from *Hertia intermedia* was used for pain of stomach (Tareen et al., 2010). In Tunisia, local people use the infusion of vegetative part (leaves + stems) from *H. cheirifolia* to reduce hyperglycemia. But, there is no scientific reference in the literature for such use. Previous studies showed that *H. cheirifolia* have important chemicals and biological activities such as spasmolytic, anti-inflammatory (Ammar et al., 2009) and acaricidal effects (Attia et al., 2012). How- ever, to our knowledge and according to literature survey, there are no reports on the enzyme inhibition effects of essential oils from *H. cheirifolia*. The present work aims to investigate the chemical composition by GC–MS, antioxidant and α-glucosidase inhibitory activities of the essential oils obtained from H. cheirifolia.

2. Materials and methods

2.1. Chemicals and reagents

Hexane, chloroform and methanol were purchased from Merck (Darmstadt, Germany). 1,1-diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), potassium ferricyanide [K_3 Fe(CN)$_6$], ferric chloride ($FeCl_3$), trichloroacetic acid (TCA), β-carotene, linoleic acid, hydrochloric acid (HCl), Sodium hydroxide (NaOH), α-Glucosidase (isolated from Aspergillus niger), 4-p-nitrophenyl-α-D-glucopyranoside (4-pNPG) were purchased from Sigma–Aldrich.

2.2. Plant material and extraction of essential oils

H. cheirifolia was collected at the flowering stage in February 2012 from Thala in Tunisia. A voucher specimen (*H. cheirifolia*) (Hc 112) was deposited in the Laboratory of Medicinal Chemistry and Natural Products at the Faculty of Science of Monastir, Tunisia. Fresh flowers, vegetative part (leaves + stems) and roots, were separately cut in small pieces, weighed before extraction and subjected to hydrodistillation for 3 h using a Clevenger-type apparatus (Clevenger, 1928). The essential oils were collected by decantation, dried over sodium sulphate, weighed and stored in sealed glass vials in a refrigerator at 4–5 ° C for further analysis.

2.3. Analysis of the essential oils

Analytical GC: essential oils compositions were determined using gas chromatograph: HP 5890-series II equipped with flame ionization detectors (FID), HP-5 (30 m × 0.25 mm ID, 0.52 μm film fused silica capillary column, carrier gas nitrogen (1.2 mL/min). The temperature oven was programmed from 50 ° C (1 min) to 280 ° C at 5 ° C/min (1 min). Injector and detector temperatures were 250 ° C and 280 ° C, respectively. Volume injected was 0.1 μL of 1% hexane solution. The identification of the components was performed by comparison of their retention times with those of pure authentic samples and by mean of their linear retention indices (L.R.I) relative to the series of n-hydrocarbons.

Analytical GC–MS: GC/EIMS analyses were performed with a Varian CP-3800 gas-chromatograph equipped with a HP-5 capillary column (30 m × 0.25 mm; coating thickness 0.25 μm) and a Varian Saturn 2000 ion trap mass detector. The analytical conditions were: injector and transfer line temperatures 220 and 240 ° C respectively; oven temperature programmed from 60 ° C to 240 ° C at 3 °

C/min; carrier gas helium at 1 mL/min; injection of 0.2 □L (10% hexane solution); split ratio 1:30. Identification of the constituents was based on comparison of the retention times with those of authentic samples, comparing their linear retention indices relative to the series of n-hydrocarbons, and on computer matching against commercial (NIST 98 and ADAMS) and home-made library mass spectra built up from pure substances and components of known essential oils and MS literature data (Stenhagen et al., 1974; Massada, 1976; Jennings and Shibamoto, 1980; Davies, 1990; Adams, 1995). Moreover, the molecular weights of all the identified substances were confirmed by GC/CIMS, using MeOH as CI ionizing gas.

2.4. Antioxidant activity

2.4.1. Scavenging effect on DPPH

The DPPH assay is known to provide reliable information concerning the antioxidant capacity of specific compounds or extracts across a short time scale (Huey-Chun et al., 2012). The hydrogen atoms or electrons donation ability of the corresponding samples were measured from the bleaching of purple colored methanol solution of DPPH (Cuendet et al., 1997). According to Hatano et al. (1988), the effect of EOs on DPPH radical was estimated. In fact, 0.5mL of each sample concentration was mixed with the same volume of DPPH methanolic solution. The mixture was shaken vigorously and allowed standing for 30 min in darkness and at a temperature of 25 ° C; the absorbance of the resulting solution was measured at 520 nm with a spectrophotometer. All measurements were performed in triplicate. A mixture of 0.5 mL of DPPH solution and 0.5 mL of methanol was taken as a control. Inhibition of free radical DPPH in percent (I%) was calculated in following way: $I\% = [(A_{blank} - A_{sample})/A_{blank}] \times 100$; where A_{blank} is the absorption of the control reaction (containing all reagents except the test com- pound), and A_{sample} is the absorption of the test compound. The IC_{50} value, which is the sample concentration providing 50% inhibition, was determined by plotting the inhibition percentage versus extract concentrations.

2.4.2. Reducing power

Many reports demonstrated that the reducing power of the natural plant extracts might be strongly correlated with their antioxidant activities (Liu et al., 2009).

1 mL of extract and 0.75 mL of distilled water were mixed with 1 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 1 mL (1%) of potassium ferricyanide [$K_3 Fe(CN)_6$]. The mixture was incubated at 50 ° C for 20 min. Then acidified with 1 mL of trichloroacetic acid (10%). Finally, 0.25 mL of $FeCl_3$ (0.1%) were added to this solution. Absorption of this mixture was measured at 700 nm using a UV spectrophotometer. (Oyaizu, 1986). The EC_{50} , which is the effective concentration of the sample at which the absorbance is 0.5, was determined.

2.4.3. β-Carotene-linoleic acid method

In this assay antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation (Dapkevicius et al., 1998). β-Carotene bleaching inhibition of *H. cheirifolia* essential oils was determined according to the method of Ikram et al. (2009). Briefly, 2 mL of β-carotene solution (1.5 mg β-carotene/2.5 mL chloroform) were added to 20 µL of linoleic acid and 200 µL of Tween-20. The chloroform was removed at 40 ° C under vacuum using a rotary evaporator. Immediately, 50 mL of distilled water were added to the dried mixture to form a β-carotene-linoleic acid emulsion. In order to

determine the β-carotene bleaching activity of the extract, 5 mL of emulsion were added to 500 μL of samples. The mixtures were incubated in a water bath at 50 °C for 60 min and the absorption of the reaction mixtures was read at 470 nm. The antioxidant activity (AA) of the samples was calculated by using the following equation: $AA\% = [A_2 / A_1] \times 100$; were A_2 is the absorption of β-carotene content after 2 h assay and A_1 is the absorption of initial β-carotene content. The IC_{50} value, which is the sample concentration providing 50% inhibition, was determined by plotting the inhibition percentage versus extract concentrations. The essential oil antioxidant activity was comparable to Butylated hydroxytoluene (BHT).

2.5. α-Glucosidase inhibition assay

The α-glucosidase inhibition assay was performed according to the method of Tao et al. (2013) with some modifications. The α-glucosidase reaction mixture, contained 2.5mM 4-p-nitrophenyl-α-D-glucopyranoside (4-pNPG), 250 μL of extract (varying concentrations) in DMSO and 0.3 U/mL α-glucosidase in phosphate buffer pH 6.9, was incubated in a water bath at 37 °C for 15 min. Control tubes contained only DMSO, enzyme and substrate, while in positive controls Acarbose replaced the plant extracts. Absorbance of the resulting p-nitrophenol (pNP) was determined at 405 nm and was considered directly proportional to the activity of the enzyme. Each sample was performed in triplicate. Percentage inhibition by extracts and acarbose (I%) were calculated using the following equation: $(I\%) = (1 - (DO_{sample} / \Delta DO_{control})) \times 100$. The IC_{50}, which is the concentration of the sample required to inhibit 50% of the enzyme was determined for each sample.

2.6. Kinetics study of α-glucosidase

Lineweaver–Burk plot analysis was performed to determine the inhibition mode of essential oils, and kinetics were measured using increasing concentrations of 4-pNPG as a substrate in the presence of various concentration of oils. The inhibition constant K_i values were calculated from the secondary plots constructed using slopes or y-intercepts of Lineweaver–Burk plots. K_i expresses the equilibrium constant for the binding of oils to α-glucosidase. The initial rates of reaction were determined using calibration curves constructed using varying concentrations of 4-pNPG.

2.7. Statistical analysis

The results were given as the average ± SE for at least three replicates for each sample. The IC_{50} (α-glucosidase inhibition, DPPH, and β-carotene/linoleic acid methods) and the EC_{50} (reducing power) values were calculated by linear regression analysis. The data were subjected to ANOVA, and Duncan's multiple range test was used to compare means. Statistical analyses were per-formed with the SPSS statistical software program (SPSS v.16). p values <0.05 were regarded as significant.

3. Results and discussion

3.1. Chemical composition of the essential oils

The chemical composition of the essential oils obtained from the fresh parts: flowers, vegetative part (leaves + stems), and roots of *H. cheirifolia* are presented in Table 1. The flowers oil revealed the presence of thirty-five components, representing 98.1% of the total oil. The major constituents of the flowers oil were α-pinene (70.4%), germacrene D (6.7%), α-cadinol (3.2%) and sabinene (2.3%). Thirty compounds were identified in the vegetative part (leaves+stems), representing 95.9% of the total oil. The main components of the vegetative part oil were α-pinene (62.5%), germacrene D

(9.5%), α-cadinol (2.7%), sabinene (2.1%) and β-caryophyllene (1.7%). Twenty-eight components accounting 90.3% of constituents of the roots oil were identified and the major com- pounds were α-pinene (22.1%), valencene (13.2%), β-caryophyllene (11.8%), germacrene A (7.6%), α-terpinyl acetate (6.9%), germacrene D (5.9%), β-elemene (3.9%) and caryophyllene oxide (2.5%). It has been found that the highest quantitative classified components from aerial part were monoterpene hydrocarbons. α-Pinene was the most abundant compound among all constituents of essential oils of different parts of *H. cheirifolia* (flowers (70.4%), vegetative part (62.5%) and roots (22.1%)).

The comparison of the chemical composition from the aerial parts of *H. cheirifolia* with that of *Hertia angustifolia* (DC.) O. Kuntze from Iran (Afsharypuor et al., 2000) showed that the compounds have varied according to species. Indeed, the main constituents of the essential oil from the aerial parts of H. angustifolia were β-pinene (51.5%), nevertheless, the predominance of α-pinene in our oils seems to characterize *H. cheirifolia* in its chemical aspect. This difference in compositional constituents of the essential oils of this plant was associated with climatic conditions, geographical location of collection sites, and other ecological and genetic factors (Afoulous et al., 2013).

3.2. Antioxidant activity

The antioxidant activities related to the contents of essential oils of *H. cheirifolia* were determined by DPPH free radical scavenging, reducing power and carotene/linoleic acid methods. The results are summarized in Table 2. All EOs showed higher scavenging ability to increase the free radical scavenging potential which IC_{50} varied from 0.016 ± 0.003 mg/mL to 0.024 ± 0.001 mg/mL). This strong inhibitory capacity of the EOs can be explained by the presence of hydroxylated compounds such as terpenoids (Kadri et al., 2013). Indeed, Lu and Foo (2001) reported that most natural antioxidative compounds often work synergistically with each other to produce a broad spectrum of antioxidative properties that create an effective defense system against free radicals. The reducing power assay was often used to measure the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) in the presence of antioxidants, this assay was often used for the analysis of total antioxidant activity of plant extracts.

The results of reducing power of the EOs may serve as significant indicator of its potential antioxidant activity. In fact, all EOs were able to catalyze the reduction of Fe^{3+} and the EO of flowers are the strongest antioxidant activity with (EC_{50} = 0.021 ± 0.001 mg/mL).

The β-carotene bleaching method measures the ability of a compounds or a mixture to inhibit lipid peroxidation. In the assay, the EO of flowers presented an IC_{50} lower than the one of BHT (synthetic antioxidant used as reference), which is an indicator of the great capacity of this oil to inhibit the lipid peroxidation to inhibit the lipid peroxidation with IC_{50} = 0.034 ± 0.003 mg/mL, even better than BHT.

From the results of the antioxidant activity, it was obtained that the essential oils of *H. cheirifolia* had the best antioxidant activity in DPPH, reducing power and β-carotene-linoleic acid bleaching assays. Thus, these EOs samples could be regarded as a kind of valuable antioxidant natural source.

3.3. α-Glucosidase inhibition assay

□-Glucosidase is key enzyme in hydrolysis of oligosaccharide (Gray, 1995). The inhibition of this enzyme is an important strategy in the management blood glucose level. But a main drawback of currently used α-glucosidase inhibitor (Acarbose, etc) is their side effects such as abdominal distention, flatulence and diarrhea (Dong et al., 2012). To develop alternative compounds with low toxicity and side effects for diabetes mellitus, it is important to evaluate the anti-diabetic properties of medicinal plants and their products.

As shown in Table 3, the α-glucosidase inhibitor effectiveness of the different *H. cheirifolia* EOs was compared on the basis of their resulting IC_{50} values. The high IC_{50} values indicated the low inhibition activity. In fact, the highest α-glucosidase inhibitory activity was recorded in the EOs of flowers with (IC_{50} = 0.24 ± 0.01 mg/mL). The anti-diabetic activity of the EOs can be related to main components. Furthermore, the terpenes such as α-pinene which exist in *H. cheirifolia* essential oils might inhibited key enzymes related to type 2 diabetes principally α-glucosidase. It was reported that administration of terpenes to diabetic exerts blood glucose lowering effect and high antioxidant activity in alloxan-induced diabetic rat (Hamden et al., 2011). However, essential oils are complex mixtures of numerous molecules, and one might wonder if their biological effects are the result of a synergism of all molecules or reflect only those of the main molecules present at the highest levels according to gas-chromatographic analysis.

According to literature survey, there are no reports on the anti α-glucosidase activity in vitro for the genus *Hertia*. However, in the Asteraceae family, there are several species used for the treatment of hyperglycemia, such as, the flowers of *Chrysanthemum morifolium* (Thi Luyen et al., 2013) and the ethyl alcohol extract of *Artemisia herba-alba* (Awad et al., 2012).

3.4. Mode of α-glucosidase inhibition by EOs

To investigate the type of enzyme inhibition and to determine the inhibition constants (Ki) for each oil, the α-glucosidase activity was assayed in the presence of different concentrations of the substrates (1.25 mM, 2.5 mM, 5 mM and 10 mM) and different concentrations of EOs (1 mg/mL, 0.5 mg/mL and 0.25 mg/mL).

The Lineweaver–Burk plot analysis indicated that the EOs inhibited α-glucosidase in a non-competitive mode (Fig. 1). In fact, the plots intersect the X-axis, as well as the maximum reaction velocity (V_{max}) was changed and Michaelis–Menten constant (K_m) kept the same value. The non-competitive mode illustrated that these EOs bind to a site other than the active site of the enzyme, without competing with the substrate, to retard the substrate conversion.

The K_i values derived from secondary plots (Fig. 1(a–c)) for flowers, (leaves and stems) and roots were 0.34 mg/mL, 0.64 mg/mL and 1.04 mg/mL, respectively, indicating that EO of flowers tended to bind more easily to the α-glucosidase. Indeed, smaller value of inhibition constant indicates stronger inhibition, which indicates that the inhibitor-enzyme binding affinity exceeds the binding affinity of the enzyme-substrate.

In conclusion, this study can be considered as the first detailed report of the effects of the EOs extracted from *H. cheirifolia* on enzymatic inhibition and in vitro antioxidant activity. The results indicate that these EOs inhibit α-glucosidase by non-competitive inhibition. The α-glucosidase inhibitory exhibited by the EOs of this plant show the potential of these oils for use in the diabetes treatment as α-glucosidase inhibitory agent. However, further in vivo studies are needed to study the complete properties of the EOs of this species.

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Table 1
Identified components in the essential oils of *Hertia cheirifolia* L.

Compound[a]	L.R.I[b]	Flowers (%)[c]	Leaves + stems (%)[c]	Roots (%)[c]
α-Pinene	941	70.4	62.5	22.1
Camphene	955	tr	tr	–
Sabinene	978	2.3	2.1	0.1
β-Pinene	982	1.3	1.2	tr
Myrcene	993	0.9	0.7	–
α-Terpinene	1020	tr	–	–
p-Cymene	1028	tr	0.5	–
Limonene	1032	0.8	0.7	tr
γ-Terpinene	1064	tr	–	–
Terpinolene	1090	0.1	–	–
α-Pinene oxide	1097	tr	0.7	–
cis-verbenol	1141	tr	0.7	0.9
4-Terpineol	1179	0.8	tr	tr
α-Terpineol	1191	tr	–	tr
Verbenone	1205	tr	0.1	tr
α-Terpinyl acetate	1352	tr	0.9	6.9
(*E*)-β-damascenone	1383	0.1	tr	–
β-Elemene	1392	0.9	1.3	3.9
β-Caryophyllene	1419	0.6	1.7	11.8
α-Guaiene	1440	–	–	tr
α-Humulene	1456	0.6	1.1	1.1
Drima-7,9(11)-diene	1471	–	–	1.2
β-Chamigrene	1476	–	–	1.7
γ-Muurolene	1478	–	0.5	–
Germacrene D	1482	6.7	9.5	5.9
Valencene	1493	0.6	1.1	13.2
Bicyclogermacrene	1496	0.8	0.6	–
Germacrene A	1505	–	–	7.6
β-Bisabolene	1508	0.8	0.7	–
δ-Cadinene	1524	0.9	1.1	1.6
Spathulenol	1577	0.6	1.3	–
Caryophyllene oxide	1582	0.7	0.8	2.5
Globulol	1584	0.7	0.7	–
Humulene epoxide II	1607	–	0.5	–
epi-10-γ-eudesmol	1622	0.7	0.7	0.9
1-*epi*-cubenol	1629	0.6	–	1.6
T-Cadinol	1641	1.0	0.8	1.1
α-Cadinol	1654	3.2	2.7	–
14-Hydroxy-9-*epi*-(*E*)-caryophyllene	1678	–	–	1.0
Acorenone	1688	0.8	0.7	1.6
Drimenone	1792	–	–	1.4
Manoyl oxide	1991	0.7	–	–
Abietadiene	2082	0.5	–	–
n-Heneicosane	2100	–	–	1.0
n-Tricosane	2300	–	–	1.2
Identified compounds		98.1	95.9	90.3
Monoterpene hydrocarbons		75.8	67.7	22.2
Oxygenated monoterpenes		0.8	2.4	7.8
Sesquiterpene hydrocarbons		11.9	17.6	48.0
Oxygenated sesquiterpenes		8.3	8.2	10.1
Diterpenes		1.2	–	–
Others		0.1	–	2.2

–: not detected.
tr: trace (<0.1%).
[a] Identification of compounds was made by the calculation of their L.R.I and by GC–MS analysis.
[b] LRI: linear retention indices (HP-5 column).
[c] %: percentage calculated by GC-FID on non-polar capillary column HP-5.

Table 2
Antioxidant activity of essential oils of *Hertia cheirifolia* L. by DPPH, reducing power and β-carotene/linoleic acid tests.

	IC_{50} (mg/mL) of DPPH	EC_{50} (mg/mL) of reducing power	IC_{50} (mg/mL) of β-carotene/linoleic acid
Flowers	0.016 ± 0.003^{c}	0.021 ± 0.001^{b}	0.034 ± 0.003^{b}
Leaves and stems	0.024 ± 0.001^{a}	0.049 ± 0.001^{b}	0.055 ± 0.005^{a}
Roots	0.019 ± 0.002^{b}	0.062 ± 0.002^{a}	0.068 ± 0.003^{c}
BHT	0.018 ± 0.001	0.02 ± 0.001	0.04 ± 0.001

Values were expressed as mean ± SE ($n = 3$).
The different letters indicate a significant difference between the oils ($p < 0.05$).
IC_{50} (mg/mL): the concentration at which 50% is inhibited.
EC_{50} (mg/mL): effective concentration at which the absorbance is 0.5.

Table 3
α-Glucosidase inhibition by essential oils of *Hertia cheirifolia* L.

Extracts	IC_{50} (mg/mL) α-glucosidase inhibition
Flowers	0.24 ± 0.01
Leaves and stems	0.29 ± 0.04
Roots	0.45 ± 0.02
Acarbose	0.28 ± 0.01

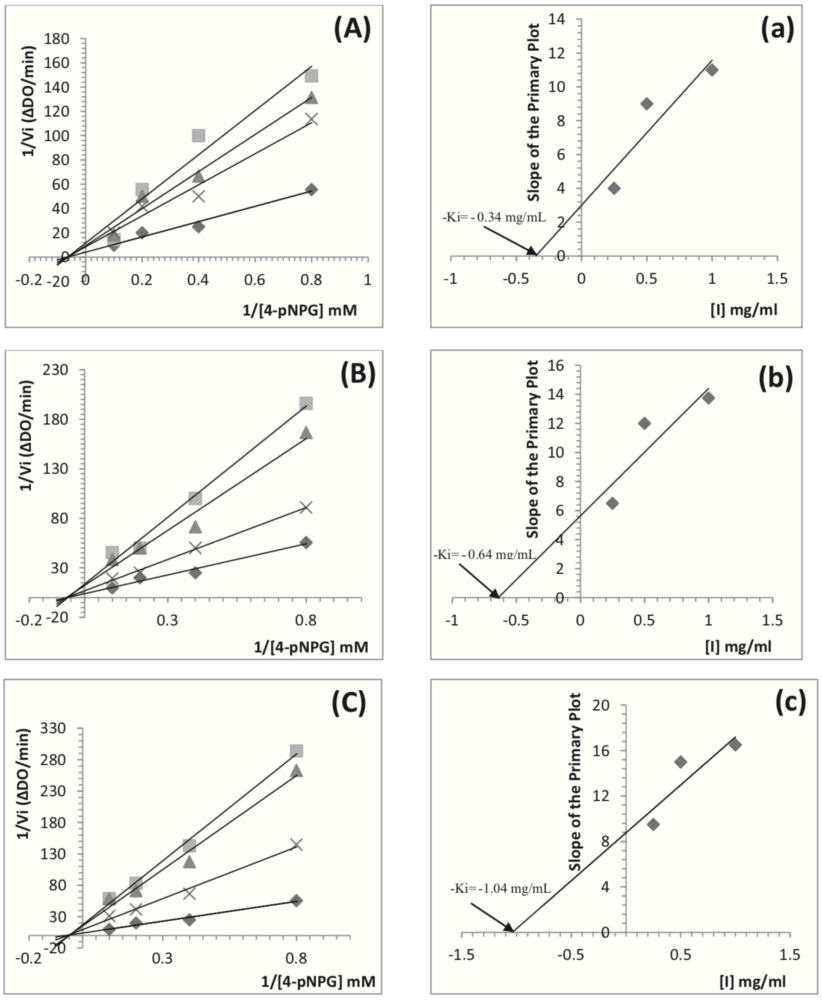

Fig. 1. Double reciprocal plots of α-glucosidase inhibition at different 4-pNPG concentrations in absence (♦) or presence of various concentrations (■: 1 mg/mL; ▲: 0.5 mg/mL; ×: 0.25 mg/mL) of flowers (A); leaves and stems (B) and roots (C) essential oils. Secondary plots of slopes (a); (b) and (c) against inhibitor concentrations to calculate K_i.