http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 11 (9): 1246-1251, 2008
ISSN 1028-8880

Use of CLSA and SPME-Headspace Techniques Followed by GC-MS Analysis to Extract and Identify the Floral Odorants

[1,3]Mourad Shonouda, [2]Sergio Angeli, [2]Stefan Schutz and [3]Stefan Vidal
[1]AgroEntomology Section, Department of Crop Sciences, University of Göttingen, Germany
[2]Institute for Forest Zoology and Conservation, University of Göttingen, Germany
[3]Department of Zoology, Faculty of Science, Alexandria University, Egypt

Abstract: Flowers of *Ziziphus spina christi* are known to be attractive for parasitoids and predators. In Y-tube olfactometer experiments, the dried flowers attracted significantly ($p<0.001$) the female parasitoids *Aphelinus abdominalis*. The flower volatile compounds were analyzed to understand which compounds could be specifically responsible for this attractiveness. The volatile compounds of *Ziziphus* flowers were extracted by closed-loop-stripping-analysis (CLSA) and also by solid phase microextraction (SPME) followed by gas chromatography-mass spectrometry (GC-MS) analysis. The main chemical classes of the volatile compounds are aldehydes, monoterpene-alcohols, ketones and hydrocarbons. Flower extract and some specific compounds will be further tested for their responsiveness to predators and parasitoids in behavioural and electrophysiological experiments.

Key words: *Aphelinus abdominalis*, behavioral response, floral volatile compounds, *Ziziphus spina christi*, allelochemicals

INTRODUCTION

Ziziphus spina christi L. is considered one of the most important tropical and subtropical fruit crops due to it has nutritional and medicinal values (Mandavillae, 1990). Its edible fruit is highly nutritious and rich in vitamin C. Also, the fruits are used in traditional medicine to treat different disease such as bronchitis, coughs and tuberculosis (Hutchens, 1973). The dried leaves have long been used as a head wash for elongation the hair in eastern Arabia, while, the ash of leaves is used by Bedouin to treat the wounds of snack bite (Boulos, 1980; Sudhersan and Hussain, 2003). Plant leaves are also used in folk medicine as an antiseptic, antifungal and anti-inflammatory agent (Abdelaaty *et al*., 2001). The biological activities of flowers are not known except to produce of wild bee honey (Ghazanfar, 1994).

Recently, it is found the flowers are important source of volatile compounds that attract a wide variety of insects especially the beneficial insects (Shonouda, 2003). Generally, some plants may be using volatile compounds to induce indirect defense by attracting natural enemies (Tooker and Hanks, 2006). The chemical composition of leaves, fruits and seeds of *Z. spina christi* L. was investigated in different studies. The oil of *Z. spina christi* L. leaves had the major components: geranyl acetone, methyl hexadecanoate, methyl octadecanoate, farnesyl acetone, hexadecanol and ethyl octadecanoate (Dweck, 2005). Younes *et al*. (1996) reported that the main constituents of the essential oil from *Ziziphus* leaves were α-terpineol and linalool. Nazif (2002) found that linoleic acid, linolenic acid, cholesterol and β-sitosterol represent the major constituents of the fruits and seeds of *Z. spina christi* and these compounds have antimicrobial activity.

As far as we know, the present work is the first study to give a complete profile of the volatile compounds emitted from the flowers of *Z. spina christi* because floral odors have received little attention to study comparing to other odors especially from infested plants. Present objective is to extract and identify volatile compounds produced by flowers of *Z. spina christi* that attract natural enemies.

MATERIALS AND METHODS

Flowers material: Flowers of *Z. spina christi* L. were collected from a new cultivated area of the coastal region of western desert, 30 km west from Alexandria, Egypt. The flowers were collected freshly during the day and left to dry completely at room temperature in laboratory. After that, the flowers were kept in a freezer at -20°C.

Corresponding Author: Mourad L. Shonouda, Department of Zoology, Faculty of Science, Alexandria University, 21511 Mharam Bey, Egypt Tel: 02/012 2836 811

Y-tube olfactometer bioassay: A Y-tube olfactometer was used to assess the responses and attractiveness of female parasitoids *Aphelinus abdominalis* (Hymenoptera: Aphelinidae) to the source of flower volatiles. The minute size of adult female parasitoids necessitated a Y-tube apparatus of small dimensions (4 cm diameter × 16 cm long stem glass × 13 cm arm glass). One arm was containing 10 g dried flowers and the other was left clean. Purified and humidified air enters each arm of the olfactometer and flows over the respective flower odor in one glass arm and in the other glass arm remains clean air as control. One naiive female parasitoid (2 days old) introduced to the main stem and was left to work until choose one arm. The female parasitoid directed to the end of one arm and stayed without return back was recorded. After each five females the two arms are replaced their position to avoid a directional bias. The experiment was repeated with twenty female parasitoids under room conditions and with fluorescent light (400 watt) over the olfactometer. The data was analysed by using Chi-square test (Zar, 1984).

Extraction of volatiles by CLSA: Samples for GC-MS analysis were collected using the closed-loop-stripping-analysis (CLSA) method (Boland *et al*., 1984). Eighteen gram of dried flowers were enclosed in a polyester cooking bag. The outlet was closed with a PTFE-stopper. Stainless steel capillary (i.d. 1 mm) was fed through the stopper. A miniature 12 V vacuum pump circulated air from the plastic bag to an adsorbent trap loaded with 1.5 mg activated carbon filter. Sampling was performed for 3 h with a flow of 1 l/min at room temperature. Three replicates of dried flower bags were done in addition to an empty plastic bag as control. Volatiles were eluted from the carbon traps with 500 μL of a mixture consisting of methylene chloride (two parts) and methanol (one part). Samples were stored in 1 mL glass vials in deep freezer at -80°C.

Extraction of volatiles by SPME: Solid-phase microextraction (SPME) was applied to extract the volatile chemical compounds (VOCs) emitted from the flowers of *Ziziphus* plant. The sample (6 g dried flowers) was maintained in glass vial (80 mL) adapted for SPME device. An 85 μm Carboxen™/Polydimethylsiloxane (CAR/PDMS) StableFlex™ fiber type (Supelco, Bellefonte, USA) was used as sample preparation. Fiber of SPME device was inserted into the glass vial and exposed to the headspace above the flowers for 1 h at room temperature in order to adsorb the released VOCs. The SPME fiber was injected directly into the GC-MS for separation and identification of compounds. Three replicates of flower samples were done in addition to an empty vial sample as control.

The closed-loop-stripping analysis (CLSA) was selected as a method to identify and quantify the relative abundance of each chemical compound, while solid phase microextraction (SPME) allowed us to other chemical volatiles with low affinity with CLSA-carbon filter. Moreover, the solventless sample method (SPME), was able to verify if CLSA extracts could contain by-products, due to the presence of the methanol- dichloromethane solvents.

GC-MS analysis: The system consisted of a gas chromatography (GC) Agilent, model 6890N connected to a Mass Spectrometer (MS) model 5973N quadrupole. The GC was equipped with a type 7163 autosampler and a split/splitless injector. Data acquisition was done with the MS ChemStation software (Agilent). A HP-INNOWax fused silica column (polar column: 30 m × 0.25 mm (ID) ×0.25 μm film thickness; HP) was used for chemical separation with a helium flow as a carrier gas, set to 1 mL min^{-1}. Samples were injected in a quantity of 1 μL into the injector in the pulsed splitless mode at a temperature of 250°C. The temperature for CLSA samples was programmed for an initial temperature of 50°C, held for 1.5 min, ramp 7.5°C min^{-1} until the temperature of 200°C was reached and held for 5 min. The temperature for SPME samples was programmed for an initial temperature of 40°C, held for 1.5 min, ramp 7°C min^{-1} until the temperature of 200°C was reached and held for 5 min. Helium is used as carrier gas. The GC-MS interface was set at 280°C and the heating sleeve of the ODP was set to 230°C. Preliminary peak identification was made by mass spectra comparison with NIST mass spectral library (National Institute of Standards and Technology, Gaithersburg, MD USA). Authentic standards were then purchased and diluted with methylene chloride to a concentration of 10^{-4}. Mass spectra and retention times of compounds were identified and compared with those of authentic standards.

RESULTS

Behavioral experiments: In Y-tube olfactometer experiments, the female parasitoids were positively attracted to the arm contains dried flowers more than the clean arm. In olfactometer bioassays, 70% (n = 14) of female parasitoids were chosen the flower arm while only 30% (n = 6) of female parasitoids were chosen the clean arm and the difference was highly significant ($\chi^2 = 62.72$, $p<0.001$). The present results showed that dried flowers emitted volatile chemical compounds that induce a behavioural response in the female parasitoids by attracting and directing them to the source of volatiles. According to the obtained positive results, we tried to

collect and to identify the chemical volatile compounds of *Z. spina christi* flowers.

GC-MS analysis: Twenty-six volatile chemical compounds were characterised in CLSA extracts, the identified compounds with their chemical classes were shown in Table 1. The percent area of each chemical compound was calculated as the peak area of individual compound relative to the total peak area. The main types of compounds were six monoterpene-alcohols (22.78%); two hydrocarbons (21.64%); four aldehydes (19.69%); four ketones (18.12%); two esters (3.80%) and four benzene compounds comprising naphthalene, naphthalene derivatives and methyl salicylate (4.98%). D-limonene was the only compound found belongs to monoterpene (6.43%). Additionally, three miscellaneous compounds (2.57%) were also characterised. The most dominant compound was linalool (16.34%), followed by tetradecane (15.97%), 2-undecanone (13.22%) and nonanal (11.56%).

Concerning the second extract method, thirty-three volatile chemical compounds were characterised in SPME samples, the identified compounds with their chemical classes were shown in Table 2. Contrary to the CLSA extract, the main fraction was the one of aldehydes, where eight aldehydes were found (41.20%). The monoterpene-alcohols fraction was composed of 6 compounds (18.71%), where 1-8 cineole was not detected but epoxylinalool was identify only in SPME. Six ketones including 3 new ones were found (14.72%). D-limonene was the only monoterpene found in SPME samples (4.59%) as in CLSA extract. The hydrocarbon fraction was less abundant and only tetradecane was identified (4.65%). The same four benzene compounds (1.58%) were identify as in CLSA extracts. The alcohol fraction was more abundant in SPME including 3 alcohols (3.24%). A new ester, acetic acid hexyl ester, was identified (4.78%), although neither of the two CLSA esters was found. The most dominant compounds were nonanal (16.69%); linalool (12.53%); hexanal (11.69%) and 2-undecanone (7.44%).

DISCUSSION

Y-tube olfactometer was used to measure the attractiveness of the parasitoid *A. abdominalis* to *Z. spina christi* dried flowers. Y-tube olfactometry is an effective bioassay technique for parasitoids species because adults are relatively sedentary and respond to attractants by walking (Tooker *et al*., 2005). Present results demonstrated that *A. abdominalis* is highly attracted to the volatile bouquet of *Z. spina christi* dry flowers, confirming previous studies, where attractiveness to *Z. spina christi* flowers was observed and proved in open-filed experiments (Shonouda, 2003). Therefore the extraction of volatiles of dried flowers was done by using two different methods, which allowed a broad identification of the volatiles emitted by the *Z. spina christi* flowers. A comparative analysis of the two methods showed similarity in the main compounds. There are 22 chemical compounds represented in both analyses (88.22% in CLSA and

Table 1: Identified compounds of flower extract by CLSA

Compound	Retention time	Area (%)	Odor	Chemical class
Hexanal[a]	6.67	0.75	New cut green grass	Aldehyde
Dodecane[b]	8.65	5.67	Odourless	Hydrocarbon
D-limonene[c]	8.67	6.43	Fruity lemon	Monoterpene
1,8 cineole[c]	8.85	1.19	Eucalyptus	Monoterpene-alcohol
3-hydroxy, 2-butanone[d]	10.16	0.89	Butter, fatty	Ketone
6-methyl, 5-haptene-2-one[d]	11.06	0.36	Fruity, sweet	Ketone
1-hexanol[b]	11.17	0.89	New cut green grass	Alcohol
2-nonanone[d]	11.98	3.65	Fruity	Ketone
Nonanal[b]	12.08	11.56	Faint elder flower	Aldehyde
Tetradecane[b]	12.21	15.97	Odourless	Hydrocarbon
Trans-linalooloxide (furanoid)[d]	12.86	1.36	Flowery, sweet	Monoterpene-alcohol
Acetic acid[d]	13.08	0.84	Volatile acidity, vinegar	Carboxylic acid
Cis-linaloloxide (furanoid)[d]	13.35	0.34	Flowery, sweet	Monoterpene-alcohol
Diallyl disulphide[a]	13.64	0.84	Garlic	Sulphide
Decanal[b]	13.89	4.62	Citrus	Aldehyde
Benzaldehyde[b]	14.37	2.76	Candy, sweet	Aldehyde
Nonanoic acid, ethyl ester[a]	14.43	0.85	Wine ether	Ester
Linalool[c]	14.49	16.34	Flowery, sweet	Monoterpene-alcohol
2- undecanone[a]	15.48	13.22	Strong fruity	Ketone
Lavandulol[e]	16.55	2.59	Fresh floral or fruity	Monoterpene-alcohol
α-terpineol[a]	16.97	0.96	Flowery, faint sweet	Monoterpene-alcohol
Naphthalene[c]	17.87	3.01	Coal tar, strong smell	Benzene compound
Methyl salicylate[b]	18.31	0.66	Minty, sweet	Benzene compound
Naphthalene, 2-methyl[a]	19.50	0.79	Coal tar, strong smell	Benzene compound
Naphthalene, 1-methyl[d]	20.01	0.52	Coal tar, strong smell	Benzene compound
Hexadecanoic acid, methyl ester[d]	24.61	2.95	Butter smell	Ester

[a-e]Represents the chemical company of authentic standards as follows: a) Aldrich, b) Acros, c) Merck, d) Fluka, e) ABCK

Table 2: Identified compounds of flower extract by SPME

Compound	Retention time	Area (%)	Chemical class
Hexanal	8.07	11.69	Aldehyde
Heptanal	10.38	4.39	Aldehyde
D-limonene	10.72	4.59	Monoterpene
Acetic acid, hexyl ester	12.35	4.78	Ester
3-hydroxy, 2-butanone	12.64	2.66	Ketone
Octanal	12.76	2.71	Aldehyde
6-methyl, 5-haptene-2-one	13.81	1.74	Ketone
1-hexanol	13.99	1.76	Alcohol
2-nonanone	14.98	1.63	Ketone
Nonanal	15.10	16.69	Aldehyde
Tetradecane	15.33	4.65	Hydrocarbon
Trans-linalooloxide (furanoid)	16.12	2.28	Monoterpene- alcohol
Acetic acid	16.34	3.82	Carboxylic acid
Cis-linaloloxide (furanoid)	16.67	1.19	Monoterpene- alcohol
Diallyl disulphide	17.02	2.64	Sulphide
Decanal	17.35	1.68	Aldehyde
Benzaldehyde	17.92	2.82	Aldehyde
Linalool	18.17	12.53	Monoterpene- alcohol
Lilac aldehyde	18.51	0.94	Aldehyde
2,3-butanediol	18.65	1.13	Alcohol
2- undecanone	19.35	7.44	Ketone
Butyrolactone	20.04	0.73	Ketone
Lavandulol	20.75	1.49	Monoterpene- alcohol
α-terpineol	21.18	0.45	Monoterpene- alcohol
Naphthalene (Tar camphor)	22.22	0.85	Benzene compound
Epoxylinalool	22.34	0.77	Monoterpene- alcohol
Methyl salicylate	22.83	Trace	Benzene compound
Cuminaldehyde	22.91	0.28	Aldehyde
α-ketone	24.10	0.30	Ketone
Naphthalene 2-methyl	24.24	0.36	Benzene compound
Benzyl-alcohol	24.44	0.35	Alcohol
Naphthalene 1-methyl	24.87	0.37	Benzene compound
β-ionone	25.61	0.22	Ketone

82.60% in SPM). Additionally, there are 4 compounds present only in CLSA while there are 11 compounds present only in SPME. The main groups represented in each method are monoterpene-alcohols (mainly linalool); aldehydes (mainly nonanal in addition to hexanal only in SPME); ketones (mainly 2-undecanone) and hydrocarbons (mainly tetradecane). These four chemical classes represent about 82.25% of the identified compounds in CLSA, while represent 79.28% of the identified compounds in SPME. It seems that *Z. spina christi* trees are characterized by linalool and α-terpineol compounds because they were also detected as major components in the oil of plant leaves (Younes *et al*., 1996). Other chemical compounds belonging to different chemical classes were represented in both methods such as: one monoterpene (D-limonene); one carboxylic acid (acetic acid); one sulphide (diallyl disulphide); four benzene compounds (naphthalene, 1-methyl-naphthalene, 2-methyl-naphthalene, methyl salicylate); two esters (nonanoic acid, ethyl ester and hexadecanoic acid, methyl ester) in CLSA while one ester only (acetic acid, hexyl ester) in SPME. In addition to one alcohol (1-hexanol) in CLSA, other two alcohols were found in SPME (2,3-butanediol and benzyl-alcohol). The comparative analysis showed that the two different methods did not substantially affect the quality of chemical components, however, the quantity of chemical components, in term of relative abundance, were affected by the type of methods. For instance, the dominant chemical class in CLSA was monoterpene-alcohol (22.78%) while in SPME was aldehyde (41.20%). Within the aldehyde fraction the SPME showed a higher affinity for low molecular weight aldehydes, with a strong increase in the relative abundance of hexanal from 0.75% in CLSA to 11.69% in SPME. Similar tendency is true for nonanal from 11.56% in CLSA to 16.69% in SPME, while, decanal present in a lower percent in SPME (1.68%) in compare to CLSA (4.62%).

The smell description of each identified volatile compound was also included (Table 1) because *Z. spina christi* flowers released a characteristic unique odor. Most of the identified volatile compounds are characterized by flowery, fruity and sweet smell odors. Some of the identified volatiles are actually used in fragrance industry and perfumery as the monoterpene alcohols (Dweck, 2005).

The strong odor of a bouquet of volatile chemicals emitted from *Z. spina christi* flowers may be responsible for modifying the behavior of different natural enemies. According to a previous work, natural enemies belong to order Diptera and Hymenoptera are the most attracted

insects to *Ziziphus* plant during flowering season (Shonouda, 2003). Also, in *Z. mauritiana* flowers it was reported that the strong scent attracted hundreds of insects (Alves *et al*., 2005). The major constituent of *Z. mauritiana* flower was benzaldehyde while the minor constituents were aliphatic carboxylic acids, benzoids, aldehydes, hydrocarbons and oxygenated monoterpenes. In the present study a variety of chemical compounds belongs to different chemical classes with allelochemical effects on natural enemies were identified. The most interesting identified compound in *Z. spina christi* flower volatiles is methyl salicylate, which plays an important role in external plant stress signaling (Kessler and Baldwin, 2001; Bi *et al*., 2007). It has been demonstrated that several plant species may release minute amount of this volatile when they are under attack of herbivorous insects as an allelochemical for the recruitment of beneficial insects, therefore the phenomenon, is known as cry for help (Forouhar *et al*., 2005). The second important compound, 6 methyl, 5-haptene-2-one, was found in the volatiles of *Ziziphus* flowers. This compound is usually induced by cis-jasmone when the plant attacks by aphids and is increasing foraging by parasitoids (Pickett *et al*., 2005). However, *Ziziphus* plant emits this compound without any infestation by aphids. Plants may be using several lines of defense based on biosynthesis pathways to protect themselves against herbivores insects (Thaler *et al*., 2002). It seems that *Z. spina christi* employ naturally indirect defense by secreting methyl salicylate and 6 methyl, 5-haptene-2-one in minor amount to attract variety of natural enemies. In addition to these two interesting compounds, there is also linalool and linalool oxide compounds which are characteristic for most flowers and their allelochemical effect were proved on different beneficial insects (Du *et al*., 1998; Georgieva *et al*., 2005).

We could conclude that *Z. spina christi* is adopting a peculiar ecological strategy, by calling natural enemies even if no pest insects are present, as an opportunistic ecological safety measurement. However, to demonstrate this phenomenon more studies have to be carry out, in term of chemical interactions between first trophic level (host plant) and third trophic level (natural enemies). A current research is now conducted to study the electophysiological and behavioural responses of different natural enemies to the *Z. spina christi* flower extract and its chemical volatiles.

ACKNOWLEDGMENT

We would like to thank the Arab Fund for Economic and Social Development in Kuwait for supporting this research project.

REFERENCES

Abdelaaty, A.S., L. Pieters, S. Apers, N. Nazeif, N. Abdel-Azim, D. Berghe and A. Vlietink, 2001. Chemical and biological investigations on *Ziziphus spina christi* L. Phytother. Res., 15 (7): 593-597.

Alves, R.J.V., A.C. Pinto, D.V.M. Coste and C.M. Rezende, 2005. *Ziziphus mauritiana* Lam. (Rhamnaceae) and the chemical composition of its floral fecal odor. J. Braz. Chem. Soc., 16 (3B): 654-656.

Bi, H.H., R.S. Zeng, L.M. Su, M. An and S.M. Luo, 2007. Rice allelopathy induced by methyl jasmonate and methyl salicylate. J. Chem. Ecol., 33 (5): 1089-1103.

Boland, W., P. Ney, L. Jaenicke and G. Gassmann, 1984. A Closed-loop-stripping Technique as a Versatile Tool for Metabolic Studies of Volatiles. In: Analysis of Volatiles, Schreier P. (Ed.). Walter de Gruyter and Co, Berlin, pp: 371-380.

Boulos, L., 1980. Medicinal Plants of North Africa. Reference Publications Inc., Michigan, pp: 151-153.

Du, Y., G.M. Poppy, W. Powell, J.A. Pickett, L.J. Wadhams and C.M. Woodcock 1998. Identification of semiochemicals released during aphid feeding that attract parasitoid *Aphidius ervi*. J. Chem. Ecol., 24 (8): 1355-1368.

Dweck, A.C., 2005. A review of *Ziziphus spina christi*. Personal Care Mag., 6 (3): 53-55.

Forouhar, F., Y. Yang, D. Kumar, Y. Chen, E. Fridman, S. Park, Y. Chiang, T. Acton, G. Montelione, E. Pichersky, D.F. Klessig and L. Tong, 2005. Structural and biochemical studies identify tobacco SABP2 as a methyl salicylate esterase and implicate it in plant innate immunity. Proc. Nat. Acad. Sci., 102: 1773-1778.

Georgieva, E., N. Handjieva, S. Popov and L. Evstatieva 2005. Comparative analysis of the volatiles from flowers and leaves of three *Gentiana* species. Biochem. Syst. Ecol., 33 (9): 938-947.

Ghazanfar, S.A., 1994. Handbook of Arabian Medicinal Plants. Boca Raton, CRC Press.

Hutchens, A.R., 1973. Indian Herbalogy of North America. Shambhala: Boston, pp: 382.

Kessler, A. and I.T. Baldwin, 2001. Defensive function of herbivore-induced plant volatile emissions in nature. Science, 291: 2141-2144.

Mandavillae, J.P., 1990. Flora of Eastern Saudi Arabia. London: Kegan Paul International.

Nazif, N., 2002. Phytoconstituents of *Ziziphus spina-christi* L. fruits and their antimicrobial activity. Food Chem., 76: 77-81.

Pickett, J.A., M.A. Birkett, T.J.A. Bruce, K. Chamberlain, R. Gordon-Weeks, M.C. Matthes, C.B. Moraes, J.A. Napier, L.E. Smart, L.J. Wadhams and C.M. Woodcock, 2005. Cis-Jasmone as an allelopathic agent through plant defence induction. Proceeding of the Fourth World Congress on Allelopathy, August 21-26, Waaga Waaga, Australia, at www.regional org.au/allelopathy/2005/1/3/2481.

Shonouda, M.L., 2003. Insects associated with *Ziziphus* plants during the flowering and non-flowering seasons. Allelopathy J., 12 (2): 215-220.

Sudhersan, C. and J. Hussain, 2003. *In vitro* clonal propagation of a multipurpose tree, *Ziziphus spina christi* (L.) Desf. Turk. J. Bot., 27: 167-171.

Thaler, J.S., M.A. Farag, P.W. Pare and M. Dicke, 2002. Jasmonate-deficient plants have reduced direct and indirect defences against herbivores. Ecol. Lett., 5: 764-774.

Tooker, J.F., A.L. Crumrin and L.M. Hanks, 2005. Plant volatiles are behavioral cues for adult females of the gall wasp *Antistrophus rufus*. Chemoecology, 15: 85-88.

Tooker, J.F. and L.M. Hanks, 2006. Tritrophic interactions and reproductive fitness of the prairie perennial *Silphium laciniatum* Gillette (Asteraceae). Environ. Entomol., 35 (2): 537-545.

Younes, M.E., M.S. Amer and A.D.E. El-Messallami, 1996. Phytochemical examination of the leaves of the Egyptian *Ziziphus spina christi* (Nabc). Bull. Nat. Res. Center (Cairo), 21 (1): 35-40.

Zar, J.H., 1984. Biostatistical Analysis. 2nd Edn. Prentice-Hall Inc., Englewood Cliffs, New Jersey.