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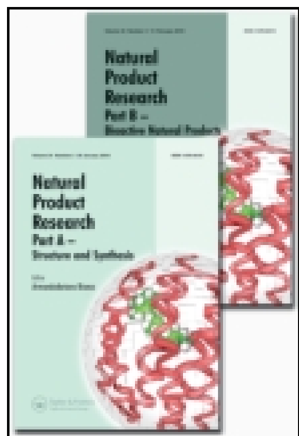

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Studies on the potential antioxidant properties of Senecio stabianus Lacaita (Asteraceae) and its inhibitory activity against carbohydrate-hydrolysing enzymes

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Studies on the potential antioxidant properties of *Senecio stabianus* Lacaita (Asteraceae) and its inhibitory activity against carbohydrate-hydrolysing enzymes

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This study showed for the first time the antioxidant and hypoglycaemic properties of the methanol, *n*-hexane and ethyl acetate extracts from *Senecio stabianus* Lacaita, a plant that belongs to the Asteraceae family. The antioxidant activities were carried out using two different *in vitro* assays, namely 2,2-diphenyl-1-picrylhydrazyl (DPPH) test and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulphonate) (ABTS) test. The ethyl acetate extract showed the highest activity with inhibitory concentration 50% (IC_{50}) values of 35.5 and 32.7 $\mu g\,mL^{-1}$ on DPPH test and ABTS test, respectively. This activity may be related to a good total phenol and flavonoid content. All extracts were also tested for their potential inhibitory activity of α-amylase and α-glucosidase digestive enzymes. The *n*-hexane extract exhibited the highest α-amylase inhibition with an IC_{50} value of 0.21 $mg\,mL^{-1}$. Through bioassay-guided fractionation processes seven fractions (A–G) were obtained and tested. Based on the phytochemical analysis, the activity of *n*-hexane extract may be related to the presence of monoterpenes and sesquiterpenes.

Keywords: *Senecio stabianus* Lacaita; antioxidant activity; α-amylase inhibition; α-glucosidase inhibition; terpenes

1. Introduction

Interest in the search for new natural antioxidants has grown dramatically over the past years because reactive oxygen species (ROS) production and oxidative stress have been shown to be linked to a large number of human degenerative diseases, including cancer, cardiovascular diseases, inflammation and diabetes (Waris & Ahsan, 2006). Also, the restrictions on the use of synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) due to their toxicity has been an important incentive for such research.

Type 2 diabetes is a heterogeneous disease resulting from a dynamic interaction between defects in insulin secretion and insulin action. Such a deficiency results in

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increased concentrations of glucose in the blood, which in turn damage many of the body's systems, in particular the blood vessels. These disorders include retinopathy, nephropathy, neuropathy and angiopathy. Patients with type 2 diabetes are insulin resistant and often have a metabolic syndrome, a multifactorial intervention including aggressive treatment of arterial hypertension and dyslipidaemia (Stolar, 2010). As most subjects are overweight or obese, the initial treatment is optimisation of the meal plan and enhancement of physical activity in order to obtain sustained weight reduction. In case of failure in following lifestyle changes, various oral hypoglycaemic agents may be used. The objective of antidiabetic therapy is to reach normoglycaemia and to reduce insulin resistance, thereby improving metabolic control with the intention of preventing diabetic late complications. These may be inhibited or lowered by maintaining blood glucose values close to normal. Some are targeting defective insulin secretion, while others are targeting insulin resistance. One therapeutic approach for treatment in the early stage diabetes is to decrease postprandial hyperglycaemia. This is done by retarding the absorption of glucose through the inhibition of carbohydrate-hydrolysing enzymes, α-amylase and α-glucosidase, in the digestive tract. Inhibitors of these enzymes delay carbohydrate digestion and prolong overall carbohydrate digestion time, causing a reduction in the rate of glucose absorption and consequently blunting of the postprandial plasma glucose rise (Chen, Zheng, & Shen, 2006; Chiasson, 2006). These drugs also have certain adverse effects like causing hypoglycaemia at higher doses, liver problems, lactic acidosis and diarrhoea. Apart from currently available therapeutic options, many herbal medicines have been recommended for the treatment of diabetes. Traditional plant medicines are used throughout the world for a range of diabetic presentations.

Many *Senecio* species are used in folk medicine as emmenagogues, anti-inflammatory agents and vasodilators (Bautista Peres, Stubing, & Figuerola, 1991; Christov et al., 2002). South American traditional medicine reported the use of *Senecio* species as a remedy for altitude sickness ('soroche'), and to relieve stomach pain (Abdo et al., 1992; Loyola, Pedreros, & Morales, 1985). *Senecio graveolens* is used in Argentina as emenagogue, and digestive and cough suppressant (Pérez, Agnese, & Cabrera, 1999). *Senecio latifolius* leaves were used by Zulu as an emetic and in the treatment of chest complaints (Steenkamp, Stewart, van der Merwe, Zuckerman, & Crowther, 2001). *Senecio* genus is also important in the folk medicine of south and southeast Spain (Torres, Mancheño, Chinchilla, Asensi, & Grande, 1988). Interestingly, in Mongolia, *Senecio nemorensis* subsp. *fuchsii* was used as herbal tea for the treatment of diabetes (Wiedenfeld, Narantuya, Altanchimeg, & Roeder, 2000).

In our previous studies, we have evaluated the antioxidant and hypoglycaemic properties of *Senecio inaequidens*, *Senecio vulgaris*, *Senecio leucanthemifolius* and *Senecio gibbosus* (Conforti, Loizzo, Statti, Houghton, & Menichini, 2006a; Conforti, Marrelli, Statti, & Menichini, 2006b; Tundis et al., 2007).

In particular, *S. gibbosus*' methanol and ethyl acetate extracts showed a good radical scavenging activity evaluated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) test with inhibitory concentration 50% (IC_{50}) values of 0.02 and 0.01 $mg\,mL^{-1}$, respectively. The ethyl acetate extract of *S. inaequidens* showed a great antioxidant activity compared with *S. vulgaris* (61.60% and 44.57% of inhibition, respectively, at a concentration of 0.312 $mg\,mL^{-1}$). A recent study (Tundis et al., 2009) on

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Senecio stabianus evaluated its potential cytotoxicity against different human cancer cell lines, demonstrating an interesting cytotoxic activity of the *n*-hexane extract with IC_{50} values of 62.7 and 71.1 $\mu g\,mL^{-1}$ against amelanotic melanoma (C32) and hormone-dependent prostate carcinoma (LNCaP), respectively.

Therefore, given the interesting biological properties reported for *Senecio* species and following our previous studies, the objective of this study is to investigate for the first time the antioxidant properties by DPPH and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulphonate) (ABTS) tests, and the hypoglycaemic activity *via* the inhibition of the carbohydrate-hydrolysing enzymes, α-amylase and α-glucosidase, of *S. stabianus* extracts and to relate the biological activity found to the chemical composition. The search for more effective and safer hypoglycaemic compounds has an important area of active research, and after the recommendations made by the World Health Organisation on diabetes, investigation on hypoglycaemic extracts and/or compounds from medicinal plants has become an important aspect of this project.

2. Results and discussion

As part of our continuous interest in the research of biological activities and chemical composition of *Senecio* genus, this article reports for the first time the *S. stabianus* antioxidant properties and its hypoglycaemic activity *via* the inhibition of α-amylase and α-glucosidase enzymes.

The antioxidant activity of *S. stabianus* extracts was evaluated employing two established *in vitro* systems (DPPH and ABTS tests). In both systems, the *S. stabianus* activity followed a concentration-dependent relationship (Figure 1a and b).

A rapid, simple and inexpensive method to measure the antioxidant capacity involves the use of the free radical, DPPH. DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity. The IC_{50} values for DPPH scavenging by *S. stabianus* extracts are given in Table 1.

The screening showed that the ethyl acetate extract possessed the highest antioxidant activity with an IC_{50} value of 35.5 $\mu g\,mL^{-1}$. Interestingly, this result is better than that obtained with the positive control ascorbic acid that showed an IC_{50} value of 50.0 $\mu g\,mL^{-1}$. In addition, the antioxidant ability of *S. stabianus* extracts to scavenge the blue–green coloured $ABTS^{+}$ radical cation was measured relative to the radical scavenging ability of Trolox. The ethyl acetate extract also possessed the highest antioxidant activity in the ABTS system with an IC_{50} value of 32.7 $\mu g\,mL^{-1}$.

This extract showed a total phenol contentof 76.3 mg chlorogenic acid equivalent per gram of plant material, which was evaluated by the Folin–Ciocalteu method. The flavonoid content, determined using a method based on the formation of a flavonoid–aluminium complex, was 11.8 mg quercetin equivalent per gram of plant material. The methanol extract showed IC_{50} values of 66.0 and 72.3 $\mu g\,mL^{-1}$ on DPPH test and ABTS test, respectively.

Previously, Conforti et al. (2006b) evaluated the antioxidant activity of some *Senecio* species. In particular, *S. gibbosus* methanol and ethyl acetate extracts showed a good radical scavenging activity evaluated by the DPPH test with IC_{50} values of 20 and 10 $\mu g\,mL^{-1}$, respectively. A lower antioxidant activity of inhibition was

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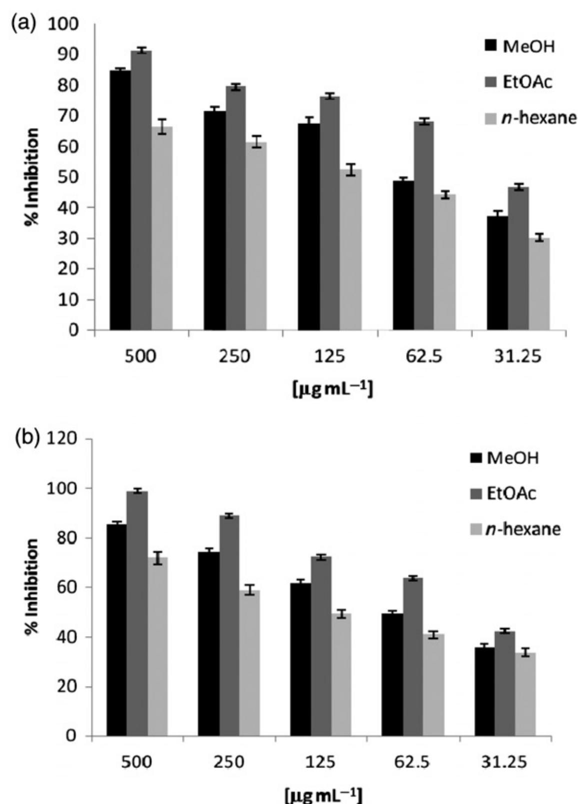

Figure 1. Antioxidant activity of *S. stabianus* extracts using (a) DPPH test and (b) ABTS test.

Table 1. Antioxidant activity of *S. stabianus* extracts.

S. stabianus extract	DPPH assay (IC_{50} μg mL^{-1})	ABTS assay (TEAC value)
MeOH	66.0 ± 1.6**	72.3 ± 1.0**
n-Hexane	88.2 ± 1.8**	65.9 ± 1.0**
Ethyl acetate	35.5 ± 3.5**	32.7 ± 1.5**
Ascorbic acid	50.0 ± 0.8	0.9 ± 0.03

Notes: Data are expressed as mean ± SD ($n = 3$). Differences within and between groups were evaluated by one-way ANOVA test followed by a multicomparison Dunnett's test.
**$p < 0.01$ compared with the positive control (ascorbic acid).

demonstrated for *S. inaequidens* and *S. vulgaris* (61.60% and 44.57%, respectively, at a concentration of 312.0 μg mL^{-1}) (Conforti et al., 2006a).

α-Amylase and α-glucosidase are the main enzymes responsible for the breakdown of starch into simple sugars. Although the activity of these enzymes has not been directly involved in the aetiology of diabetes, α-amylase and α-glucosidase inhibitors have been thought to improve glucose tolerance in diabetic

Table 2. Hypoglycaemic activity *via* the α-amylase and α-glucosidase enzymes inhibition of *S. stabianus* extracts.

	IC_{50} (mg mL^{-1})	
S. stabianus extract	α-Amylase	α-Glucosidase
Methanol	0.29 ± 0.03*	>1
n-Hexane	0.21 ± 0.05*	>1
Ethyl acetate	0.76 ± 0.09*	>1

Notes: Data are given as the mean of at least three independent experiments $\pm$ SD. Differences within and between groups were evaluated by one-way ANOVA test completed by a with a multicomparison Dunnett's test. Acarbose was used as positive control (IC_{50} 0.05 mg mL^{-1}). *$p < 0.01$ compared with the control experiment.

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patients (Lebovit, 1998). In the recent years, extensive efforts have been made to find effective hypoglycaemic agents, with the objective of obtaining better control of diabetes.

Our results (Table 2) suggested a significant and selective α-amylase inhibitory activity of *S. stabianus* methanolic extract with an IC_{50} value of 0.29 mg mL^{-1}. Further studies were conducted on this extract that was subjected to bioassay-guided fractionation process. As the first step of fractionation, methanolic extract was consecutively partitioned with *n*-hexane and ethyl acetate. *n*-Hexane extract exhibited the highest activity with an IC_{50} value of 0.21 mg mL^{-1}. With the purpose to identify the putative active compounds, the *n*-hexane extract was subjected to further fractionation processes to obtain seven main fractions (A–G). When A–G fractions were assayed for hypoglycaemic activity, all the fractions showed a selectivity of action against α-amylase. The results are given in Table 3. Among the tested fractions, A and F showed the highest activities with IC_{50} values of 0.16 and 0.52 mg mL^{-1}, respectively.

The chemical composition of these fractions were analysed by GC–MS (Table 3). The most active fraction A (IC_{50} of 0.16 mg mL^{-1}) was characterised by the presence of some monoterpenes (borneol and terpinen-4-ol) and different sesquiterpenes, namely β-caryophyllene, α-ylangene, α-humulene, aromadendrene, β-patchoulene, α-patchoulene, α-muurolene, nerolidol, β-selinene, δ-cadinol, isobicyclogermacrene, neophytadiene, α-copaene, β-elemene, epi-bicyclosesquiphellandrene, *trans*-β-farnesene, α-gurjunene, γ-cadinene, α-amorphene and sandaracopimaradiene. The activities of fractions F and G (IC_{50} of 0.52 and 0.70 mg mL^{-1}, respectively) could be attributed to the presence of sterols. No inhibitory α-glucosidase activity was detected for extracts up to 20 mg mL^{-1}, indicating a selectivity of action against the other carbohydrate-hydrolysing enzyme α-amylase.

A previous study on *S. inaequidens* and *S. vulgaris* showed that the methanolic extract of both *Senecio* species possessed a good activity with α-amylase inhibition of 93% and 82% at 1 mg mL^{-1} concentration for *S. inaequidens* and *S. vulgaris*, respectively (Conforti et al., 2006a). Interestingly, the *n*-hexane extract of both species did not inhibit α-amylase.

Table 3. Chemical composition and α-amylase inhibitory activity of *S. stabianus n*-hexane chromatographic fractions (A–G).

Fraction	Identification	IC_{50} ($mg\,mL^{-1}$)
A	Terpenes: borneol, terpinen-4-ol, β-caryophyllene, α-ylangene, α-humulene, aromadendrene, β-patchoulene, α-patchoulene, α-muurolene, nerolidol, β-selinene, δ-cadinol, isobicyclogermacrene, neophytadiene, α-copaene, β-elemene, epi-bicyclosesquiphellandrene, *trans*-β-farnesene, α-gurjunene, γ-cadinene, α-amorphene and sandaracopimaradiene. Alkanes: octadecane, tetracosane, pentacosane, eicosane, heptacosane, nonacosane, triacontane and heneicosane	0.16 ± 0.04*
B	Terpenes: eremophilene, γ-gurjunene, γ-cadinene, fonenol, δ-cadinene and neophytadiene. Fatty acids: methyl laurate, methyl myristate, methyl pentadecanoate, methyl palmitoleate, methyl palmitate, palmitic acid, ethyl palmitate, methyl linoleate, methyl heptadecanoate, 8,11-octadecadienoic acid, methyl ester, methyl linolenate, methyl stearate, linoleic acid, ethyl linoleate, ethyl linolenate, methyl nonadecanoate, methyl arachidate, methyl eicosanoate, ethyl stearate, methyl heneicosanoate and methyl erucate.	0.86 ± 0.07*
C	Terpenes: γ-gurjunene and α-cyperone. Fatty acids: methyl palmitate, palmitic acid, methyl linoleate and methyl linolenate. Miscellaneous: γ-tocopherol	0.74 ± 0.06*
D	Terpenes: linalool, spathulenol, veridiflorol, α-cedrol, δ-cadinene, α-bergamotene, phytol and taraxasterol. Fatty acids: methyl behenate, methyl nervonate, methyl lignocerate, 2-hydroxy-1-(hydroxymethyl) and ethyl linoleate. Alkanes: cyclotetracosane and cyclohexadecane. Miscellaneous: α-tocopherol, 1-octadecanol and 3-eicosene	0.79 ± 0.09*
E	Terpenes: veridiflorol, phytol, squalene, dammaradienol, α-amyrin, β-amyrin and taraxasterol	>1
F	Sterols: stigmast-7-en-3-ol, stigmasterol and β-sitosterol	0.52 ± 0.03*
G	Sterol: stigmasta-5,23-dien-3-ol	0.70 ± 0.08*

Notes: Data are given as the mean of at least three independent experiments ± SD. Differences within and between groups were evaluated by one-way ANOVA test completed by a with a multicomparison Dunnett's test. Acarbose was used as positive control (IC_{50} 0.05 $mg\,mL^{-1}$). *$p < 0.01$ compared with the control experiment.

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Plants are an excellent source of chemical structures with a wide variety of biological activities, including antioxidant and hypoglycaemic properties. The phytochemical and biological investigations should be encouraged, especially in view of the urgent need to discover new bioactive extracts and isolated molecules with greater efficacy and fewer side effects than existing drugs. With this objective, this study showed for the first time the biological properties of different extracts from *S. stabianus*.

Our results clearly show that the ethyl acetate extract has potent antioxidant properties. It is generally accepted that free radicals play an important role in the development of tissue damage and pathological events. A potent antioxidant exhibits a significant peroxyl radical scavenging ability by the donation of its hydrogen atom to the radical species. Natural products with antioxidant activity could retard the

oxidative damage of the tissue by increasing those defences in different degenerative diseases such as diabetes. In this regard, the results obtained by *S. stabianus* in the evaluation of the hypoglycaemic activity *via* the inhibition of the carbohydrate-hydrolysing enzymes, α-amylase and α-glucosidase, are also particularly important. *Senecio stabianus n*-hexane extract showed a good and selective α-amylase inhibitory activity. Although we have been able to consider monoterpenes and sesquiterpenes responsible to the hypoglycaemic activity, further studies could be done on the identification of effective active compounds in *S. stabianus*. Moreover, additional *in vivo* studies, to confirm the *in vitro* obtained results, are warranted.

3. Experimental

3.1. *Plant materials*

The aerial parts of *S. stabianus* Lacaita were collected from Calabria in July 2003 and authenticated by Dr N.G. Passalacqua, Botany Department, University of Calabria (Italy). A voucher specimen was deposited in the Botany Department Herbarium, University of Calabria (CLU no. 4482).

3.2. *General experimental procedures*

Gas chromatography–mass spectroscopy (GC–MS) analyses were recorded on a Hewlett-Packard 6890 gas chromatograph equipped with a HP-5 nonpolar capillary column (30 m × 0.25 mm i.d. × 0.25 mm film thickness) and interfaced with a Hewlett-Packard 5973 Mass Selective Detector operating in EI (70 eV). The injector and detector temperatures for the gas chromatograph were 250°C and 280°C, respectively. Column temperature was initially kept at 60°C for 5 min, then gradually increased to 280°C at 16°C min^{-1} and finally held for 10 min at 280°C. Silica gel (VWR International, Italy, 70–230 and 230–400 mesh) was used for column chromatography. Thin-layer chromatography (TLC) analysis was carried out on silica gel GF_{254} plates (VWR International, Italy). Spots were visualised using ultraviolet (UV) light (254 and 365 nm), by spraying them with Dragendorff's reagent (DRG) (Sigma–Aldrich, Italy) and 50% H_2SO_4 followed by heating for 2 min at 120°C.

3.3. *Chemicals*

Methanol, chloroform, dichloromethane, ethanol, *n*-hexane, dimethyl sulphoxide (DMSO), ethyl acetate, H_2SO_4, NH_3, diethyl ether, isopropanol, HCOOH, CH_3COOH, NH_4OH, $AlCl_3$, NaOH, Na_2CO_3 and Zn powered thin-layer chromatography was obtained from VWR International (Milan, Italy). *O*-Dianisidine (DIAN), peroxidase/glucose oxidase (PGO) system, α-amylase from porcine pancreas (EC 3.2.1.1) and α-glucosidase from *Saccharomyces cerevisiae* (EC 3.2.1.20), Folin–Ciocalteu reagent, chlorogenic acid, ABTS, sodium nitrite, DPPH, ascorbic acid and potassium persulphate were purchased from Sigma–Aldrich (Milan, Italy). Acarbose from *Actinoplanes* sp. was obtained from Serva (Heidelberg, Germany). All chemicals and solvents used were of analytical grade.

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3.4. *Extraction procedure*

Dried and powdered aerial parts of *S. stabianus* (200 g) were extracted exhaustively with methanol (48 h × 3) at room temperature to give the crude extract (16.1 g; 8.1%). The crude extract was suspended in H_2O and fractioned with *n*-hexane (3.4 g; 1.7%) and ethyl acetate (0.8 g; 0.4%). The residue was acidified with 2.5% H_2SO_4 and stirred overnight with Zn powder to reduce native or artificial pyrrolizidine alkaloid (PA) *N*-oxides. The aqueous acid solution was basified and extracted with $CHCl_3$ until no more alkaloids could be detected in aqueous phase (TLC, silica gel, eluent CH_2Cl_2–MeOH–NH_3 85 : 14 : 1, detection by DRG and H_2SO_4 50% (v/v)). The combined organic solutions were dried over anhydrous sodium sulphate and evaporated to dryness to yield 0.4 g of alkaloid fraction (0.2%).

3.5. *TLC analysis*

TLC of *S. stabianus* extracts was performed on silica gel 60 (VWR plates, 0.25 mm) in EtOAc–HCOOH–CH_3COOH–H_2O (100 : 11 : 11 : 26, v/v), *n*-hexane–diethyl ether (8 : 2, v/v), EtOAc–isopropanol–NH_4OH (65 : 35 : 20, v/v). The developed TLCs were examined under UV 254 nm and UV 366 nm prior to spraying. Flavonoids were detected in ethyl acetate extract using aminoethanol diphenylborate–PEG 400 mixture and visualised at 365 nm (Brasseur & Angenot, 1986). DRG (Wagner & Bladt, 1996a) was used for alkaloid detection.

Anisaldehyde/H_2SO_4 reagent was used to reveal the presence of terpenoids (Wagner & Bladt, 1996b).

3.6. *Determination of total phenol content*

The amount of total phenolics of *S. stabianus* ethyl acetate extract was determined by the Folin–Ciocalteu method (Gao, Ohlander, Jeppsson, Björk, & Trajkovski, 2000). Briefly, the extract was mixed with 0.2 mL Folin–Ciocalteu reagent, 2 mL of distilled water and 1 mL of 15% Na_2CO_3. The absorbance was measured at 765 nm using a UV-Vis Jenway 6003 spectrophotometer after 2 h incubation at room temperature. The levels of total phenolics content were determined in triplicate.

Chlorogenic acid was used as a standard and the total phenolics content was expressed as chlorogenic acid equivalents in milligram per 100 g of fresh material.

3.7. *Determination of total flavonoid content*

The flavonoid content was determined spectrophotometrically using a method based on the formation of a flavonoid–aluminium complex (Yoo, C.H. Lee, H. Lee, Moon, & C.Y. Lee, 2008). Briefly, 1 mL of the ethyl acetate extract, 5 mL of distilled water and 0.3 mL of 5% (w/v) sodium nitrite were added to a volumetric flask. After 5 min, 0.6 mL of 10% (w/v) $AlCl_3$ was added. Then, at 6 min 2 mL of 1 mol NaOH and 2.1 mL distilled water were added to the mixture and the absorbance at 510 nm was immediately read. Quercetin was chosen as a standard and the levels of total flavonoid content were determined in triplicate and expressed as quercetin equivalents in milligram per 100 g fresh material.

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3.8. n-*Hexane extract fractionation and analysis*

In order to identify the compounds present within the bioactive *n*-hexane extract, this extract was fractionated by silica gel (Merck, 20–45 mm) column chromatography using a stepwise gradient of *n*-hexane and dichloromethane (starting with pure *n*-hexane, ending with dichloromethane, and using the following mixtures (v/v) in between: 95 : 5, 90 : 10, 80 : 20, 70 : 30, 50 : 50). Column fractions were collected and combined by TLC similarity (silica gel plates, *n*-hexane/dichloromethane 9 : 1 and 8 : 2) into seven main fractions A–G, that were analysed by GC–MS.

Identification of the compounds was based on the comparison of the mass spectral data on computer matching against Wiley 138 and Wiley 275 and confirmed by the determination of the retention index. Identification of the constituents was based on the comparison of the mass spectral data on computer matching against NIST 98 and confirmed by the determination of the retention index; modified Van den Dool and Kratz formula was used to calculate the retention index by interpolation between bracketing C_9–C_{31} *n*-alkanes (Adams, 2001).

3.9. *DPPH test*

Radical scavenging capacity was determined according to the technique reported by Wang et al. (1998). Briefly, an aliquot of 1.5 mL of 0.25 mmol DPPH solution in ethanol and 1.5 mL of *S. stabianus* extracts at concentrations ranging from 25 to 400 $\mu g\, mL^{-1}$ were mixed. The mixture was shaken vigorously and allowed to reach a steady state at room temperature for 30 min.

Decolourisation of DPPH was determined by measuring the absorbance at $\lambda = 517$ nm with a UV-Vis Jenway 6003 spectrophotometer.

The DPPH radical scavenging activity was calculated according to the following equation: scavenging activity $= (A_0 - A_1/A_0) \times 100$, where A_0 is the absorbance of the control (blank, without extract) and A_1 the absorbance in the presence of the extract.

3.10. *Antioxidant capacity determined by radical cation*

ABTS assay was based on the method of Re et al. (1996) with slight modifications. ABTS radical cation ($ABTS^+$) was produced by reacting 7 mmol ABTS solution with 2.45 mmol potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The $ABTS^+$ solution was diluted with ethanol to an absorbance of 0.70 ± 0.05 at 734 nm. After addition of 25 µL of sample or Trolox standard to 2 mL of diluted $ABTS^+$ solution, at exactly 6 min absorbance was measured at $\lambda = 734$ nm. The decrease in absorption was used for calculating Trolox equivalent antioxidant capacity (TEAC) values.

A standard curve was prepared by measuring the reduction in absorbance of $ABTS^+$ solution at different concentrations of Trolox. Appropriate blank measurements were carried out and the values recorded. Results were expressed as TEAC.

3.11. *Bioassay for α-amylase inhibition*

The α-amylase inhibition assay method was performed using the method described previously (Tundis et al., 2007). Briefly, a starch solution (0.5% w/v) was obtained

by stirring 0.125 g of potato starch in 25 mL of 20 mmol sodium phosphate buffer with 6.7 mmol sodium chloride, pH 6.9 at 65°C for 15 min. The α-amylase (EC 3.2.1.1) solution was prepared by mixing 0.0253 g of α-amylase in 100 mL of cold distilled water.

Samples were dissolved in buffer to give final concentration from 1 mg mL^{-1} to 12.50 μg mL^{-1}. The colorimetric reagent was prepared mixing a sodium potassium tartrate solution (12.0 g of sodium potassium tartrate, tetrahydrate in 8.0 mL of 2 mol NaOH) and 96 mmol 3,5-dinitrosalicylic acid solution. Control and extracts were added to starch solution and left to react with α-amylase solution at 25°C for 5 min.

The reaction was measured over 3 min. The generation of maltose was quantified by the reduction of 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid, the product being detectable at 540 nm. In the presence of an α-amylase inhibitors, less maltose will be produced and the absorbance value would decrease. Preliminary experiments were carried out to establish optimal conditions and they were found to be: starch 0.25% (w/v); α-amylase 1 unit mL^{-1}; inhibitor concentration 1 mg mL^{-1}.

The α-amylase inhibition was expressed as the percentage of inhibition and calculated by the following equation:

$$\% \text{ Inhibition} = 100 - \left(\frac{[\text{maltose}]\text{test}}{[\text{maltose}]\text{control}} \times 100\right) \pm \text{SD}.$$

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3.12. *Bioassay for α-glucosidase inhibition*

The α-glucosidase inhibition was measured through a modified Sigma–Aldrich bioassay method (Kapustka, Annala, & Swanson, 1981). A maltose solution (4% w/v) was prepared by dissolving 12 g of maltose in 300 mL of 50 mmol sodium acetate buffer. The enzyme solution (EC 3.2.1.20) was prepared by mixing 1 mg of α-glucosidase in 10 mL of ice-cold distilled water. Pepper extracts and lipophilic fractions are dissolved in DMSO to give a final concentration from 1 mg mL^{-1} to 5 μg mL^{-1}.

The colorimetric reagent DIAN solution was prepared by dissolving one tablet in 25 mL of distilled water, while the PGO system-colour reagent solution was prepared fresh by dissolving one capsule in 100 mL of ice-cold distilled water. In the first step, both control and samples were added to maltose solution and left to equilibrate at 37°C. The reaction was started by adding α-glucosidase solution and tubes were left to incubate at 37°C for 30 min. After that time, perchloric acid solution (4.2% w/v) was added to stop the reaction. In the second step, the generation of glucose was quantified by the reduction of DIAN. The supernatant of tube of first step was mixed with DIAN and PGO and was left to incubate at 37°C for 30 min. The absorbance of DIAN was measured spectrophotometrically at 500 nm. The α-glucosidase inhibition was expressed as the percentage of inhibition and calculated by the following equation:

$$\% \text{ Inhibition} = 100 - \left(\frac{[\text{glucose}]\text{test}}{[\text{glucose}]\text{control}} \times 100\right) \pm \text{SD}.$$

3.13. *Statistical analysis*

All experiments were carried out in triplicate. Data were expressed as means ± SD. Differences were evaluated by the one-way analysis of variance (ANOVA) test completed by a multicomparison Dunnett's test. Differences were considered significant at $^{**}p < 0.01$.

The IC_{50} was calculated by a nonlinear regression curve with the use of Prism Graphpad Prism Version 4.0 for Windows, GraphPad Software, San Diego, CA, USA. The dose–response curve was obtained by plotting the percentage of inhibition *versus* the concentrations.

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