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PHARMACOLOGICAL, BIOLOGICAL STUDY AND GC/MS ANALYSIS OF THE ESSENTIAL OIL OF THE AERIAL PARTS AND THE ALCOHOL SOLUBLE FRACTION OF THE N. HEXANE EXTRACT OF THE FLOWERS OF *REICHARDIA TINGITANA* L.

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ABSTRACT

The essential oil of *Reichardia tingitana* L. of the aerial parts (stems and leaves) and flowers extract were obtained by hydrodistillation and extraction methods, respectively, then were analyzed by Gas Chromotography-Mass Spectrometry (GC / MS). Twenty-two components comprising 93.446% of the oil of the aerial parts and forty-five components comprising 98.056% of flowers extract were identified. The major components of aerial parts oil were cinnamaldehyde (29.78%), and undecane (12.99%). The flowers extract contained lanoleic acid ethyl ester (34.146%), hexadecanoic acid ethyl ester (20.6%) and linoleic acid methyl ester (8.05%) as the major components. Both oil and extract were examined for their anti-microbial activities against gram positive, gram negative bacteria and fungi. The results revealed the potent anti-microbial activity of the oil. Both oil and extract were studied for their cytotoxic activities against three different human cell lines. The oil showed potent cytotoxic activity against tumor cells.

Keywords: *Reichardia tingitana* L., Asteraceae, essential oils.

INTRODUCTION

Reichardia tingitana L. (family: Asteraceae) is a bitter annual herb (Galawein, Moraar) with branched stems from the base, cauline sessile leaves, and ligulate golden deciduous yellow flowers, which are purplish on lower surface (Tächolm, 1974). *Reichardia tingitana* L. grows wild in desert wadis, Mediterranean region, Southwest Asia, Tropical East Africa and Australia (Trease and Evans, 2002; Pengelly, 2003; Daniel, 2006; Bolus, 2002). The plant exhibited anti-inflammatory, anti-oxidant, anti-diabetic, anti-feedant and insecticidal activity besides its ornamental value (Stalińska *et al*., 2005; The Local Food-Nutraceuticals Consortium, 2005; Daniewski *et al*., 1988). *Reichardia tingitana* L. contains a number of medicinally important constituents vis. Sesquiterpene lactones, flavonoids, volatile oils, sterols, triterpenoids and phenolic compounds (Daniewski *et al*., 1988; Mañez *et al*., 1994; El Masry *et al*., 1980). No literature was reported on the essential oil of aerial parts and flowers extract of *Reichardia tingitana* L. Thus it was interesting to study this essential oil and extract in the Egyptian plant.

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MATERIALS AND METHODS

Plant material
Fresh flowers, stems and leaves of *Reichardia tingitana* L. were collected in March 2011 from North coast (Alexandria). They were identified by Dr. Abd El Halim Abd El Mogly Mohamed, Agricultural museum, Giza, Egypt.

Preparation of essential oil
The dry aerial parts (stems and leaves) 500 g were subjected to hydrodistillation method. The isolated essential oil was dried over anhydrous sodium sulphate and stored at 4° - 6°C (Egyptian pharmacopoeia, 2005).

Preparation of the alcohol soluble fraction
The alcohol soluble fraction of n. hexane extract of the flowers was prepared by soaking about 500g of the freshly collected flowers in successive amounts of n. hexane, the solvent was evaporated under reduced pressure at a temperature not exceeding 40°C and the obtained concentrate was cooled in ice chest. The cooled concentrate was extracted by maceration in ice cold absolute ethanol to dissolve the extract and remove waxes. The alcoholic extract was filtered and the residue

was cooled again and extracted with ice cold absolute ethanol and so on. The process was repeated until the residue dissolved completely in cooled absolute ethanol.

Analysis of the essential oil and extract

The obtained essential oil and extract were divided into three portions.

First portion

Analysis of essential oil of aerial parts and the alcohol soluble fraction of n. hexane extract of the flowers were carried out on Agilent 6890 equipped with a mass spectrometric detector (MSD), model Agilent 5973, equipped with an HP-5MS column (30m x 0.25mm, 0.25µm); programming from 80°C (3 min) to 260°C at 8°C / min, 10 min. hold ; carrier gas, helium ; flow rate, 1.0 mL / min.; injection in split mode (60:1); injector and detector temperatures 225° and 300°, respectively . The EIMS mode at 70 eV; electron multiplier, 2500 V; ion source temperature, 250°C; mass spectral data were acquired in the scan mode in the m /z range 50-700.

The essential oil and the alcohol soluble fraction of the n. hexane extract components were identified by comparing their mass fragmentation patterns with that available references (Adams, 2009; www.lipidlibrary.com, www.massbank.com and www.nist.com). Also compounds identification was confirmed by electronic Wiley and NIST mass spectral database. The retention indices (RI) of the components were determined relative to the retention times of series of hydrocarbons. Results are tabulated (Tables 2,3).

Second portion

Both oil and extract were studied for their anti-microbial activities. The anti-microbial activity was tested using agar diffusion technique. 100 µL of each essential oil were aseptically inoculated into wells, each of 1 cm diameter. The plates were incubated at 37°C for 24 hours in case of bacteria and at 25°C for 48 hours in case of fungi and results are tabulated (Table 5).

Third portion

The potential cytotoxicity of the essential oil of aerial parts and the alcohol soluble fraction of n.hexane extract of the flowers of *Reichardia tingitana* L .was tested using the method of (Skehan and Storeng, 1990) on three cell lines; liver, larynx and colon cancer human cell lines. Also IC_{50} for each sample was reported and results are tabulated (Tables 6, 7, 8 and Figs.1-6).

RESULTS AND DISCUSSION

Table 1 shows the characteristics of the volatile oil of aerial parts in comparison to those of the alcohol soluble fraction of the n. hexane extract of the flowers.

GC / MS of the essential oil and extract

From the GC / MS of the essential oil of aerial parts (Table 2), 22 components were identified representing 93.446% of the oil yield. Major components were Cinnamaldehyde with percentage of 29.779% of the oil yield, Carvotanacetone whose percentage is 5.517% of the oil yield, Undecane representing 12.993% of the total oil yield. The hydrocarbons represented 41.538% of the oil, the main of which is Undecane constituting 12.993% of the oil. The aldehydes represented 30.640%, the main of which is Cinnamaldehyde whose percentage is 29.779% of the oil yield. Ketones represented 9.137% of the oil; the main is Carvotanacetone whose percentage is 5.517% of the oil yield. The alcohols represented 11.275%, the main of which is 2-isopropenyl-5-methyl cyclohexanol with percentage of 7.85 of the total oil. The only oxide is Myroxide representing 2.99% of the oil yield.

From the GC / MS of the alcohol soluble fraction of n. hexane extract of flowers (Table 3), 45 components were identified representing 98.056% of the extract yield. Major components were lanoleic acid ethyl ester with percentage of 34.146% of the extract yield, hexadecanoic acid ethyl ester whose percentage is 20.6% of the extract yield, linoleic acid methyl ester representing 8.05% of the

Table 1. Physical properties of the essential oil of aerial parts and alcohol soluble fraction of the n. hexane extract of flowers of *Reichardia tingitana* L.

Oil / Physical properties	Essential oil of aerial parts	Alcohol sol.fraction of n.hexane extract of flowers
Yield % (w/w)	2	1
Colour	Yellowish green	Yellow
Odour	Aromatic	Aromatic
Refractive index	1.3975	1.3866
Specific gravity	0.57	0.75
Miscibility	Completely miscible with petroleum ether, hexane, benzene, chloroform and ether.	Completely miscible with petroleum ether, hexane, benzene, chloroform and ether.

total extract yield. Esters representing 86.084% of the extract, the main of which was lanoleic acid ethyl ester with percentage of 34.146% of the extract yield. Acids represented 3.414% of the extract, the main of which was 9-octadecenoic acid representing 1.44% of the extract.

Table 2. GC / MS analysis of the essential oil of aerial parts of *Reichardia tingitana* L.

Peak No.	Rt. (min)	Mass Spectral Data			Compound	Kovats index	Percentage%
		M^+	Base Peak	Other Peaks			
1	3.78	112	57	69, 43, 97	Methyl Cycloheptane	860	0.278
2	4.51	112	55	113, 111, 93	1-Heptene-3-one	953	3.62
3	5.23	142	57	71, 85, 97	Decane	1000	7.23
4	5.44	126	55	69, 81, 97	1-isobutyl 3-methyl cyclopentane	-	0.42
5	5.85	118	57	55, 69, 83	3-methyl 1,5-pentane diol	1060	0.41
6	6.61	144	57	55, 69, 83	n-octane-1ol	1068	0.58
7	7.22	154	55	71, 81, 95	3-methyl-3-undecene	1017	0.57
8	8.16	136	121	93, 79, 67	α-terpinene	1017	0.57
9	8.76	156	57	85, 71, 113, 127	Undecane	1100	12.99
10	9.45	154	55	69, 83, 111, 93	2-isopropenyl-5-methyl cyclohexanol	1104	7.85
11	9.85	154	55	71, 57	Myroxide	1145	2.99
12	10.08	126	57	71, 85, 112	3-nonene	1189	4.01
13	10.84	170	57	71, 81, 95	Dodecane	1200	5.22
14	11.39	152	82	93, 108, 121	Carvotan acetone	1247	5.517
15	12.33	132	131	103, 77, 51	Cinnamaldehyde	1270	29.78
16	13.03	182	55	69, 83, 97	1-tridecene	1291	2.25
17	13.50	168	55	67, 69, 81, 83	Undec(9E)en-1-al	1312	0.86
18	14.12	206	79	133, 105, 91	1,3,11(13) Elematrien-12-ol	-	0.29
19	15.68	-	57	191	Non identified	-	0.04
20	16.43	208	77	193, 118, 105	2-hydroxy phenoxy 1-phenyl ethyl	-	0.79
21	19.54	220	79	55, 57, 93	β-Acoradienol	1763	2.145
22	24.435	252	55	155, 93	Δ5-octadecene	1775	5.01
23	25.50	242	57	185,29	Bis 6-methyl heptyl hexanoate	-	0.066

Table 3. GC/MS analysis of the alcohol soluble fraction of the n.hexane extract of the flowers of *Reichardia tingitana* L.

Peak No.	Rt. (min)	Mass Spectral Data			Compound	Kovats index	Percentage%
		M^+	Base Peak	Major Peaks			
1	8.20	158	59	136, 121, 93	Beta Fenchol	1116	0.28
2	8.58	150	71	138, 123, 95, 81	Menthol	1171	0.493
3	8.72	148	148	121, 91, 71	Estragole	1196	0.429
4	9.57	152	82	108, 91	Carvotanacetone	1247	1.106
5	10.15	132	131	103, 77, 51	Cinnamaldehyde	1270	0.611
6	10.32	148	148	133, 117, 105, 77	Anethole	1284	1.272
7	12.03	200	55	155, 101, 88	Decanoic acid ethyl ester	1395	0.121
8	12.485	204	91	133, 93, 79, 69	Caryophylline-9-epi E	1466	0.205
9	12.84	-	98	155, 144, 101	Unidentified	-	0.161
10	13.85	214	74	87, 55	Dodecanoic acid metyl ester (Azelaic acid)	1510	0.194

Continued…

Table 3 continue….

Peak No.	Rt. (min)	Mass Spectral Data			Compound	Kovats index	Percentage%
		M^+	Base Peak	Major Peaks			
11	14.03	204	159	131, 119, 91	Cis calamenene	1529	0.148
12	14.915	216	152	199, 105, 111, 83	Dimethyl azelate	1548	0.194
13	15.06	200	73	157, 129, 60	Dodecanoic acid (Lauric acid)	1566	0.378
14	15.47	224	55	69, 83, 97, 111	Δ-Hexadecene	1593	0.242
15	16.42	244	199	157, 111, 55	Diethyl azelate	-	0.378
16	16.52	238	81	95, 123, 93	3,3-dimethyl 1,4-pentadecadiene	-	0.188
17	16.62	228	88	101, 183, 73	Dodecanoic acid ethyl ester (ethyl laurate)	1595	1.223
18	16.816	212	97	88,55, 138, 167	8-Nonenoic acid ethyl ester	-	0.173
19	17.590	228	73	60, 101, 129	Myristic acid (Tetradecanoic acid)	1720	0.387
20	17.736	242	74	87, 55, 143	Methyl tetradecanoate (methyl myristate)	1723	0.301
21	18.14	270	74	87, 129, 143	Pentadecanoic acid methyl ester	1820	0.155
22	18.46	184	101	73, 55	4,5-dimethyl 2-cyclohexyl 1,3-dioxolane	-	0.218
23	18.87	270	70	55, 239	Isoamyldecanoate	1845	0.552
24	19.01	256	88	101, 157, 213	Tetradecanoic acid ethyl ester (ethyl myristate)	1885	1.172
25	19.16	282	55	69, 83, 97	9-Hexadecenoic acid methyl ester	1896	0.423
26	19.45	270	88	55, 71, 101	13-methyl tetradecanoic acid ethyl ester	-	0. 405
27	19.64	300	55	69, 83, 97, 111	11-Hexadecenoic acid ethyl ester	1915	1.161
28	20.34	270	74	55, 87	Hexadecanoic acid methyl ester	1921	5.467
29	20.342	160	117	88, 71, 55	3-hydroxy hexanoic acid ethyl ester	-	0.157
30	20.5	272	55	227, 83, 97, 111	2-hydroxyhexadecanoic acid	1960	0.535
31	20.89	282	55	69, 83, 97	9-Hexadecenoic acid ethyl ester	1977	2.022
32	21.23	284	88	157, 73, 55	9 -Hexadecanoic acid ethyl ester	1993	20.6
33	21.23	294	67	55, 81, 95, 109	9,12-octadecadienoic acid methyl ester	2085	8.05
34	21.485	292	79	67, 55, 95, 105	9,12,15-octadecatrienoic acid methyl ester	2098	5.127
35	21.57	292	79	69, 55, 93	Methyl 3,11,14-octadecatrienoate	2098	0.782
36	21.751	298	74	55, 67, 143	Methyl octadecanoate	2125	0.522
37	22.317	306	55	69, 79, 95	Ethyl 9,12,15-octadecatrienoate	2139	0.975
38	22.539	308	55	67, 81, 95	Ethyl linoleate (9,12-octadecadienoic acid ethyl ester)	2141	34.146
39	23.182	292	55	69, 83, 97, 111	9-octadecenoic acid	2161	1.44
40	23.493	280	67	55, 83, 110, 263	9,12-octadecadienoic acid(linoleic acid)	2173	0.674
41	23.671	-	73	-	Unidentified	-	0.782
42	24.514	312	88	55, 101, 157	Ethyl octadecanoate	2196	3.286
43	24.747	324	57	71, 85	Tricosane	2300	0.519
44	25.00	322	55	67, 81, 151	9,12 Nonadecadienoic acid ethyl ester	-	0.234
45	25.12	166	81	55, 95, 125, 151	1,6-dimethyl decahydronaphthalene	-	0.367
46	25.39	322	55	73, 85, 263	Propyl 9,12-octadecadienoate	-	0.378
47	25.57	166	151	95, 123, 138	1,1-dimethyl decahydronaphthalene	-	0.367

Table 4. The calculated percentages of different classes of compounds of *Reichardia tingitana* L. volatile oil of aerial parts and extract of flowers.

Components	Yield (%) in the essential oil of aerial parts	Yield (%) in the extract of flowers
Total hydrocarbons	41.538	2.036
Monoterpenes	0.570	-
Alcohols	11.275	0.773
Phenols	0.79	1.701
Aldehydes	30.640	0.611
Ketones	9.137	1.494
Esters	0.066	87.809
Acids	-	3.414
Oxygen containing compounds	-	0.218
Total identified components	93.446	98.056
Unidentified components	0.04	0.943

Table 5. Anti-microbial activities of essential oil of aerial parts and alcohol soluble fraction of n.hexane extract of flowers of *Reichardia tingitana* L.

Test Organism / Oil	Gram negative bacteria		Gram positive bacteria		Fungi		
	E. Coli	*Pseudomonas aerugiosa*	*Staphylococcus aureus*	*Bacillus Subtillus*	*Candida albicans*	*Saccharomyces cerevisiae*	*Aspergillus niger*
Essential oil of aerial parts (100µL)	30	22	25	20	20	20	–
Alc.sol.fraction of flowers (100µL)	–	–	–	–	–	–	–
Chloramphenicol (100µL of 5mg)ml solution	21	20	22	20	–	–	–
Griseofulvin (100µL of 5mg)ml solution	–	–	–	–	20	20	20

Table 6. Cytotoxic activity of alcohol soluble fraction of n.hexane extract of flowers of *Reichardia tingitana* L.

Conc.of extract (µg)	Tumor cells surviving fraction		
	Liver carcinoma cell line	Larynx carcinoma cell line	Colon carcinoma cell line
0.0	1.000	1.000	1.000
5.0	0.9215	0.995	0.850
12.5	0.8352	0.686	0.396
25.0	0.411	0.338	0.372
50.0	0.346	0.355	0.198

Table 7. Cytotoxic activity of essential oil of aerial parts of *Reichardia tingitana* L.

Conc.of oil (µg)	Tumor cells surviving fraction		
	Liver carcinoma cell line	Larynx carcinoma cell line	Colon carcinoma cell line
0.0	1.000	1.000	1.000
5.0	0.599	0.780	0.708
12.5	0.576	0.344	0.434
25.0	0.134	0.288	0.234
50.0	0.139	0.243	0.166

Table 8. IC_{50}^{*} of the essential oil and the alcohol soluble fraction of n.hexane extract of *Reichardia tingitana* L.

Sample	Tumor cells(IC_{50}^{*})		
	Liver carcinoma cell line	Larynx carcinoma cell line	Colon carcinoma cell line
Extract of flowers	1.000	1.000	1.000
Essential oil of aerial parts	0.9215	0.995	0.850

IC_{50}^{*}: the dose of the sample which reduces survival to 50 %.

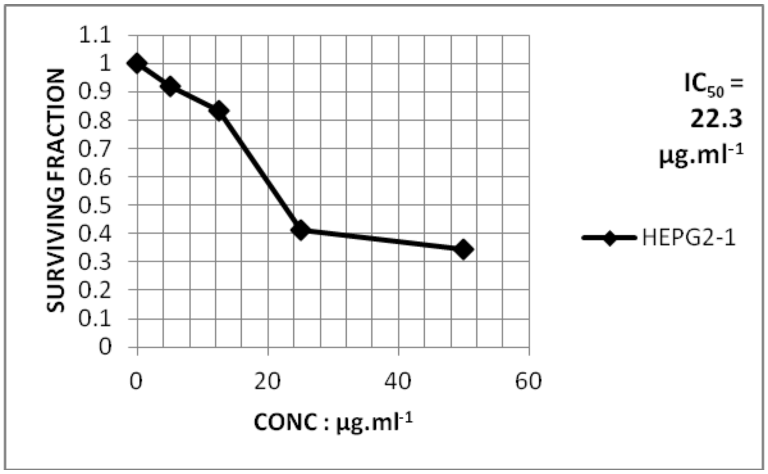

Fig. 1. Cytotoxic activity of alcohol soluble fraction of flowers of *Reichardia tingitana* L. on liver carcinoma.

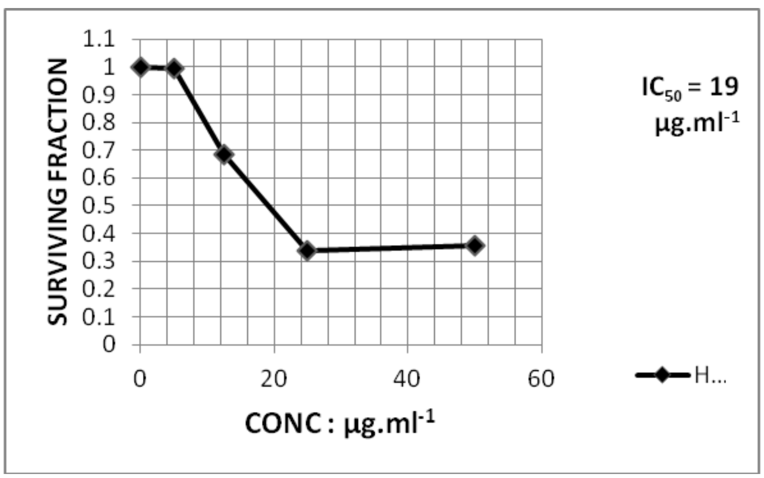

Fig. 2. Cytotoxic activity of alcohol soluble fraction of flowers of *Reichardia tingitana* L. on Larynx_carcinoma.

The hydrocarbons represented 2.036% of the extract. Alcohols represented 0.773% of the extract. The only sesquiterpene is Caryophylline 9 epi representing 0.205% of the extract yield. The only aldehyde was Cinnamaldehyde constituting 0.611% of the extract yield.

From table 4, it is observed that the yield of total hydrocarbons in the essential oil of aerial parts (41.538%) is higher than that of the flower extract. The percentage of alcohols (11.275%), aldehydes (30.640%) and ketones (9.137%) are vigorously higher than that of flower extract. On the other hand the total esters in the flower extract represent (87.809%), while the only ester found in the oil of aerial parts represents (0.066%). Acids representing (3.414%) of the flower extract, are not detected in essential oil of aerial parts.

As shown in table 5, only the essential oil of aerial parts of *Reichardia tingitana* L. exhibits anti-microbial activity, while the extract of the flowers showed no antimicrobial activity. The anti-bacterial activity of essential oil of aerial parts were higher than Chloramphenicol activity used as a reference standard except with *Bacillus subtillus*, they exerted the same effect.

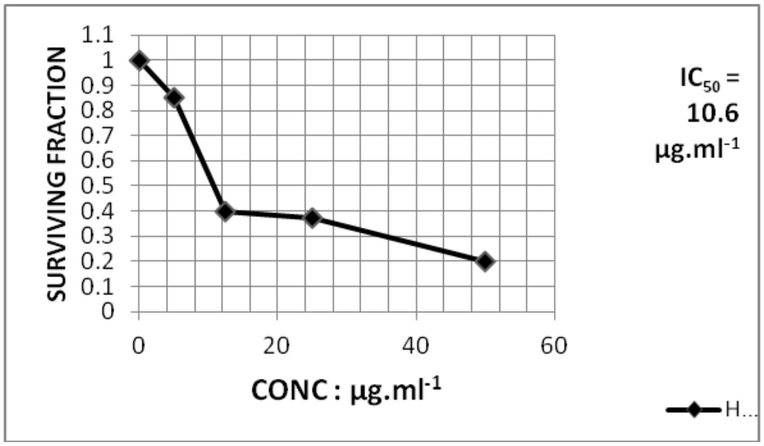

Fig. 3. Cytotoxic activity of alcohol soluble fraction of flowers of *Reichardia tingitana* L. on colon carcinoma.

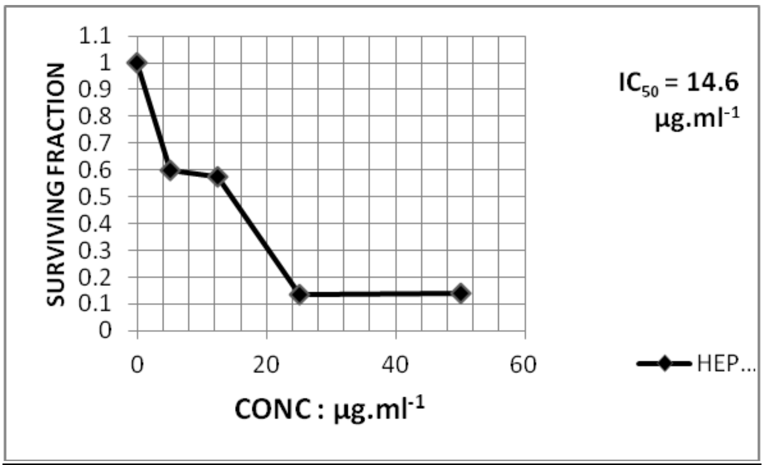

Fig. 4. Cytotoxic activity of aerial parts of *Reichardia tingitana* L. on Liver carcinoma.

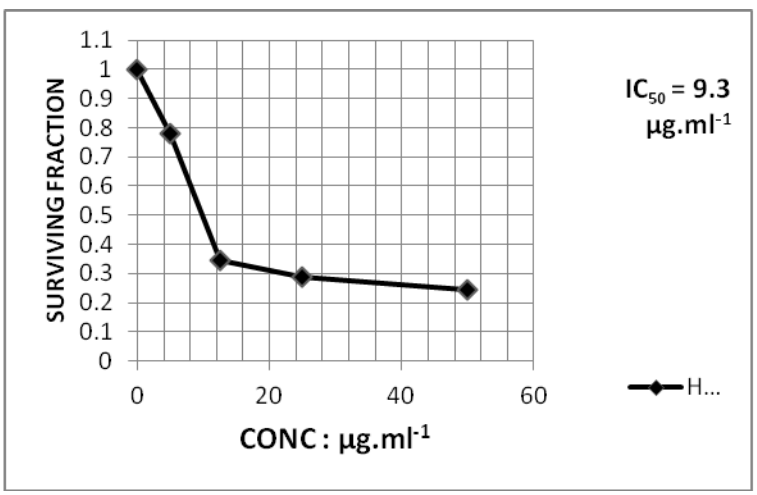

Fig. 5. Cytotoxic activity of aerial parts of *Reichardia tingitana* L. on Larynx carcinoma.

The anti-fungal activity of the essential oil of aerial parts of *Reichardia tingitana* L. was similar to Griseofulvin activity used as reference standard with Candida albicans and Saccharomyces cerevisiae.

The essential oil of aerial parts of *Reichardia tingitana* L. showed NO effect against Aspergillus niger. The high potency of the aerial parts essential oil may be due to the content of aldehydes specially (cinnamaldehyde), alcohols and ketones which contribute more than (50%).

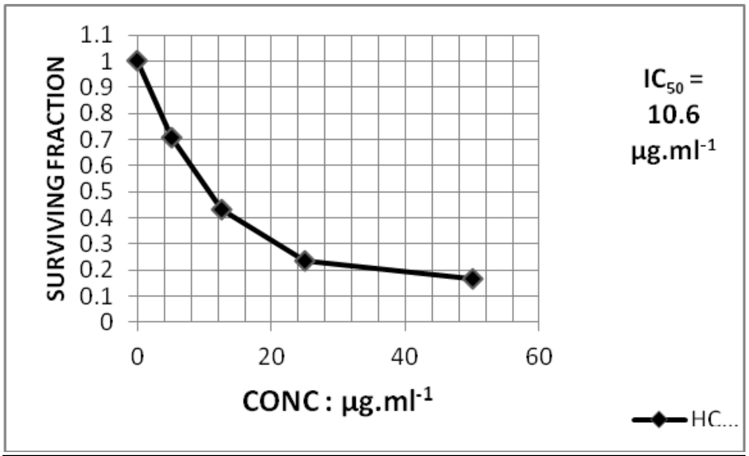

Fig. 6. Cytotoxic activity of aerial parts of *Reichardia tingitana* L. on Colon carcinoma.
IC_{50}^{*} : Inhibition concentration that stops (inhibits) growth of 50 % of cells.

Cinnamaldehyde has an anti-microbial activity against gram positive, gram negative bacteria and fungi (Ooi *et al*., 2006; Satya *et al*., 2012).

From the previous figures 1-6 and tables 6,7,8, it is obvious that the essential oil of aerial parts shows more potent cytotoxic activities on larynx and colon carcinoma rather than liver carcinoma. On the other hand, alcohol soluble fraction of flowers showed less cytotoxic activities on liver and larynx carcinoma. Both essential oil of aerial parts and alcohol soluble fraction of flowers showed similar cytotoxic activity on colon carcinoma cell lines. Thus it's observed that the essential oil of the aerial parts showed a potent cytotoxic activity against tumor cells. Cinnamaldehyde impairs melanoma cell proliferation, invasiveness and tumor growth (Cabello *et al*., 2009).

CONCLUSION

This is the first report to study the constituents of the essential oil of aerial parts and extract of flowers of *Reichardia tingitana* L. growing in Egypt. The potent anti-microbial, anti-fungal and cytotoxic activities of the essential oil of aerial parts should be taken in consideration i.e. it can be used in pharmaceutical formulations after biological and clinical studies.

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