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Determination of Antioxidant Activities of Two Endemic *Allium* Species From Turkey: *A. sibthorpianum* and *A. Stylosum*

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Abstract
In this study, two endemic *Allium* species, *A. sibthorpianum* and *A. stylosum* from Denizli and Mugla regions were examined for their total phenolic content and antioxidant activities. The total phenolic content in different solvent extracts obtained from bulbs and leaves of two *Allium* species was determined colorimetrically by using the Folin-Ciocalteau reagent. The antioxidant activities of the extracts (ethanol, methanol, acetone and petroleum benzine) were determined by their ability to inhibit linoleic acid peroxidation (β-carotene/linoleic acid assay) and DPPH radical scavenging activity. *A. stylosum* was subjected to EI-MS and H-NMR analysis and Apigenin 7,4'-di-methyl ether flavonol glucoside was detected. *A. sibthorpianum* methanol bulbs extract has the highest phenolic content (6.55 mg/gGAE). According to the results of antioxidant activity, bulbs extracts exhibited higher antioxidant activity than leaves extracts from all types of solvent. These results indicated that *A. sibthorpianum* has strong antioxidant properties and this species can be used as a natural antioxidant in food processing and pharmaceutical industries.

Keyword: *Allium stylosum, A. sibthorpiaum,* Antioxidant activity, Total phenolic content, DPPH and β-carotene/linoleic acid.

INTRODUCTION

The species of *Allium sibthorpianum* and *A. stylosum* are the members of *Alliaceae* family. The genus *Allium* comprises of 700 species of bulbous perennials and biennials that occur in temperate regions of the northern hemisphere [18] and 164 of which are available in the Turkish flora; 65 of them being endemic [11, 12,15]. *Allium* L. which a genus the important of between geophyta is creates a group of natural antioxidants. Since ancient times, many *Allium* species, such as onion, garlic, leek and chives, have been used as foods, spices and herbal remedies in widespread areas of the world, especially in the northern hemisphere. It is well known that the *Allium* genus, with about 500 species, is a rich source of steroidal saponins, as well as sulfur-containing compounds [27]. The *Allium* genus is one of the major sources of polyphenolic compounds and the antioxidative activity of some *Allium's* species has been reported and has been mainly attributed to a variety of organo-sulfurous compounds as well as their precursors [24,5]. In particular, *Allium* species are rich source of flavonols, among which quercetin 3,4-O-diglucoside and 4-O-glucoside are the major components. Quercetin is known for its antioxidant and free radical scavenging power and its capability in protecting against cardiovascular disease [6]. Some *Allium* species such as *A. nevsehirense, A. sivasicum, A. dictyoprosum, A. scrodoprosum* subsp. *rotundum,* A.*atroviolaceum, A. cepa* and *A. oschaninii,* A. *sphaerocephalon, A. porrum* were studied biologic activites in previous studies [30, 32, 19, 22].

Free radicals play a vital role in various pathological conditions such as tissue injury, inflammation process and neurodegenerative diseases. Antioxidants have an important role to protect the human body against damage by the free radicals [7]. In the presence of antioxidants, the oxidative rates decrease due to an increased activation energy for reaction, thus increasing the "lifetime" of the substrate, serving as a parameter for the evaluation of the antioxidant activity [8]. Our bodies has a system that recognize and neutralize of free radicals. This system is composed of enzymes and antioxidants, free radicals, cell membranes, and attracts and binds himself attacking the cell components. The importance of antioxidants is increasing in natural food as importance protective medico. Plants and their products are rich sources of phytochemicals and have been found to possess a variety of biological activities including antioxidant potential. Natural antioxidants are shown as the most important source in the treatment of oxidative diseases [2, 33].

In the present study, total phenolic content of extracts prepared from the bulbs and leaves of *A. stylosum* and *A. sibthorpianum* that it is an endemic for the flora of Turkey were determined as mg/gGAE. These extracts (ethanol, methanol, acetone and petroleum benzine) were tested for their antioxidant activity by using two methods namely β-carotene-linoleic acid test system and DPPH free radical scavenging assay. This study examined the antioxidant activities of these species for the first time.

MATERIALS AND METHODS

Plant Material

Both the bulbs and leaves of *A. stylosum* O. Schwarz and *A. sibthorpianum* Schultes & Schultes fil. (Alliaceae) species were collected from Muğla in May and Denizli in August-September 2011. The species identified by the Laboratory of Botanic of Pamukkale University. Their bulbs and leaves were dried, chopped up with a blender and prepared for the experiment.

Preparation of Plant Extracts

10 g of air-dried parts of *A. stylosum* and *A. sibthorpianum* were extracted with with 100 mL of four different solvents (methanol, ethanol, acetone and petroleum benzine) in a shaker water bath at 55°C for 6 h. The extraction was repeated twice at the same condition. These extracts were filtered and the solvents were removed in vacuum by a rotary evaporator at 42-49°C. The water in each extract was frozen in freeze-drying machine and then drawn out. Anhydrous extracts were stored at -20°C until analysis.

Identification of Active Constituent

The active substance of methanol extract of *A. stylosum* was determined using EI-MS and HNMR analysis after thin layer chromatographic separation. EI-MS and H-NMR spectra were used chromatographic method for the determination structure of the purified compound. Active compound was conducted in 30% CH_3COOH system by applying cellulose plates. Plaque stain shows that can be found dark purple under the UV light (366 nm), by keeping NH_3 vapor ejected and marker NA is analyzed.

Determination of Total Phenolic Content

The concentrations of total phenolic content in the crude extracts were expressed as as gallic acid equivalents (mg/gGAE extract) were determined with Folin-Ciocalteu reagent (FCR) according to the method of Slinkard and Singleton [28]. Briefly, 1 ml of the solution extracts (1 mg) was added to 46 ml of distilled water and 1 ml of FCR and was mixed thoroughly. After 3 min, the mixture was added to 3 ml of sodium carbonate (2%) and shaken intermittently for 2 h. The absorbance was measured at 760 nm with spectrophotometer (Shimadzu UV- 1601, Japanese). The total phenolic contents were calculated using standard gallic acid graph. The results were expressed as gallic acid equivalent in microgram per gram dry weight.

β –Carotene/Linoleic Acid Assay

The antioxidant activity of the crude extracts was evaluated using the β-carotene-linoleic acid test system with slight modifications [3]. β-Carotene (0.2 mg) in 1 mL of chloroform was added to 20 µL of linoleic acid and 200 mg of Tween-20 emulsifier mixture. The mixture was then evaporated at 40°C for 10 min by means of a rotary evaporator to remove chloroform. After evaporation of chloroform, 100 mL of distilled water saturated with oxygen, 4.8 mL of this emulsion was placed into test tubes which had 0.2 mg of the sample and 0.2 of the extract in them. For control, 0.2 mL of solvent (methanol, ethanol, acetone and petroleum benzine) was placed in test tubes instead of the extract. As soon as the emulsion was added into the test tubes, initial absorbance was measured with a spectrophotometer (Shimadzu UV- 1601, Japanese) to be at 470 nm. The measurement was carried out at 0.5 h intervals for 2 h. All samples were assayed in triplicate. BHT was used as standards. The antioxidant activity was measured in terms of successful bleaching of β-carotene by using the following equation [1, 14]. The measurements were made using the equation below:

Antioxidant Activity: $[1- (A_0-A_t / A_0^o - A_t^o)] \times 100$

where A_0 and A_0^o are the absorbance values measured at the initial incubation time for samples and control, respectively, While A_t and A_t^o are the absorbance values measured in the samples or standards and control at t½ 2 h.

Scavenging Activity on DPPH (2,2-diphenyl-1-picrylhydrazyl) Free Radical

Free radical scavenging activity of the extracts was determined using the free radical DPPH [20, 31]. In its radical form, DPPH absorbs at 517 nm, but upon reduction by an antioxidant or a radical species its absorption decreases. Briefly, 0.1 mM solution of DPPH in methanol was prepared and 4 mL of this solution was added to 1 mL (0.2 - 1.0 mg) of extracts in methanol, ethanol, acetone and benzine at different concentrations. Thirty minutes later, the absorbance was measured at 517 nm. Synthetic antioxidant BHT (butylated hydroxytoluene) was used as a positive control. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. Free radical scavenging activity was measured using the equation below:

Scavenging activity = $[A_{blank} - A_{sample} / A_{blank}] \times 100$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound.

RESULTS AND DISCUSSION

The ethanol, methanol, acetone and petroleum benzine extracts prepared from the bulbs and leaves were screened for their possible antioxidant activity by using two complementary methods, namely the β-carotene/linoleic acid and DPPH free radical scavenging assays. The total phenolic content of the ethanol and methanol extracts was evaluated by Folin-Ciocalteu assay. Results obtained in the present study revealed that the total phenolic contents in the extracts were considerable (Table 1). Polyphenolic compounds are known to have antioxidant activity and it is likely that the activity of the extracts is due to these compounds [9]. As seen in Table 1, the total phenolic contents of the bulbs ethanol extracts are higher than leaves extracts. The results showed that the total phenolic content from different extracts of leaves and bulbs ranging from 4.18 to 6.55 (mg/gGAE).

Table 1. Total phenolic contents (mg/gGAE) of *A. stylosum* and *A. sibthorpianum* extracts from different parts using various solvents

Extracts	Total Phenolic content (mg/gGAE)			
	A. stylosum		*A. sibthorpianum*	
	Bulbs	Leaves	Bulbs	Leaves
Ethanol	5.26	4.18	5.46	5.04
Methanol	6.39	5.58	6.55	6.18

The highest antioxidant activity was found in the extracts obtained with ethanol solvent from *A. sibthorpianum* bulbs (85.87%). Also, the lowest antioxidant activity was found in the extracts obtained with petroleum benzine solvent from the leaves (45.72%) (Figure 1). The

reason why the extracts obtained from the same plant with different solvents have very different antioxidant activity can be seen in the polarities of the solvents.

Figure 2 and Figure 3 shows the concentration-dependent of DPPH free radical scavenging activity of the methanol, ethanol, acetone and petroleum benzine extracts from leaves and bulbs of these species and standart compounds (BHT). Maximum scavenging activity was found at concentration of 1.0 mg/ml while the minimum scavenging activity was found at 0.2 mg/ml. All extracts possessed free radical scavenging activity but at different levels. The antioxidant activity of the extracts increased with their concentration. The highest scavenging activity was observed in the methanolic bulb extracts of *A. sibthorpianum.* The lowest scavenging activity was recorded on petroleum benzine-leaf extract (15.45%) at 0.2 mg/ml concentration. The effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability. Though the DPPH radical scavenging abilities of the extracts were less than those of BHT (92%) at 1.0 mg/ml, the study showed that the extracts have the proton donating ability and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants. Antioxidants have of great importance for the prevention of oxidative stress that to cause in many illness. Antioxidants can inhibit oxidative reactions in vivo, and aid in functional performance of enzyme systems for self-defence mechanisms within cells [26].

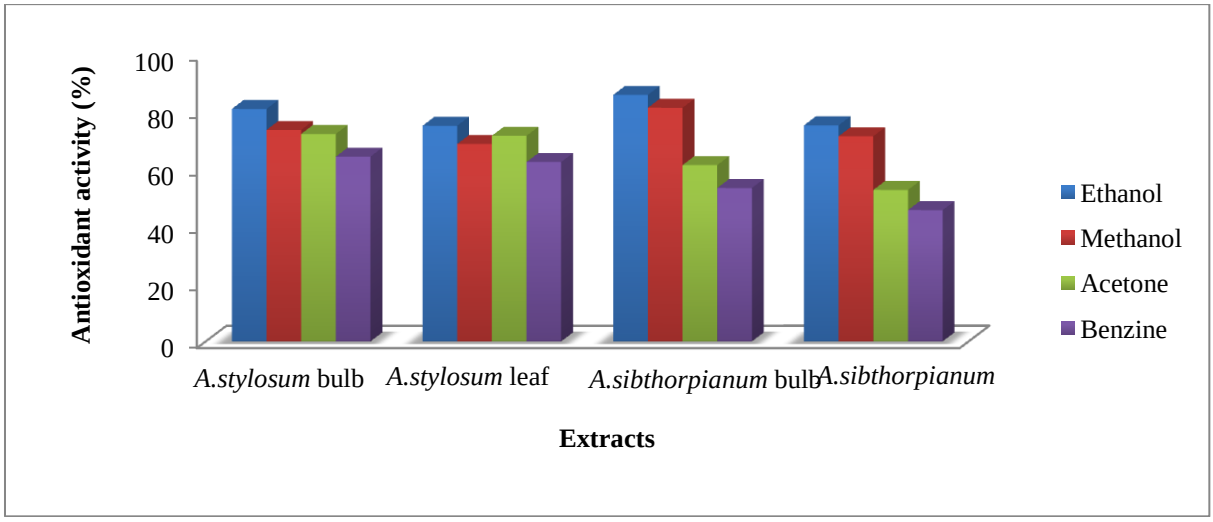

Figure 1. Antioxidant activities efficiency in the methanol, ethanol, acetone and petroleum benzine extracts of of *A.stylosum* and *A. sibthorpianum* extracts by β-carotene/linoleic acid method.

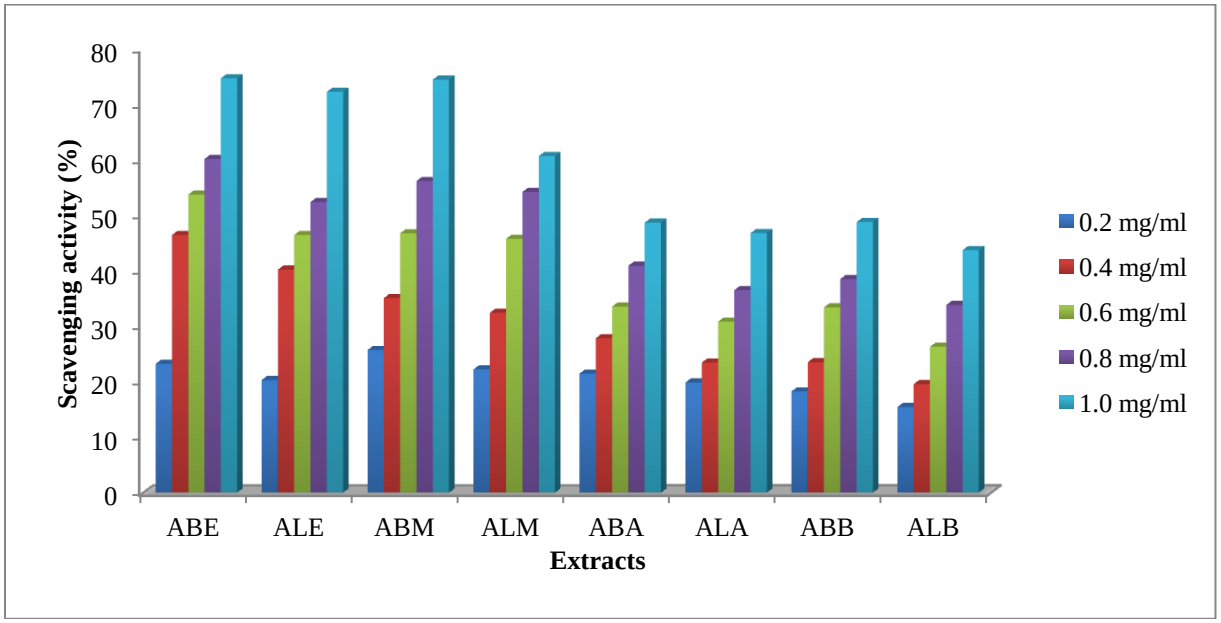

Figure 2. The DPPH free radical scavenging activity of the extracts with methanol, ethanol, acetone and petroleum-benzine through DPPH method. *A.sibthorpianum* (A), Bulb-Methanol (ABM), Bulb-Ethanol (ABE), Bulb-Acetone (ABA), Bulb-petroleum benzine (ABB), Leaf-Methanol (ALM), Leaf-Ethanol (ALE), Leaf-Acetone (ALA), Leaf-petroleum benzine(ALB).

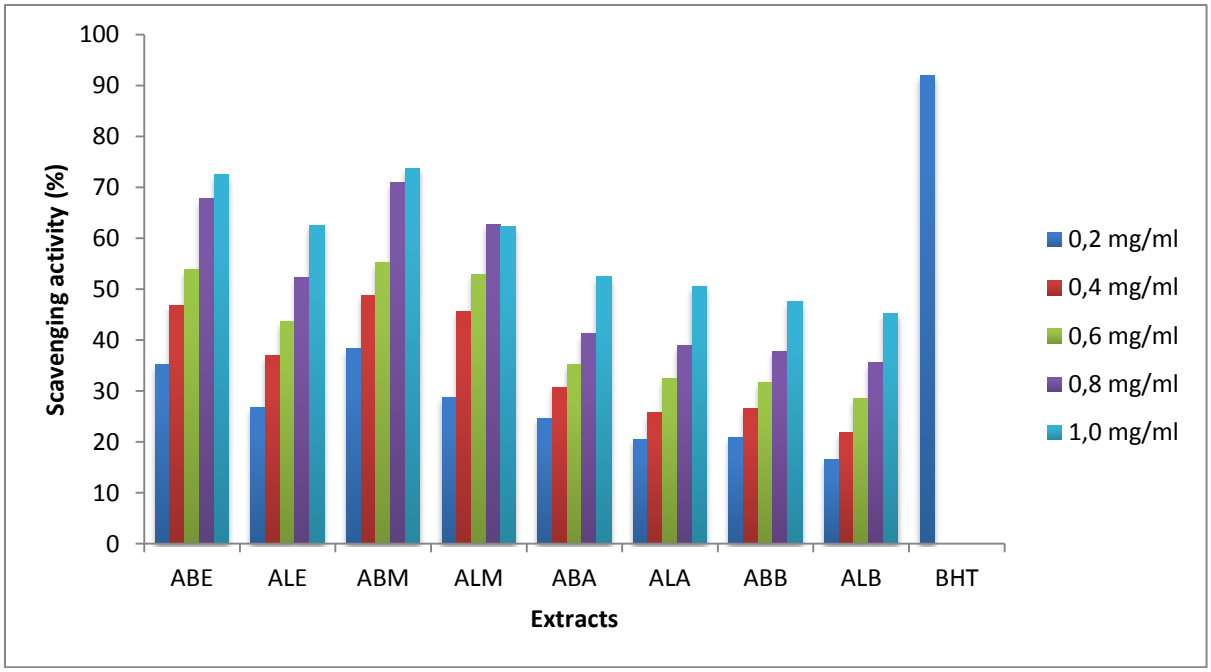

Figure 3. The DPPH free radical scavenging capacity of the extracts with methanol, ethanol, acetone and petroleum-benzine through DPPH method. *A. stylosum* (A), Bulb-Methanol (ABM), Bulb-Ethanol (ABE), Bulb-Acetone (ABA), Bulb-Benzine (ABB), Leaf-Methanol (ALM), Leaf-Ethanol (ALE), Leaf- Acetone (ALA), Leaf-Benzine(ALB).

Analysis of the EI-MS and ^{1}H-NMR spectra data identified a phenolic compound, Apigenin 7,4′-di-metil eter (5 Hidroksi-7,4'-dimetoksiflavon) (Figure 4). Figure 4 (a,b and c) shows the EI-MS and ^{1}H-NMR spectra of flavonol glucosid Apigenin 7,4′-di-metil eter that have been identified as flavonol in the extract under study of *A. stylosum* species. The determination of the active substance was used ^{1}H-NMR and mass spectroscopy methods. ^{1}H-NMR spectrum indicated the presence of the two methoxy groups in the molecule (400 MHz, CDCl3); δ 3.82 (3H, s) and δ 3.83 (3H, s) from the monitored signals. Peak followed at 12.78 ppm (1H, s) by intramolecular C-5 OH groups in the carbonyl group at C-4 in response to make hydrogen bonding. Peak at 6.52 ppm (^{1}H, s) H-3 protons, peak at 6.32 ppm (^{1}H, s) peak at 6.42 ppm H-6 (^{1}H, s) and H-8 protons depicts. dd observed at the 6.96 and 7.78 ppm are the signal protons H-2 ', H-3' (2.35, 11.72 Hz) and H-5 ', H-6' (2.35,11.91 Hz) at position in ring B in. As a result this isolation methods, Apigenin compound 7,4'-di-methyl ether was detected. The structure of the compound is given in Figure 4.

Allium species has analyzed the flavonol composition of the edible portion of six different varieties and consumption typologies of onion bulbs and two varieties of shallot bulbs, employing the high-performance liquid chromatography–diode array detector (HPLC–DAD) coupled with electron spray mass spectrometry (ESI-MS), a fast and accurate technique that allows quantitative and qualitative analyses of these compounds [27].

Phenolic compounds belong to a group of natural substances found in dietary products, and these compounds have gained considerable attention due to their potent antioxidant activity [21]. We demonstrate that the total phenolic content of bulbs extracts is higher than that of leaf extracts. Amount of total phenolic contents were found higher bulbs extract according to the leaf extracts in both plants. Total antioxidant and DPPH free radical scavenging activity high presence in species caused by the presence flavonoid as phenolic compounds especially arise from of the component Apigenin 7,4′-di-metil eter. A previous study suggest that flavonoids possess significant antioxidant potential because of presence of phenolic hydroxyl groups. In addition, many studies are available, which relate analgesic, anti-inflammatory and antioxidant activity with flavonoids by interacting with prostaglandins and superoxides [29]. Ghahremani-majd *et al.* [14] reported that total phenolic contents of *Allium hirtifolium* were found to be 6.5 and 8.4 mgGAE/g extract in the assay [34]. Yumrutas *et al.* were shown that they determined possible *ın vitro* antioxidant activities of MeOH extracts A. tuncelianum. According to their study, A. tuncelianum were shown marked *ın vitro* antioxidant activities [35].

Five *Allium* species (A. *obliquum*, *A.senescens* subsp. *montanum*, *A. schoenoprasum* subsp. *schoenoprasum*, *A.fistulosum* and *A. ursinum*) were analysed in order to determine the presence of 19 polyphenolic compounds through an HPLC method coupled with UV and mass spectrometry detection. Luteolin and apigenin were identified before and after hydrolysis only in *A.obliquum* [36]. Polyphenols, anthocyanins, flavonoids, quercetin, kaempferol and their glycosides have been reported in some Allium species[10, 13].

Researchers have studied the antioxidant activity of many plants [25]. Especially, garlics and different garlic extracts have been shown to have antioxidant activity in different in vitro models. The antioxidant activity of *Allium* plants has been mainly attributed to a variety of sulphur-containing compounds and their precursors [16]. Previous study on the antioxidant activity of *A. roseum* leaves methanolic extract IC_{50} value was 17 times that of BHA. When compared to the synthetic antioxidant BHA, the *A. roseum* leaves methanolic extract exhibited moderate antioxidant abilities to reduce DPPH radicals. According to Nuutila *et al.* [25] the linear correlation between antioxidant activity and polyphenol content underlie the fact that phenolic compounds of *Allium* plants contribute to their antioxidative effects [4, 17, 23].

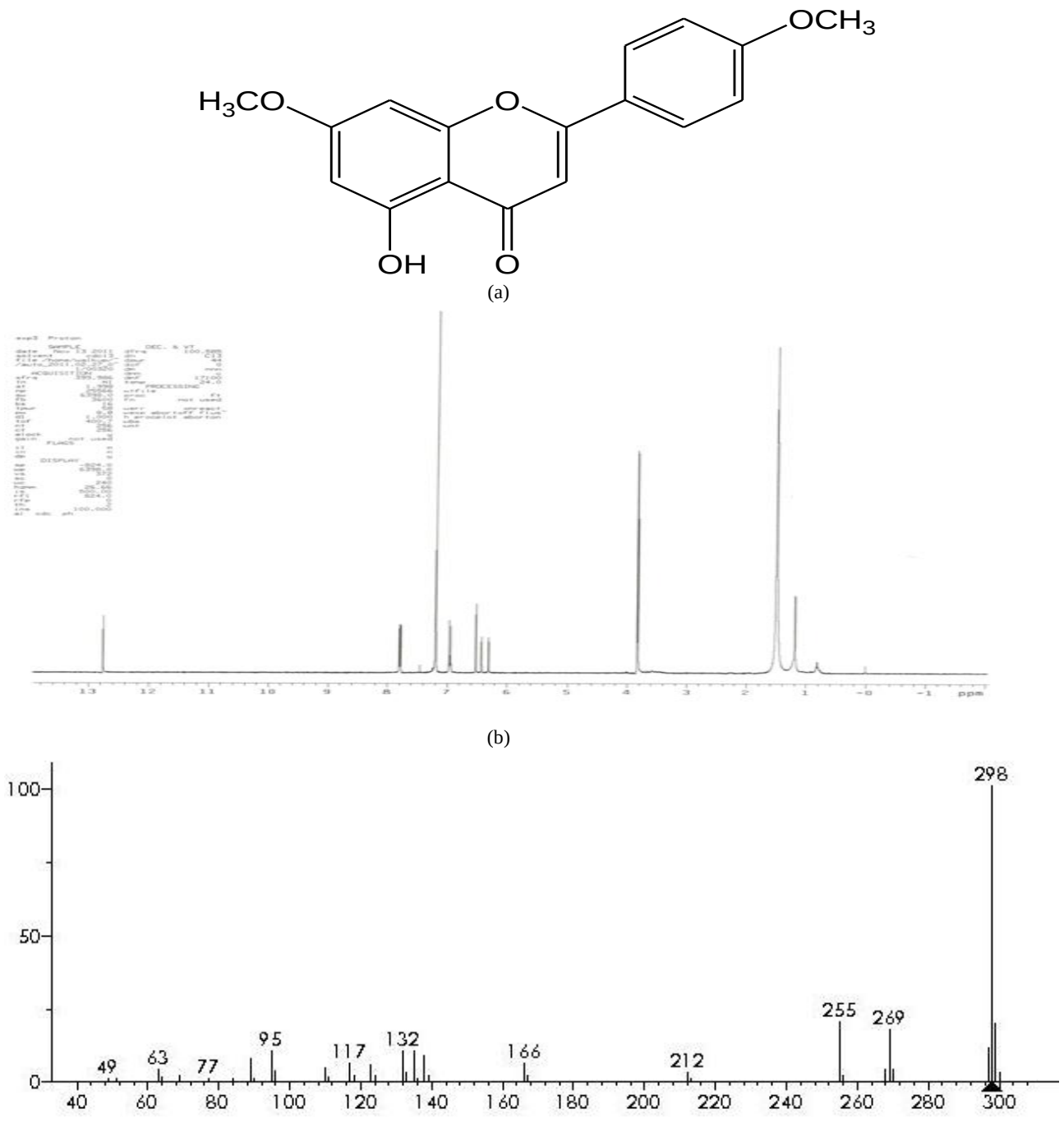

Figure 4. Compound isolated from *A. stylosum*,(a:Apigenin 7,4'-di-metil eter; b:^{1}H-NMR spektrum of active compound; c: EI-MS spektrum of active compound).

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