Journal of Medical Sciences

ISSN 1682-4474

science alert

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Research Paper

J. Med. Sci., 2 (3): 119-123
May - June, 2002

JMS (ISSN 1682-4474) is an International, peer-reviewed scientific journal that publish original article in experimental & clinical medicine and related disciplines such as molecular biology, biochemistry, genetics, biophysics, bio-and medical technology. JMS is issued six times per year on paper and in electronic format.

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Phytochemical Investigation of Biologically Active Fractions of *Astragalus spinosus* Roots Grown in Egypt

Fikria A. Darwish

The research work was undertaken to asses activity directed isolation of plant extracts, column fractions and isolated glycosides of *Astragalus spinosus* roots (Fabaceae) in the treatment of induced hepatic, renal and cardiac toxicities. A single subcutaneous dose of CCl_4 (5.93 ml kg^{-1} b.wt. which represents 24h LD_{100}) served as a toxicant, was used during this study. Ethyl acetate fraction was the most active fraction, then butanol, aqueous and dichloromethane fraction. By subjecting the ethyl acetate fraction to column chromatography (CC) it afforded two column fractions A and B which have effect in reducing CCl_4-induced mortalities. Rechromatography of fractions A and B on medium pressure liquid chromatography (MPLC) and CC, respectively, afforded (a mixture of isoastragaloside I and trigonoside I) and cycloastragenol-6-0-glucoside. The structure of the isolated glycosides was determined by ^1H, ^{13}C NMR, EIMS and FABMS spectral data, together with co-TLC with reference materials. The efficacy of the isolated glycosides (isoastragaloside I and trigonoside I), cycloastragenol-6-0-glycoside and adsurgenic acid (recently isolated from the ether extract) in reducing CCl_4-induced mortalities and some disturbances in biochemical parameters (liver transaminases AST, ALT and creatine kinase CK activities, serum urea and serum creatinine) were investigated in adult female *Bufo regularis* (Egyptian toads). The bioassay studies revealed that the effect of the glycosides on reducing CCl_4-induced mortality have the following order (cycloastragenol-6-0-glycoside > isoastragaloside I and trigonoside I > adsurgenic acid. The biochemical studies confirmed the bioassay studies. This is the first report on the identification of trigonoside I in *Astragalus spinosus* plant and the presence of isoastragaloside I and cylcoastragenol-6-0-glycoside in *A. spinosus* roots. This suggested that these saponin glycosides may be promising in modulating CCl_4-induced lethality and most of its toxic effects.

Key words: A. *spinosus roots, Bioassay*, biochemical activity, isoastragaloside I, trigonoside I and cycloastragenol-6-0-glycoside adsurgenic acid

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Introduction

Astragalus L. (Fabaceae) is one of the largest and most widely distributed genera of about 2000 species, mainly in the North Temperate regions and Tropical African mountains (Evans, 1998 and Boulous, 1999). The genus *Astragalus* has 37 species indigenous to Egypt. *Astragalus spinosus* Vahl. is a perennial spiny herb growing in Egypt (Hutchinson, 1973 and Tackholm, 1974).

Some *Astragalus* species are used as forage for livestock and wild animals particularly in Asia, others are used as foods, cosmetics, source of vegetables (Rizk, 1986). A number of *Astragalus* species are used in Chinese traditional medicine as antiperspirant, antihypertensive, antidiabetic, diuretic and tonic (Konoshima, 1996; Hikino *et al.*, 1976). They also have hepatoprotective, antioxidative, immunostimulant and antiviral properties, whereas others have shown toxic activity (Hartwell, 1970) and have been used for the treatment of leukemia and uterine cancer. In many cases the cytotoxic principles can pass to humans through milk and meat (Rios and Waterman, 1997).

From the aerial parts of *A. spinosus*, five astragalosides were previously isolated namely, *astragaloside* I, *isoastragaloside* I, *astragaloside* II, *astragaloside*IV and cycloastragenol-6-0-glucoside (EL-Sebakhy *et al.*, 1990 and Abdallah *et al.*, 1993). Recently, adsurgenic acid was isolated for the first time from the ether extract of the same plant (Darwish, 2002).

Carbon tetrachloride (CCl_4) is commonly used as a typical toxicant reflecting the various aspects of toxicity. Doses of CCl_4 in the range of several ml kg^{-1} body weight produce liver necrosis within one or two hours (Edwards *et al.*, 1993). Also, it was known to cause damage to lungs, kidneys, adrenal and central nervous system in humans and experimental animals (Rechnagel, 1989; Zhao and O' Brien, 1996; Wong *et al.*, 1998).

The study was planned to asses the ability of the fractionated plant extracts, column fractions, and isolated glycosides of the dried roots of *Astragalus spinosus* to prevent or reduce the CCl_4-induced mortality, hepato-renal dysfunctions and cardiac toxicity.

Materials and Methods

The research work was conducted at Faculty of Pharmacy, Faculty of Science, Alexandria University, Alexandria, Egypt during 1999 to 2002.

Plant material: Fresh flowering and fruiting plant of *A. spinosus* Vahl. were obtained from area around Alexandria-Cairo Desert Highway at Kilo/65. The plant was identified by Late Prof. V. Tackholm, Faculty of Science, Cairo University. Voucher specimen has been deposited at the herbarium of Faculty of Science, University of Alexandria, Egypt.

NMR spectra were recorded on Bruker AMX 500 (^{1}H NMR 500 MHZ, ^{13}C NMR 125MHz), chemical shifts are expressed in δ values with TMS as internal standard. Mass spectra were carried out on Varian MAT 311 A, FABMS using NBA as matrix. MPLC: Gilson pump, silica gel 60 (15-40 μm Merck). Column chromatography using silica gel 60 (Merck).

TLC was performed on precoated silica gel 60 F_{254} plates (Merck). Solvent system ethyl acetate-glacial acetic-water (5:1:1) and dichloromethane-methanol (9:1), (8.5:1.5) and (8:2). Detection was carried out by spraying with anisaldehyde-sulphuric and heating at 120 °C.

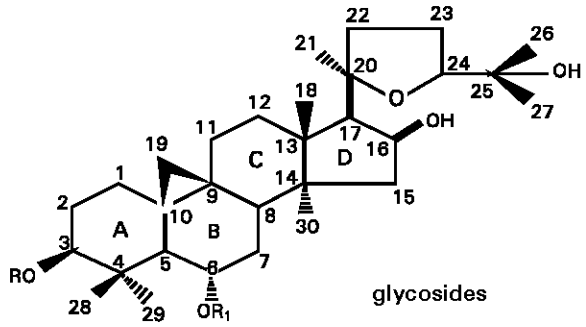
Extraction and isolation: The air-dried powdered roots of *A. spinosus* (1 Kg) were extracted with 90% ethanol. The alcoholic extract was concentrated to 200ml, added slowly with continuous stirring to water (1L), left for 5 hours at room temp. and filtered. The filtrate was partitioned into ether, dichloromethane, ethyl acetate and n-butanol, successively. Ether fraction was previously investigated and afforded a new acid analogue (adsurgenic acid) (Darwish, 2002).

Ethyl acetate soluble fraction was fractionated on silica gel column, eluted with dichloromethane-methanol increasing percentage of methanol, fraction A and fraction B eluted with 20% methanol were examined biologically.

The two fractions were biologically active:

Fraction A was subjected to medium pressure liquid chromatography (MPLC) using silica gel 60 (15-40 um, Merck) flow rate 5ml/min, MPLC: column 1x 55cm eluted with dichloromethane-methanol, Omin: 0% methanol, 40 min: 1% methanol, 60 min: 5% methanol, 120 min: 5% methanol isocratic, 180 min :12% methanol, 200min: 20% methanol. Fractions eluted at 120 min. were a mixture of two saponin glycosides; isoastragaloside I (compound I) and trigonoside I (compound 2) (Fig.1) which were identified by comparison of their spectral data (NMR, EIMS, FABMS) with the reported data.

Fraction B was subjected to silica gel CC using, dichloromethane-methanol as eluant. Fractions eluted with 30% methanol-dichloromethane were collected, concentrated and rechromatographed on another silica gel column. Fractions eluted with methanol- dichloromethane (45%) afforded cycloastragenol 6-0-glycoside (compound 3) (Fig.1) which was identified by comparison of their spectral data (NMR, EIMS, FABMS) with the reported data together with co-TLC with reference sample (Fig. 1)



Compounds	Name of glycoside	R	R_1
Compound I	Isoastrgaloside I	β-D-xyl (2,4 di Ac)	glucose
Compound II	Trigonoside I	H	xylose
Compound III	Cyloastragenol-6-0-glucoside	H	glucose

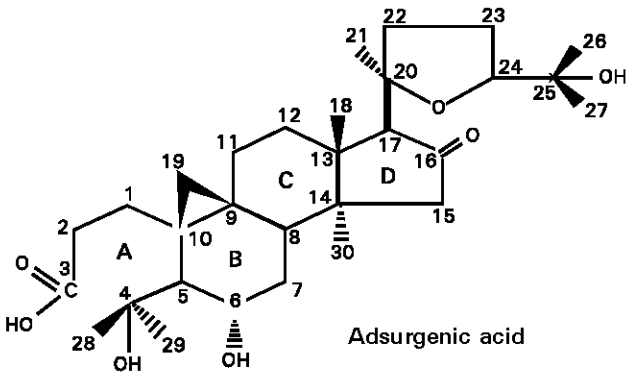


Fig. 1: Structure of glycosides and Adsurgenic acid

Compound 1 (Isoastragaloside I): FABMS (positive ion) m/z 891 (M^+ + Na) 868 (M^+) for molecular formula $C_{45}H_{72}O_{16}$, EIMS m/z 43 (100% rel. abund) for the acetyl group. ^{1}HNMR spectrum showed

signals at δ2.30 and 1.94 for the two acetyl groups of the sugar together with two signals at δ170, 171 in the ^{13}CNMR spectrum.

Compound 2 (trigonosides I): FABMS m/z 645 (M^+ + Na), 662 (M^+) corresponding to molecular formula $C_{35}H_{58}O_9$.
^{1}H and ^{13}CNMR data for compounds I and II were identical with the reported data.

Compound 3 (cycloastragenol 6-0-glycoside): FABMS (positive ion) m/z 675 (M^+ + Na), 652 (M^+) corresponding to molecular formula $C_{36}H_{60}O_{10}$, ^{1}HNMR (500MHz, DMSO):δ 0.18 (1H, d, J = 4.6Hz, H-19), 0.46 (1H, d, J = 4.6 Hz, H-19),1.09 (2x 3H,S M-21 and M-29), 0.9 (3H, S, M-27), 1.0 (3H, S, M-28), 1.11 (3H, S, M-26), 1.18(3H, S, M-18), 1.2 (3H, S, H-30) 4.88 (1H, d, J = 8.8 Hz, H-1 glucose). ^{13}CNMR (125 MHZ, DMSO), 104.47(C-1``), 80.91 (C-6), 78.6 (C-3``), 77.65 (C-3), 77.13(C-5``), 74.94 (C-2``),, 73.17 (C-16), 72.61 (C-4``), 70.9(C-25), 62.20 (C-6``), 28.36(C-29), 28.15 (C-27), 27.58 (C-26), 26.9(C-21), 20.17(C-18), 19.88 (C-28) and 15.69(C-30).

Bioassay studies

Animals and treatments: Adult female *Bufo regularis* (Egyptian toads), weighing about 25 ± 4.3 g, were used throughout the studies. The animals were housed in groups of 20 in glass cages covered with a wire galvanized meshes. The bottom of these glass cages were covered with a wet sponge. Food (earth worms) was available ad libitum. In order to minimize possible nutritional effects, both experimental and control animals were fasted for 18 h before sacrifice.

Carbon tetrachloride: The CCl_4 (Analar) was used as the toxicant agent. It was diluted with corn oil to deliver the proper dosage in a volume of 10 ml kg^{-1} b.wt. All doses were given as a single subcutaneous injection.

Experiment I: Animals were divided into groups of 20 and were administered CCl_4 at various doses. Mortalities were recorded at 24 h following CCl_4 injection. 24 h LD_{100} was estimated. It was found 5.93ml Kg^{-1}. b.wt.

Experiment II: The dried alcoholic extract of *A. spinosus* roots was dissolved in methanol to prepare the desired dose in a volume of 1 ml kg^{-1} b.wt. Preliminary experiments were carried out to determine the most effective concentration of the alcoholic extract that can reduce the mortality-induced by CCl_4. It was found to be 60mg kg^{-1} b.wt. when administered subcutaneously. The animals were divided into 5 groups, 20 animals in each group (Table 1). Mortalities were recorded after 24 h of CCl_4 injection.

Experiment III: The fractions of the alcoholic extract (dichloromethane, ethyl acetate, butanol) and the aqueous fraction left after fractionation of the alcoholic extract with the organic solvents were dissolved in methanol to prepare the desired doses in a volume of 1 ml kg^{-1} b.wt. Preliminary experiments proved that the s.c. injection of 50 mg of each fraction / Kg b.wt. was the most effective concentration in reducing CCl_4-induced lethality. The animals were divided into 10 groups 20 animals in each group (Table 2). Moralities were recorded after 24 h following CCl_4 injection. Ethyl acetate proved to be the most potent one in reducing the CCl_4-induced mortality.

Experiment IV: The ethyl acetate fraction was subjected to chromatographic separation on silica gel column (as previously mentioned) to yield fractions A and B. Preliminary experiments proved that the s.c. injection of 10 mg Kg^{-1} b.wt. of each fraction was the most effective concentration in reducing CCl_4-induced lethality. The animals were divided into 6 groups, 20 animals in each group (Table 3). Mortalities were recorded after 24 h following CCl_4 dosing.

Experiment V: The isolated glycosides (I + II) and III as well as Adsurgenic acid were used to evaluate their effectiveness in preventing both the CCl_4-induced mortality and hepato- renal perturbations. This experiment includes both lethality and biochemical studies.

Lethality studies: Preliminary experiments indicated that the s.c. administration of 2 mg Kg^{-1} b.wt. of each of (compound I, II) and compound III as well as the adsurgenic acid was the most effective concentration in protecting against CCl_4-induced lethality. The animals were divided into 8 groups 20 animals each for lethality studies (Table 4). Mortalities were recorded after 24 h following CCl_4 injection.

Biochemical studies: Animals were divided into 8 groups as those done for the lethality studies, while animal specimens were collected after 16 h only. Animals were anaesthetized with ether and blood withdrawn by cardiac puncture. Blood was collected in heparinized tubes for cellular determinations and non-heparinized tubes for serum preparation.
Serum was prepared by centrifugation of the blood at 8000 r.p.m. for 15 minutes. The activities of serum transaminases i.e., aspartate aminotransferase (AST or GOT) and alanine aminotransferase (ALT or GPT) and creatin kinase (CK), in addition to urea and creatinine concentrations in the serum were estimated by using BM/Hitachi system 7,7 Automatic Analyzer.

Statistical analysis: Measured values are presented as the arithmetic mean of seven experiments ± standard error (S.E.). Results were analyzed using student's t-test. Differences were considered significant at $p < 0.05$. The test of significance of differences were calculated between:

a Means of the control group and means of either of these treated groups: CCl_4, compound III, adsugenic acid, CCl_4 + (compound I and II), CCl_4 + adsurgenic acid were referred by letter a.
b Means of CCl_4 group and either of these experimental groups: CCl_4 + compound (I + II) CCl_4 + compound III, CCl_4 + and adsurgenic acid were referred by letter b.

Results

The simultaneous administration of the alcoholic extract of *A. spinosus* root (60 mg Kg^{-1} b.wt. s.c.) and CCl_4 (5.93 ml kg^{-1} b.wt. s.c., which represents the 24h LD_{100}) reduced the percentage of mortality that were induced by the latter to 65% (Table 1). The results indicate that the injection of the fractionated extracts ethyl acetate, butanol, aqueous and dichloromethane (50 mg kg^{-1}. b. wt. s. c.), decreased the percentages of CCl_4- induced mortality to 50, 55, 60 and 75%, respectively (Table 2).
Thus, the ethyl acetate extract (50 mg kg^{-1}. b. wt. s. c.) gave the best protective effect against CCl_4 (24 h LD_{100}) - induced lethality. So, it was used in the subsequent experiments. The data (Table 3) indicate that A is slightly more effective than B. The results (Table 4) shows that the simultaneous administration of compound I and II, compound III and adsurgenic acid decreased the percentage of CCl_4 - induced lethality to 45, 35 and 55%, respectively.
The results (Table 5) shows that CCl_4 administration resulted in marked increases in liver transaminases (AST and ALT) and CK activities.
The simultaneous administration of compound I + II, compound III and adsurgenic acid with CCl_4 offered partial protective effects against the increases in the activities of AST and ALT that were induced by the latter, since, there were a significant differences between the values of these groups, the control and CCl_4 groups. The co-administration of either compound III or adsurgenic acid provides partial protective effects against the CCl_4 -induced elevations in the CK activities. On the other hand compound I, II

Table 1: Effect of Alcoholic extract (60 mg kg^{-1} b.wt.) on the mortality of *Bufo regularis*

Studied groups**	Number of mortalities	% of mortality
Control	0	0
CCl_4	20	100
Methanol*	20	0
Alcoholic extract*	20	0
CCl_4 + alcoholic extract	13	65

*: These groups served as negative controls
**: Number of animal used in each studied group n = 20

Table 2: Effect of the fractionated extracts (50 mg kg^{-1} b.wt.) on the mortality of *Bufo regularis*

Studied groups**	Number of mortalities	% of mortality
Control	0	0
CCl_4	20	100
Dichloromethane extract*	0	0
CCl_4 + dichloromethane extract	15	75
Ethyl acetate extract*	0	0
CCl_4 + ethyl acetate extract	10	50
Butanol extract*	0	0
CCl_4 + butanol extract	11	55
Aqueous extract*	0	0
CCl_4 + aqueous extract	12	60

*: These groups served as negative controls
**: Number of animal used in each studied group n = 20

Table 3: Effect of ethyl acetate column fractions (10 mg kg^{-1} b.wt.,) on mortality of *Bufo regularis*

Studied groups**	Number of mortalities	% of mortality
Control	0	0
CCl_4	20	100
Fraction A*	0	0
CCl_4 + fraction A	9	45
Fraction B*	0	0
CCl_4 + fraction B	11	55

*: These groups served as negative controls
**: Number of animal used in each studied group n = 20

Table 4: Effect of (isoastragaloside I and trigonoside I), cycloastragenol-6-0-glycoside and adsurgenic acid. (2 mg kg^{-1} b.wt.) on mortality of *Bufo regulari*

Studied groups**	Number of mortalities	% of mortality
Control	0	0
CCl_4	20	100
Isoastragaloside I and trigonoside I*	0	0
CCl_4 + isoastragaloside I and trigonoside I	9	45
Cycloastragenol-6-0-glycoside*	0	0
CCl_4 + cycloastragenol-6-0-glycoside	7	35
Adsurgenic acid*	0	0
CCl_4 + Adsurgenic acid	11	55

*: These groups served as negative controls
**: Number of animal used in each studied group n = 20

did not provide any protective effects against these elevations in CK activities.

CCl_4 administration induced marked increases in urea and creatinine levels in the serum. The data (Table 6) also indicate that administration of either compound I, II and compound III following CCl_4 injection succeeded partially in preventing these disturbances. On the other hand, administrated dose of Adsurgenic acid increased the CCl_4 B induced elevations in serum urea and creatinine concentrations.

It is important to note that the injection of cycloastragenol-6-0-glycoside (compound III), isoastragaloside I and trigonoside I compound (I and II) alone did not induce any alterations during either the bioassay or the biochemical studies, while the administration of adsurgenic acid alone caused severe toxic effects on urea and creatinine levels.

Discussion

The investigation indicated that the 24 h LD_{100} of CCl_4 for adult female *Bufo regularis* is 5.93 ml kg^{-1} b.wt.

The data (Table 5) revealed that CCl_4 induced liver dysfunction as indicated by increases in the activities of AST and ALT. A number of reports indicated that CCl_4 can result in similar biochemical changes in both man (Ruprah *et al.*, 1985) and experimental animals including gerbils (Cal and Mehendale, 1990), Marino ewes (Vajdovich *et al.*, 1995), rats (Lin *et al.*, 1998) and mice (Wong *et al.*, 1998).

It also caused impairment in the renal function as indicated by elevations in serum urea and creatinine concentrations (Table 6). Ruprah *et al.* (1985) observed that renal damage, normally manifest within 48h, occurring up to 2 weeks after CCl_4 exposure. McGregor and Lang (1996) mentioned that the kidney is one of the primary targets in CCl_4 toxicity, moreover, Sanzgiri *et al.* (1997) detected high concentrations of CCl_4 in the kidney of Male Sprague-Dawley rats subjected to equivalent oral and inhalation CCl_4 exposures. The present results showed that CCl_4 induced significant increase in creatin kinase activity. Creatin kinase activity increases in myocardial infarction and muscular dystrophy (Julian *et al.*, 1998).

In this study, results indicated that alcoholic extract of *A. spinosus* roots was effective in reducing CCl_4-induced lethality (Table 1). Results also depicted that the ethyl acetate extract was the most effective fraction in reducing CCl_4- induced mortality. The efficacy of the three other fractionated extracts was as follows: butanol > aqueous > dichloromethane (Table 2). In addition, the data indicated that the ethyl acetate column fractions A and B were effective in reducing CCl_4- induced lethality (Table 3). In this investigation the results of the biochemical studies were co- inside with that of the bioassay studies (Table 5 and 6). The bioassay studies of the isolated glycosides indicated that they were useful

Table 5: Effect of isoastragaloside I and trigonoside I (compound I and II) cycloastragenol-6-0-glycoside (compound III) and adsurgenic acid (2 mg kg^{-1} b.wt.) on liver transaminases (AST, ALT) and creatine kinase (CK) activities

Studied groups	AST (GOT) (IU L^{-1})	AST (GPT) (IU L^{-1})	CK (IU L^{-1})
Control	148.33 ± 2.611	164.16 ± 3.101	56.16 ± 0.911
CCl_4	279.14 ± 4.421a	301.13 ± 5.121a	77.16 ± 0.891a
(isoastragaloside I and trigonoside I)	148.10 ± 2.139	163.09 ± 2.199	56.31 ± 1.021
CCl_4 + (isoastragaloside I and trigonoside I)	169.33 ± 2.028a,b	185.33 ± 2.631a,b	78.21 ± 1.201a,b
Cycloastragenol-6-0-glycoside	148.20 ± 2.187	163.88 ± 3.672	56.11 ± 0.981
CCl_4 + cycloastragenol-6-0-glycoside	192.33 ± 3.321a,b	210.18 ± 4.421a,b	67.03 ± 0.992a,b
Adsurgenic acid	150.01 ± 3.213	164.13 ± 2.961	56.15 ± 0.616
CCl_4 + Adsurgenic acid	194.21 ± 4.261a,b	270.31 ± 4.123a,b	70.08 ± 0.961a,b

a,b = statistically significant ($p < 0.005$) when compared with values of the control group or CCl_4 injected group , respectively

Table 6: Effect of isoastragaloside I and trigonoside I (compound I and II), cycloastragenol-6-0-glycoside (compound III) and adsurgenic acid on serum urea and serum creatinine concentration

Studied groups	Serum urea (mg dl^{-1})	Serum creatinine (mg dl^{-1})
Control	172.16 ± 1.247	0.19 ± 0.003
CCl_4	285.11 ± 2.689a	0.39 ± 0.001a
isoastragaloside I and trigonoside I	175.11 ± 1.691	0.17 ± 0.009
CCl_4 + isoastragaloside I and trigonoside I	260.15 ± 2.870a,b	0.30 ± 0.003a,b
Cycloastragenol-6-0-glycoside	170.11 ± 1.317	1.16 ± 0.002
CCl_4 + cycloastragenol-6-0-glycoside	218.13 ± 2.267a,b	0.24 ± 0.006a,b
Adsurgenic acid	189.11 ± 2.001a	0.25 ± 0.001a
CCl_4 + Adsurgenic acid	338.12 ± 3.121a	0.45 ± 0.003a

a,b = statistically significant ($p < 0.005$) when compared with values of the control group or CCl_4 injected group, respectively

in decreasing CCl_4 induced mortality as compared with adsurgenic acid. The order of their protective effect was as follows: cycloastragenol-6-0- glycoside > (isoastragaloside I and trigonoside I) > adsurgenic acid. The s.c. injection of cycloastragenol-6-0-glycoside succeeded partially in modulating the hepato, renal and cardiac toxicities as compared with the control values, while (isoastragaloside I and trigonoside I) offered partial protection in functions of liver and kidney only, but it failed in improving the CCl_4 induced elevation in CK activity. On the other hand adsurgenic acid caused renal toxic effects. This suggest that cycloastragenol-6-0-glycoside is promising in modulating the disturbances in liver, kidney and heart functions that were induced by CCl_4 or any other toxicants.

The present research work indicate that the co-administration of the cycloastragenol-6-0-glycoside or of (isoastragaloside I and trigonoside I) along with CCl_4 is more effective and advantageous in protecting against lethality and toxicities of the latter, than when it is given with adsurgenic acid.

Acknowledgments

The author is deeply grateful to Dr. Karima Wershana (Associate Prof. Zoology Department, Faculty of Science, University of Alexandria, Egypt) for running all the bioassay and biochemical studies and also to Prof. Ahlam Y. Daabees, Prof. of Comparative Animal Physiology, (Zoology Department, Faculty of Science, University of Alexandria, Egypt) for the valuable assistance. The author thanks Prof. R.Bauer (institute fur Pharmazeutiche Biologie der Heinrich-Heine Universilät, Düsseldorf, Germany) for NMR and Mass spectra.

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MS received 24th April, 2002; accepted 30th May, 2002