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ORIGINAL ARTICLE

Phytochemical and taxonomic evaluation of *Rhazya stricta* in Saudi Arabia

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Abstract *Rhazya stricta* Decne is an important medicinal species used in indigenous medicinal herbal drugs to cure various diseases in South Asia (Pakistan, India and Afghanistan) and in the Middle East (e.g. Saudi Arabia, Qatar, United Arab Emirates (UAE), Iran and Iraq). Some of its alkaloids have been reported to have anticancerous properties. The aim of our study is to examine the morphological and taxonomical parameters for *R. stricta* in the Saudi Arabia; concentrations and distributions of some secondary metabolites; and also to determine the antioxidant and free radical scavenging activity. The results of present study showed that there was no influence of environment on the structure of stomata and trichomes as studying species with *R. stricta*. In conclusion our study shows no trichomes on leaf of *R. stricta* it is glabrous, whereas, variations between many secondary metabolites such as flavonoids and phenolic compounds occurred in response to changing climatic conditions.

1. Introduction

Autecology is a branch of ecology that deals with the study of individual species particularly in relation to its environment (Odum, 1971). Autecological studies are necessary to acquire the basic information required for the management of the species in concern. West (1968) outlined the important autecological studies required for rangeland plants. He emphasized six categories of information necessary to understand the autecology of a plant species: (1) taxonomy, (2) genecology, (3) developmental history, (4) ecological relationships, (5) physiology and (6) economic value.

Taxonomy of *Rhazya stricta* Decne (family Apocynaceae) in Saudi Arabia is well documented (Migahid, 1978; Mandaville, 1990; Chaudhary and Al-Jowaid, 1999). It is an evergreen dwarf shrub that is widely distributed in Saudi Arabian rangelands (Mandaville, 1990; Chaudhary and Al-Jowaid, 1999). The vernacular name '*harmal*' is also applied to another noxious weed, *Peganum harmala* (family Zygophyllaceae). The second species is widely distributed in the northern regions of Saudi Arabia whereas *R. stricta* northern range of distribution does not extend that far.

R. stricta grows in depressions with silty and sandy soils sometimes forming a pure stand (Shaltout, 2002). Shaltout (2002) reported that it increased in abundance along gradient of sand. Soils supporting dense stand of *R. stricta* have also been reported to be relatively rich in available magnesium (Mady, 1996).

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No natural hybrids of *R. stricta* are known to exist in Saudi Arabia. However, somatic hybridization between *R. stricta* + *Rauwolfia serpentina* have been used for pharmacological purposes (Kitajima and Shirakawa, 1996; Kostenyuk and Lyubarets, 1992). The aims of the present investigation were to study the morphological and taxonomical parameters for *R. stricta* species in Saudi Arabia; to evaluate the variations in the concentrations and distributions of some secondary metabolites and also to determine the antioxidant and free radical scavenging activity.

2. Materials and methods

Plants were collected from samples of different herbariums. These herbariums are from King Saud University (KSU), King Abdul-Aziz University (KAU), King Abdulaziz city for science and technology (KACST), National Commission for Wild Life Conservation and Development (NCWCD), Ministry of Water (RIY) and collected from different localities of Saudi Arabia- Middle region (Riyadh) and west region.

Study of Morphological Characters

a. Description of morphological and floral characters.
b. The measurements of the whole plant.
c. The measurement of leaf area.
d. The measurement of sepals (calyx) and petals (corolla).

2.1. Study of stomata and trichomes on leaf surface (micro morphology)

Leaves are collected from plant samples of different herbariums. Dried adult leaves are cut into a fragment of 1 cm (West, 1968) in the middle of the lamina and put into a test tube which contains 10% of nitric acid (HNO_3). The test tube was placed in a water bath at 100^0 for 5–10 min. After cooling the fragment was then transferred into a Petri dish filled with distilled water. Both halves of the cuticular membrane were gently brushed to clean them from any remaining pieces of the mesophyll tissue. The fragment was then placed into a watch glass filled with 5% acetic acid for 30 min to bleach. The fragment was washed with distilled water and transferred into 50% alcohol for 2 min; alcin blue for 5 min; alcoholic series (50%, 70%, 80%, 90% and 100%) for 2 min in each series and finally in 1:1 solution of absolute alcohol and Histo clear for 2 min and then in Histo clear for 3 s. After dehydration, the fragment was transferred onto a slide greased with Histo clear and mounted with Canada balsam. Leaf stomata sculpturing and trichomes were studied with Light Microscope Olympus (CX41RF), and photographed with camera mounted on a light microscope (V-TV063XC).

Four species were scanned using Scanning Electron Microscope (SEM). Young leaves (first fully expanded leaf from the tip) and old leaves (third or fourth fully expanded leaf from the tip) were collected from each plant. Plant specimens for SEM were prepared using procedures described by McWhorter et al. (1993). Squares of leaves (with approx. 1 mm thickness of underlying tissues) were excised from the plant, using a razor blade, avoiding the midrib areas so as to give a relatively consistent surface. Leaf segments of approximately 20 mm were fixed for 12 h in 4% glutaraldehyde and rinsed three times with distilled water before dehydration in a graded ethanol series. Samples were dried in a critical point drier and were mounted on aluminum stubs using a two-sided adhesive carbon tape. The samples were then coated with a thin layer of gold. Electron micrograph images were captured using low vacuum scanning electron microscope (JEOL JSM 6060 LV). Electron images were recorded using a digital image processor.

2.2. Extraction, separation and identification of some secondary metabolites by gas chromatography-mass spectrometry

Analysis of some secondary metabolites in plants by using GC–MS (GC MS Hewlett–Packard HP-6890-Series GC System) Plate (7–14) was performed as described by Carmen et al. (2000).

2.2.1. Extraction procedure

The qualitative analysis of some bioactive compounds from plants was made by using gas chromatography (GC) and GC: MS. The liquid–liquid extraction (LLE) method was used. The extraction procedure was the following. One gram of each dried and crushed leaves was sort mixed with 20 ml ethanol and 20 ml distilled water and stored 2 days at room temperature. Then 0.6 ml from these mixtures was mixed with 0.6 ml distilled water and 0.2 ml solvent A (ethyl acetate: hexane: methylene chloride, 5:1:1, v:v:v). The new mixture was agitated for 2 min, and 4 ml of the supernatant was injected into the chromatograph. The GC and GC:MS analyses were performed on the same day with the extraction. The compounds were identified with the mass spectrometer. The extraction procedure of the standard mixture was 30 ml standard mixture in 0.9 ml solution distilled water: ethanol (1:1, v:v), 0.9 ml distilled water and 0.3 ml solvent A were mixed for 2 min and then 1 ml 3-hepten-2-one was added to the supernatant and 1 ml was injected twice by using the auto sampler injector. The standard mixture extractions (n_4) were used to measure the precision and recovery of the extraction procedure. Flavonoid was extracted from the leaves; one gram of dried plants was extracted in 20 ml ethanol for 1 h at 45 °C and 3 ml was injected into GC and analyzed by GC:MS. Standards used were: Catechin, Kaempferol, Luteolin, Apigenin, Pyrrolidine, Quercetin from SIGMA.

2.3. Evaluation of the antioxidant capacity

2.3.1. Superoxide radical scavenging assay

The reaction mixture consisting of 1 mL of nitro blue tetrazolium (NBT) solution (156 mmol L^{-1} NBT in phosphate buffer, pH 7.4), 1 mL NADH solution (468 mmol L^{-1} NADH in phosphate buffer, pH 7.4), and 1 mL of sample solution of extract was mixed. The reaction was initiated by adding 100 mL of phenazine methosulfate (PMS) solution (60 mmol L^{-1} PMS in phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25 °C for 5 min and the absorbance was measured at 560 nm against blank sample and compared with the standards (Gülçin et al., 2005; Nishikimi et al., 1972). Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated.

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2.3.2. Scavenging of hydrogen peroxide

A solution of hydrogen peroxide (40 Mm L^{-1}) was prepared in phosphate buffer (pH 7.4). Different concentrations (250–2500 μg mL^{-1}) were added to the hydrogen peroxide solution (40 Mm L^{-1}, 0.6 mL). Absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide (Gülçin et al., 2005). Percentage scavenging of hydrogen peroxide of the extract and standard compounds was calculated.

3. Results and discussion

3.1. Duration and habitat

Glabrous stout erect perennial shrub with many branches ascending from the base, evergreen, branches are densely leafy, according to earlier studies (McWhorter et al., 1993; Shaby et al., 1985; Boulos, 2000; Mandaville, 1990) (Fig. 1).

3.1.1. Stem

Smooth central stem and dense semi erect branched mainly near the base, ascending according to Western (1989) represented in Table 1.

3.1.2. Leaves

Leaves are sessile and simple, linear-oblong or elliptical, erect nearly, with entire margin and acute apex, thick, leathery and alternate blade tapering toward the base, about 10 cm long and 1.5 cm abroad, to 12 cm on short stalks, with prominent midrib, they have glabrous surface according to earlier studies (McWhorter et al., 1993; Boulos, 2000; Western, 1989) represented in Table 2.

3.1.3. Inflorescences and flowers

Flower bisexual, inflorescences are axillary cymes, found near the tips of branches, flowers are pentamercus, white, 2–2.5 cm long, short-pedicelled, with inserted stamens, flowers with white petals, calyx c. 4 mm long, deeply lobed with acute triangular lobes, corolla 1–1.4 cm, white; tube ± cylindrical; the lobes ovate, with a rounded mucronate apex, c. 12–15 mm long, and also with brownish-green tube expanded somewhat above the middle and longer than the salver form limb, partly occluded by bristles at the throat: lobes of the limb broadly obovate, obtuse, mucronate, white inside and often bluish on back stamens 5, inserted above the middle of the tube; filaments short, anthers lanceolate, disk annular, sometimes absent, style filiform, stigma globose, consistent with earlier studies (Shaby et al., 1985; Mandaville, 1990; Boulos, 2000; Migahid, 1996) represented in Table 3.

R. stricta collected from Riyadh and West region shows anomocytic stomata (Table 4 and Figs. 2 and 3). Information on type of stomata of this plant is not available, however Inamdar et al. (1975) had studied species *Catharanthus roseus* (Apocynaceae) and they found that the leaf is characterized by three types of stomata anomocytic, anisocytic and paracytic stomata.

3.2. GC–MS identification of flavonoid profiles in R. stricta from two geographical regions in Saudi Arabia

Qualitative and quantitative comparison of the flavonoid profiles of studied plants collected from different regions of Saudi Arabia was carried out in order to assess any change in profile number and intensities due to variation catchment area. Flavonoid constituents were quantified by HPLC on C-18 reverse phase column. The absorption spectra resulting from detection were used to distinguish peaks due to the major flavonoids from those of other UV absorbing compounds.

Table 5 evidences that great differences in types and quantity of flavonoid compounds between studied plants and these variations were dependent on plant geographical region. Most of isolated flavonoids were common on most studied plants named Quercetrin, Hesperetin, Kaempferol, Quercetrin-3-rhamnaside, Isoquercitrin, Rutin, Apigenin, Luteolin, Luteolin-7-glucoside, Acacetin and Apigenin-8-Cglucoside. Significant variations including the number and intensities of peak profiles of isolated flavonoids were observed and detected in *R. stricta* plant species

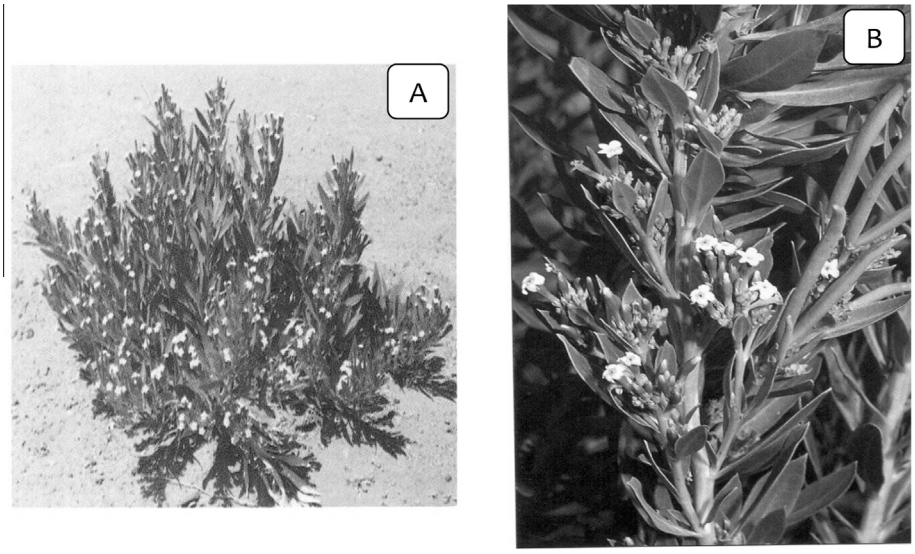

Figure 1 *Rhazya stricta in* Saudi Arabia.

Table 1 Duration, habitat and morphological characters for stem of the studied *Rhazya stricta* species in Saudi Arabia.

Species	Characters													
	Duration		Habitat			Stem								
	Perennial	Annual	Herb	Shrub	Shrublet	Orientation				Defiance				
						Twining	Prostrate	Erect	Climb	Glabrous	Tomentose	Canescent	Pubescent	Woody
Rhazya stricta	+	–	–	+	–	–	–	+	–	+	–	–	–	–

Table 2 Morphological characters for leafs of the studied *Rhazya stricta* species in Saudi Arabia.

Species	Characters																
	Blade										Arrangement		Leaf surface			Attachment	
	Apex				Shape				Margin								
	Obtus	Acute	Triangular-ovate	Oblong-linear	Elliptical	Spathulate	Linear-lanceolate	Rolled	Entire	Crenate	Antennate	Ooposite	Scarbled-hairy	Tomentose	Glabrous	Petiolate	Sessile
Rhazya stricta	–	+	–	–	+	–	–	–	+	–	+	–		–	+	–	+

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Table 3 Floral characters of the studied *Rhazya stricta* species in Saudi Arabia.

Species	Characters																		
	Inflorescence						Flower			Calyx			Corolla						
	Type					Flower attachment				Shape			Color						
	Axillary	Solitary	Terminal	Corymbose	Racemose	Pedicellate	Sessile	Unisexual	Bisexual	Lanculate	Campanulate	Oblong-ovate	Deeply lobed	Yellow	White	Blue	Pink
Rhazya stricta	+	–	–	–	–	+	–	–	+	–	–	–	+	–	+	–	–

collected from different geographical area- the middle region (Riyadh) and west region (Figs. 4 and 5). The absence of many profiles such as Hesperetin, Kaempferol, Rutin, Luteolin, Acacetin and Apigenin-8-C- glucoside was observed in *R. stricta* collected from the middle region (Riyadh), in comparison with, the same species collected from West region.

Variations of climatic conditions between different habitats could be considered as the main cause of the significant variations in the types and amounts of isolated flavonoid profiles. Thereafter, climatic conditions affect greatly the physiological and biochemical reactions that control the biosynthesis of various precursor compounds essential for the biosynthesis of many types of flavonoids. Our results are consistent with Harborne et al. (1975) and Singh et al. (2003). The presence of Quercetrin, Querectin-3-rhamnoside and apigenin in studied plants could be considered as an important taxonomic feature, especially it was found in various amounts in these plants and it is considered as an essential precursor for the formation of tannins. Ziba et al. (2003), Frederick and Hilary (1999), Martin and Broenkow (1989) concluded that, climatic factors affect greatly on the synthesis of secondary metabolites in plants including phenolic compounds, flavonoids, tannins and saponin. These results are consistent with our results and observations about the great variation in types and concentrations of flavonoid profiles in all studied plants. According to our results presented the peaks isolated and separated by GC–MS, we can conclude that, a great variations between many secondary metabolites such as flavonoids occurred in response to changing climatic condition.

A comparative percentage between studied plants showed significant variations in flavonoid contents; especially the difference was clarified in relation to the plant region (Table 6). The flavonols quercetin, kaempferol and isorhamnetin, the flavone luteolin and the hydroxycoumarin cichoriin were identified. It was seen that the aglycone pattern is useful for the delimitation of some species in the genus and correlates with morphological features.

The climatic conditions of the south and middle regions are characterized by more precipitation in comparison with other habitat. These conditions are favorable for the enhancement of photosynthesis and consequently, resulted in the formation of many metabolite precursors that could be involved in the biosynthesis of various secondary metabolites. However, the climatic conditions in the north region are extremely different from south and middle regions and as a result, the biosynthesis of flavonoids in most studied plants was significantly lower than that in the other region in response to the plant species and habitat. On the other hand, Riyadh region which is characterized by lower precipitation and high temperature may cause lower synthesis of these compounds. Our results indicated the presence of Rutin flavonoid in all studied plants, but varied with different plant locations. These results are consistent with Harborne et al. (1986) and Khamis et al. (1997) who illustrated that most plants contain Rutin. Several isolate flavonoids from *R. stricta* such as Isorhamnetin varied within the catchment region. On the other hand Bashir et al., 1994, isolated 3-(6-rhamnosylgalactoside)-7 rhamnosidase from *R. stricta*. Our analysis showed that, phenolic compounds greatly differ between plant species and are affected by the environmental conditions. Moreover the phenolic acids affect greatly on the dynamics biosynthesis of flavonoids in plants (Kumar and Singh, 1993).

Table 4 Types of stomata and trichomes of the *R. stricta* plant species in Saudi Arabia.

Species	Characters			
	Stomata type		Trichome type	
	Anomocytic	Paracytic	Glandular	Non-glandular
Rhazya stricta	+	–	–	–

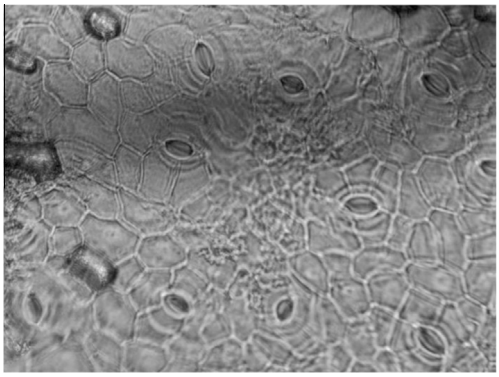

Figure 2 Light micrographs of the leaf surface showing the type stomata of *Rhazya stricta* in Saudi Arabia.

Table 5 Isolated flavonoid compounds in *Rhazya stricta*, collected from different locations in Saudi Arabia.

Rt (min)	Compound	*Rhazya stricta*	
		Ry	W
2.96	Quercetin	+ + +	+ + +
3.25	Hesperetin	–	+ +
9.90	Kaempferol	–	+ +
12.68	Querectin-3-rhamnaside	+ +	+ +
14.26	Isoquercetin	+	–
15.36	Rutin	–	+
16.31	Apigenin	+	+
17.86	Luteolin	–	+
18.30	Luteolin-7-glucoside	–	–
19.72	Acacetin	–	+
24.94	Apigenin-8-C-glucoside	–	+

(W, West; Ry, Riyadh). *[+][+ +][+ + +] The presence of varying amounts, [–]Absent.

3.3. Evaluation of the antioxidant capacity of R. stricta collected from different regions of Saudi Arabia

The extracts obtained were subjected to screening for their possible antioxidant activity. Four complementary test systems, namely superoxide radical scavenging assay, scavenging of hydrogen peroxide, total phenolic compounds, and total flavonoids were used for this purpose.

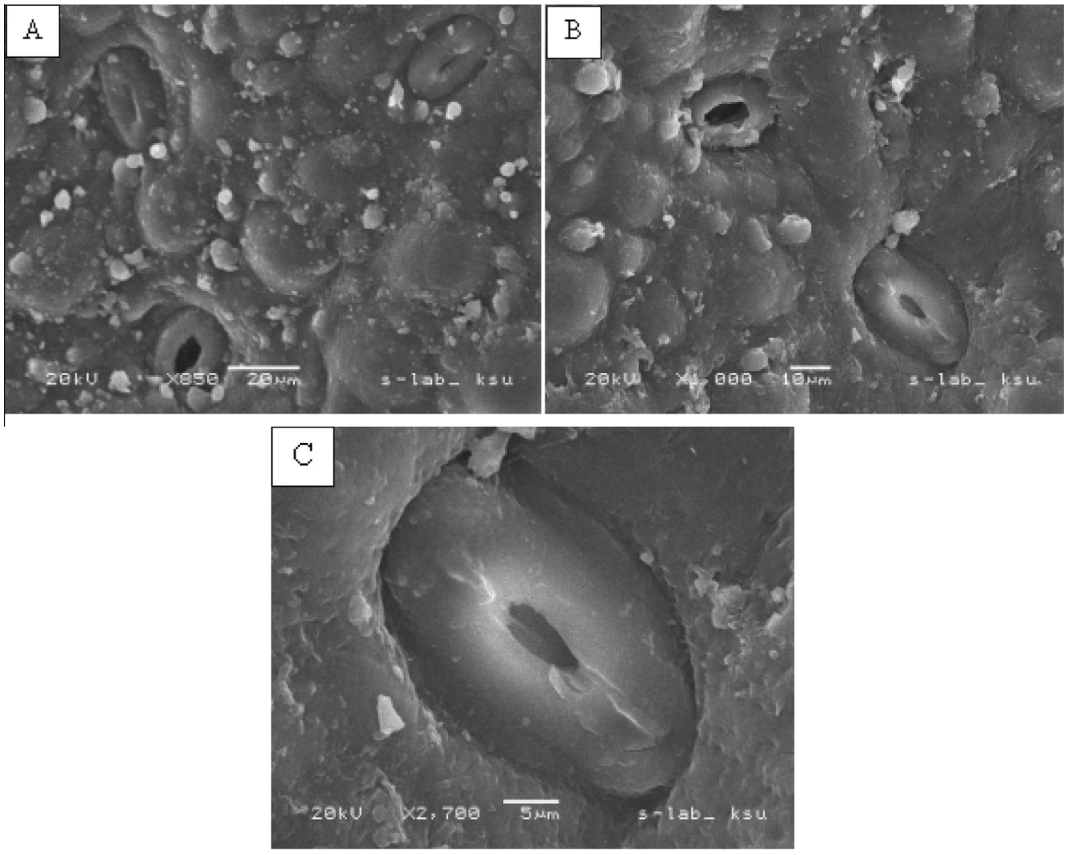

Figure 3 SEM micrographs of the leaf surface showing stomata of *Rhazya stricta* in Saudi Arabia.

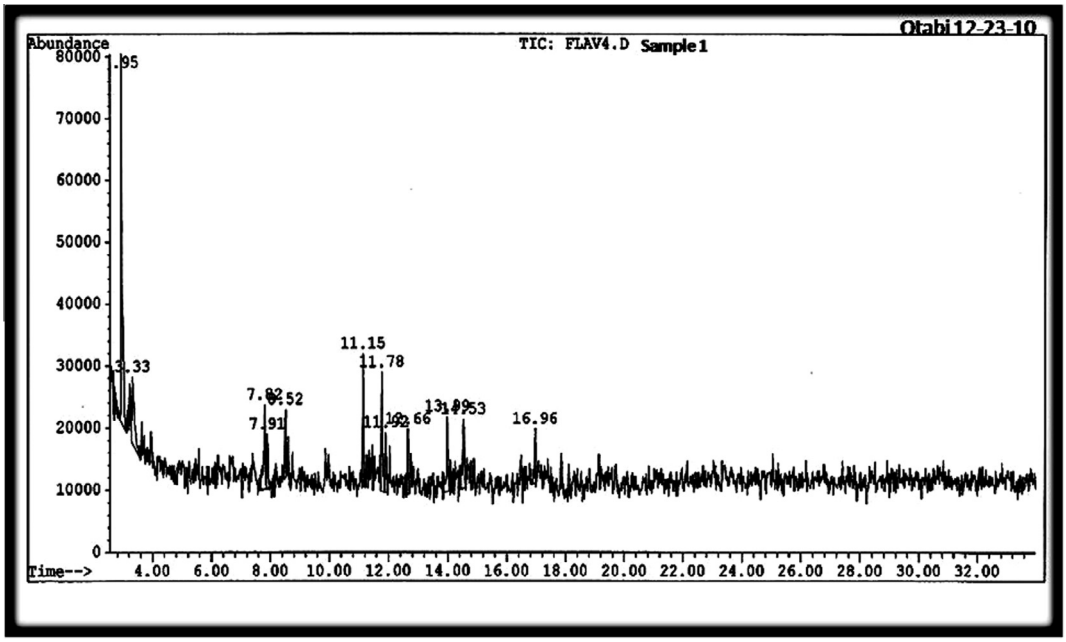

Figure 4 GC–MS of isolated flavonoid compounds in *Rhazya stricta* collected from the middle region (Riyadh) in Saudi Arabia.

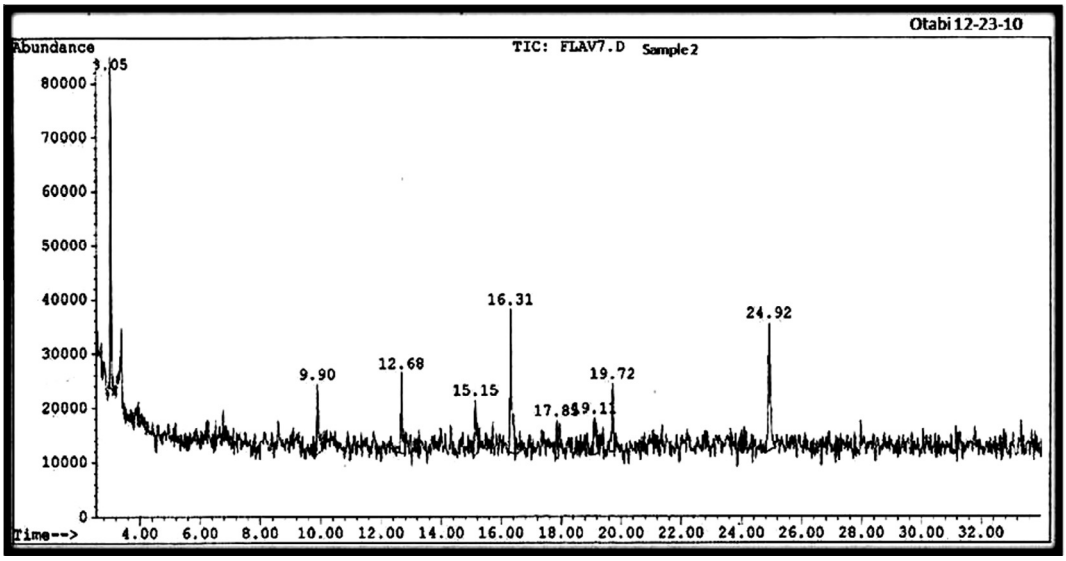

Figure 5 GC–MS of isolated flavonoid compounds in *Rhazya stricta* collected from West region in Saudi Arabia.

Table 6 Variation in Total phenolics & Flavonoids content in *Rhazya stricta*, collected from different locations in Saudi Arabia.

Sample	Location	Total phenolics mg g^{-1} DW*	Total flavonoids mg g^{-1} DW*
Rhazya stricta	The middle region (Riyadh)	62.5 ± 0.2	32.8 ± 0.04
	West region	66.63 ± 0.03	43.7 ± 0.07

*Each value is an average of three replications $p < 0.05$.

3.3.1. Scavenging of hydrogen peroxide

The results are summarized in Fig. 6. The scavenging percentage of hydrogen peroxide was varied significantly within plant types as well as, different plant habitats, for instance, *R. stricta* from Riyadh region has only 35.7% scavenging percentage. Furthermore, it was found that the change in plant habitats ranging from the middle region (Riyadh) to West enhanced the degree of scavenging of hydrogen peroxide from 35.7% to 48.3%. Plants responded to some unfavorable conditions by activating antioxidant defense system, including enzymatic and non-enzymatic constituents. Our results suggested that, the higher percentage of the scavenging of superoxide radicals,

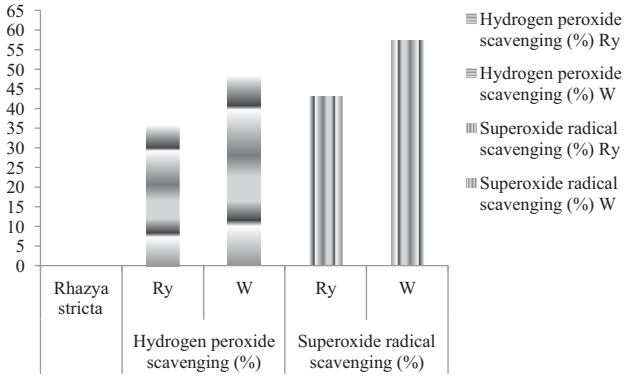

Figure 6 Evaluation of Superoxide radical scavenging assay and scavenging of hydrogen peroxide of *R. stricta* collected from different regions of Saudi Arabia (Ry: Riyadh; W: West).

as well as, the scavenging of hydrogen peroxide indicated the higher activities of antioxidant defense system in collected plants to face the presence of some conditions.

3.3.2. Superoxide radical scavenging assay

The results obtained for superoxide radical scavenging assay of plants are presented in Fig. 6. The results showed that the superoxide radical scavenging assay was varied significantly in plants collected from Riyadh and west region. The corresponding values for *R. stricta* were 43.2% for Riyadh region and it enhanced to 57.4% for west region respectively.

R. stricta (Apocynaceae) is a small shrub, it is used in the indigenous system of medicine as a bitter tonic, for sore throat and in fever. *R. stricta* is commonly used in folk medicine for liver ailments (Collenette, 1999). This plant is distributed in many parts of Saudi Arabia except the high mountains. It is known worldwide as a medicinal plant with economic potentialities (Ali et al., 2000). *R. stricta* became a dominate species in the range land in Saudi Arabia due to its allopathic effects on other rangeland species (Mossa, 1985). The growth and the survival of some rangeland plant seedlings were affected by irrigation water treated with *R. stricta* (Mossa, 1985; Assaeed and Al-Doss, 1996). *R. stricta* is famous for the presence of indole alkaloids which have been shown to possess certain biological activities such as anticancer activity (Mukhobpadhyay et al., 1981, 1983). Studies on the plant growing in Saudi Arabia showed that a total alkaloid fraction was greatly differed according to the plant species and their habitats. Previously it was reported that twenty new alkaloids were identified from *R. stricta*. Recently they have isolated three new indole alkaloids rhazizine, 15-hydroxyzine cadifformine and β-himberine acetate. SEWARINE, a new alkaloid from *R. stricta*, has been shown to be a $C_{20}H_{22}N_2O_3$ compound. *R. stricta* is a medicinal plant used traditionally in some Asian countries. Its components were found to affect some agricultural pest, and *R. stricta* extracts (alkaloids) affect the growth of various pests (Atta and Khanum, 1987; El Hag et al., 1999). *R. stricta* extracts were also found to provide nematicidal activity at a rate of 100 ppm against nematode *Meloidogyne javanica*.

In conclusion our study shows no trichomes on leaf of *R. stricta* as it is glabrous, similarly to Akyalcin et al. (2006) reported in glabrous leaf of *Rhazya orientalis* (Apocynaceae). We can also conclude that, great variations between many secondary metabolites such as flavonoids and phenolic compounds occurred in response to change in climatic conditions.

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