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Genetic Profiling, Chemical Characterization and Biological Evaluation of Two *Conyza Species* Growing in Egypt

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ABSTRACT

The present report is a comparative investigation of two *Conyza* species growing wild in Egypt namely, *Conyza dioscoridis* (L.) Desf. and *Conyza bonariensis* (L.) Cronquist. It comprises a genetic and chemical characterization of the plants, as well as an evaluation of their biological activities. The DNA fingerprints of the two species were established based on a polymerase chain reaction (PCR) procedure using ten decamer primers. Further characterization of the plants was performed *via* determination of pharmacopœial constants, phytochemical screening and estimation of phenolic content (total phenolics, tannins and flavonoids). The ethanol (70%) extracts of *C. dioscoridis* (**EECD)** and *C. bonariensis* **(EECB**) were subjected to acute toxicity study to determine their LD_{50}; the anti-inflammatory, antimicrobial and cytotoxic activities were then evaluated. Screening for potential cytotoxicity was carried out both by Brine Shrimp Lethality Test and Sulphorodamine-B assay on three human cell lines *viz*., breast carcinoma (MCF7), colorectal carcinoma (HCT116) and cervical carcinoma (HELA) cell lines. The DNA profiling revealed a similarity index of 88.89% between the investigated species. The variability observed among the pharmacopoeial constants constitute a valuable differential criterion; the total ash, acid insoluble ash, water soluble ash and crude fiber values obtained for *C. bonariensis* exceeded (17, 5, 10 and 3.5%, respectively) those for *C. dioscoridis*; meanwhile, the moisture content was higher (10%) in the latter. The phytochemical screening of EECD and EECB revealed the presence of flavonoids, steroids, terpenoids and tannins in both species. Estimation of phenolic contents (total phenolics, tannins and flavonoids expressed as gallic acid, tannic acid and rutin equivalents, respectively) showed that EECD contains higher amounts of all these constituents when compared to EECB (1.17 *vs.* 0.96 mg/g, total phenolics; 2.43 *vs.* 1.83 mg/g, tannins and 0.62 *vs.* 0.29 mg/g, flavonoids). EECD and EECB were found to be safe (LD_{50} upto 0.5g/kg). Throughout evaluation of the antimicrobial activity against a set of microbial strains and potential cytotoxicity against MCF7, EECD appeared more efficient (MIC: 200-400 µg/ml and IC_{50}: 2.97 µg/ml, respectively); meanwhile, the effect of EECB was more significant on HCT116 and HELA ($IC_{50:}$ 21 and 5.4 µg/ml, respectively). Results of *in-vivo* assessment of the anti-inflammatory activity showed that the inhibitory effect of EECD was more prominent than that of EECB (74.20% *vs.* 59.0%). However, the effect of the extracts was inversed in the Brine Shrimp Lethality test (30% *vs.* 40% lethality, respectively).

INTRODUCTION

Family Asteraceae (Compositeae, Sunflower or Aster family) is the largest family of flowering plants; it comprises about 23600 species distributed in 1620 genera and 12 subfamilies (Jeffrey, 2007). Compositous plants are distributed among the tropics and warm temperate regions of South, South-East and East Asia and Africa; some being cultivated as vegetable or food while others grow wild (Panero and Crozier, 2008). *The genus Conyza* Less., represented by approximately 60 species, is composed of annual herbaceous plants that prosper chiefly in the tropical and subtropical regions of the globe (Nesom, 1990). In Egypt, this genus is represented by about 6 species, growing in different localities. The most common *Conyza* species reported in Egypt are *Conyza dioscoridis* (L.) Desf. and *Conyza bonariensis* (L.) Cronquist; these are mainly localized in El-Fayoum and Beni Suef areas. Secondary metabolites belonging to different phytochemical groups have been reported from members of genus *Conyza* including: alkaloids, volatile oils, terpenoids, phenolic acids, flavonoids and hydrolysable tannins (Shahwar *et al.*, 2012; Bohlmann and Mahanta, 1978). *C. dioscoridis* was among the favorite desert plants for feeding insects (Junnila *et al.*, 2010).

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The plant was found to exert a molluscicidal activity against *Biomphalaria alexandrina* snails (Bakry, 2009); as well as to exhibit anti-inflammatory, antinociceptive (Atta and Abo Sooud, 2004) and antipyretic effects (Awaad *et al.*, 2011).

The growth inhibitory activity of this plant against a series of selected microbial strains has also been reported (Zain *et al.*, 2012). Likewise, *C. bonariensis* (L.) is widely used as a folk medicine in treatment of rheumatism, gout, cystitis, nephritis, dysmenorrhea, tooth pain and headache; it was also reported to have an antiulcerogenic and anticoagulant activity (Favila and Antonio, 2006); in addition, the antioxidant potential of its extracts has been assessed (Shahwar *et al.*, 2012).

During the past few decades, classical strategies of evaluating genetic variability among closely related species such as comparative anatomy, morphology, embryology and physiology have been increasingly complemented by pharmacopoeial constants and molecular approaches, particularly molecular markers (Williams *et al.*, 1990).

The scarcity of reports traced concerning the two selected species *viz.*, *Conyza dioscoridis* (L.) Desf. and *Conyza bonariensis* (L.) Cronquist, stimulated the authors to contribute to their genetical, chemical and biological characterization aiming to establish additional criteria which could be helpful for differentiation between these two plants.

MATERIAL AND METHODS

Plant Material and Extracts

Samples of *Conyza dioscoridis* (L.) Desf. and *Conyza bonariensis* (L.) Cronquist were collected from El-Fayoum, Egypt, 2009. The plants were kindly identified and authenticated by Prof. Dr. Mounir M. Abd Elghani, Botany Department, Faculty of Sciences, Cairo University, Egypt. Voucher specimens of both species are deposited in Pharmacognosy Department, Faculty of Pharmacy, Beni Suef University.

For molecular investigation, samples of leaves (50 mg, each) were freeze-dried and ground under liquid nitrogen to a fine powder. DNA was isolated by extraction with 0.8ml of cetyl trimethylammonium bromide (CTAB), precipitation with isopropanol, washing with ethanol 70% followed by solubilization in deionized water (Doyle and Doyle, 1987).

For phytochemical and biological studies, samples of the powdered whole plants (500 g, each) were exhaustively extracted by cold maceration with 70% ethanol, the solvent evaporated under reduced pressure and the residues saved for further study.

Material and Methods for DNA Fingerprinting

Apparatus

DNA thermocycler (Hybaid PCR Express) was used for amplification of DNA, agarose gel electrophoresis tool (Biorad Wide Mini Sub Cell) for separation of RAPD (Random Amplified Polymorphic DNA) fragments according to size and UV Polaroid camera for visualization of RAPD fragments.

Extraction Buffer Solutions and Gel

2% N-cetyl-N,N,N-trimethylammonium bromide (CTAB); 0.1 M Tris-HCl (Hydroxyl methyl amino methane with HCl to pH 8); 0.02 M EDTA, 1.4 M NaCl, 1% (v/v) β-mercaptoethanol (added immediately before use). Agarose gel: 1.4% agarose gel (Sigma), with running TE buffer, was used for electrophoresis.

Enzyme, Primers and Molecular Size Marker

Tak DNA polymerase (Perkin-Elmer/Cetus, USA, advanced Biotechnologies, UK) was used for DNA amplification. Ten oligonucleotide primers (Operon Technologies Inc.

Almeda, California, USA) were used for RAPD analysis with the following sequence: OPB-05, TGCGCCCTTC; OPB-07, GGTGACGCAG; OPB-14, TCCGCTCTGG; OPB-15, GGAGGGTGTT; OPB-16, TTTGCCCGGA; OPB-18, CCACAGCAGT; OPB-19, ACCCCCGAAG; OPB-20, GGACCCTTAC; OPG-12, CAGCTCACGA and OPO-17, GGCTTATGCC. Molecular size marker: a 100bp ladder (Promega Corporation, Madison, USA) was used as a standard marker.

DNA Extraction and Quantification

Extraction

DNA was extracted from the frozen powders (50mg) with 0.8ml of CTAB, precipitated, washed and redissolved in deionized water adopting the procedure of Doyle and Doyle, 1987.

Amplification of RAPD markers

The polymerase chain reactions were carried out using 100ηg of genomic DNA template and following a thermal cyclic program.

Amplified products were analyzed by gel electrophoresis on 1.4% agarose gel and finally stained with ethidinium bromide. A molecular size marker (100bp lader) was used as standard marker.

Analysis of RAPD data

This was performed to estimate the genetic distance among the tested samples. Genetic similarity (GS) was analyzed according to Jaccard's equation: $GS = 2N_{ab} / (N_a + N_b)$, where N_{ab} is the number of scored fragments between plants a and b; N_a is the number of scored fragment**.**

Material and Methods for Phytochemical Characterization

Determination of Pharmacopoeial Constants

Total ash, acid-insoluble ash, water soluble ash, as well as crude fiber and moisture contents of the cited plant species were determined following the methods mentioned in the Egyptian Pharmacopoeia (EP, 2005).

Phytochemical Screening

The phytochemical screening of EECD and EECB was carried out (Wagner and Bladt, 1996). The qualitative identification of different types of secondary metabolites was

performed based on the response (color and/or precipitate) to specific chemical tests. All solvents used were of analytical grade.

Spectrophotometric Determination of Total Phenolics, Tannins and Flavonoids

Total phenolic, tannin and flavonoid contents were determined in each of the plant extracts and expressed as mg/g gallic acid, tannic acid and rutin equivalents, respectively. Total phenolics were determined by the Folin-Ciocalteu method as reported by Jimoh *et al.*, 2007 and tannin contents by following the procedure described by Van-Burden and Robinson, 1981. The total flavonoid contents were estimated as described by Piccolella *et al.*, 2008. Standard reference samples namely, gallic acid, tannic acid and rutin were purchased from E. Merk, Darmstadt, Germany.

Instruments

UV-visible spectrophotometer, Shimadzu UV- 1650 PC was used for recording UV spectra and measuring the absorbance in UV range.

Material and Methods for Biological Studies

Animals

Albino mice (25-30 g) were used for determination of LD_{50}. Adult male albino rats (120 - 150g) were used for evaluation of the anti-inflammatory activity. Animals were obtained from a breeding colony from Faculty of Veterinary Medicine, Beni-Suef University, Egypt. Animals were kept under the same hygienic conditions, fed with well balanced normal diet and water supplied *ad Libitum*.

Microorganisms

Nine bacterial and fungal strains were used for investigation of the antimicrobial activity including: Gram positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus* "Pathogenic LMG 3240", "Non-pathogenic LMG 3242" and "Lab. Strain", Gram negative bacteria (*Escherichia coli, Mycobacterium phlei, Listeria innocua* "LMG 2710" and *Enterococcus faecalis*) and fungi (*Candida albicans*).

The tested microbial strains were obtained from stock cultures kept at the Microbiology Department, Faculty of Pharmacy, Beni Suef University. The strains were grown on Sabouraud Dextrose Agar (Fluka) medium.

Tumour cell lines

Three tumour human cell lines were used for assessment of the cytotoxic potential of the extracts *viz.*, breast carcinoma (MCF7) cell line, colorectal carcinoma (HCT116) cell line and cervical carcinoma (HELA) cell line**.** They were obtained from the National Cancer Institute (Kasr El Ainy Street, Cairo, Egypt).

Brine shrimps

Brine shrimp (*Artemia salina* L. cysts Sanders TM Great Salt Lake, Brine Shrimp Company L.C., U.S.A.) were incubated for 24 hours at room temperature (25-29°C) in filtered sea water (Aqua Marine, Thailand), newly hatched free-swimming pink-colored nauplii were harvested and used for the bioassay.

Chemicals, Drugs and Devices

Carrageenan (Sigma Chemical CO., USA) and Indomethacin (Merk, Sharp and Dohme, USA) were used during evaluation of the anti-inflammatory activity. A binocular microscope (MBS 9, USSR, 4.8 x) was used for counting the survival nauplii in the Brine Shrimp Lethality Assay. The intensity of the color developed in the Sulphorodamine-B assay, applied for assessment of the cytotoxic potential, was measured in an ELISA reader.

Acute Toxicity Study

The median lethal doses (LD_{50}) of EECD and EECB were deduced based on the method of Spearman and Karber, 1978.

Evaluation of the Anti-inflammatory Activity

The anti-inflammatory activity of EECD and EECB was assessed by the Carrageenan-induced paw œdema method as described by Winter *et al.*, 1962. Thirty male albino rats were divided into 5 groups and orally treated one hour before induction of oedema. Group 1 received saline and served as negative control. Groups 2 and 3 received EECD and EECB, respectively, at a dose of 50mg/kg b.wt. each. Group 4 received Indomethacin (30mg/kg b.wt.) and served as positive control. Induction of œdema was carried out by sub-planter injection of 0.1ml of 1% Carrageenan in saline into the pad of right paw while the left hind paw was injected with 0.1 ml saline. Four hours after drugs administration, the rats were sacrificed. Both hind paws were excised and separately weighed; the difference in weights between the paws of each individual animal represents the weight of oedema.

Evaluation of the Antimicrobial Activity:

The antimicrobial activity of EECD and EECB was assessed *in-vitro* by adopting the agar dilution method according to the procedure described by the Clinical Laboratory Standards Institute (CLSI) 2009. All tests were performed in triplicates and minimum inhibitory concentrations (MICs) recorded. Sabouraud Dextrose Agar was used as a culture medium.

Evaluation of the Cytoxicity Potential by the Brine Shrimp Lethality Test (BSLT)

The lethality noticed *in-vivo* in a simple zoological organism (*Artemia salina* L.) was the basis of the brine shrimp lethality test (BSLT), first developed by Meyer *et al.* (1982); results were taken as an estimate of toxicity and consequently of physiological activity. The investigated plant extracts *viz.*, EECD and EECB were separately dissolved in seawater containing 1% DMSO (v/v) to a concentration of 100 µg/ml. Ten of the collected nauplii were used for each test. Each experiment was carried out in triplicate. After 24 hrs, the number of surviving nauplii was recorded and the percentage mortality calculated as compared to the control.

Evaluation of the Cytotoxic Potential by Sulphorhodamine-B (SRB) assay

The cytotoxic potential of EECD and EECB was evaluated against three human cell lines namely, MCF7, HCT116 and HELA by adopting the method of Skehan *et al.,* (1990); using different concentration starting from 5 µg/ml to 50 µg/ml**.** The relation between different concentrations of each extract and the surviving fraction was plotted to get a survival curve for each tumor cell line after its incubation with the extract.

Statistical analysis

Data analysis was performed by the one way Analysis of Variance (ANOVA) as statistical tool in conjunction with Tukey's test. The *p*-values < 0.05 were selected to indicate statistical significance between the groups. The analytical software used was SPSS statistics 17.0, release (Aug. 23, 2008), Chicago, USA.

Animal Ethics

All animal procedures were performed upon approval from the Ethics Committee of Beni Suef University and in accordance with the recommendations of the proper care and use of laboratory animals.

RESULTS AND DISCUSSION

DNA Profiling

The DNA extracted from each of the two *Conyza* species was amplified using ten decamer primers of arbitrary sequences. A total of 50 different fragments were generated in *Conyza dioscoridis* (L.) while; only 47 fragments were generated in *Conyza bonariensis* (L.) Cronquist (Fig. 1a & 1b, Tables 1 and 2).

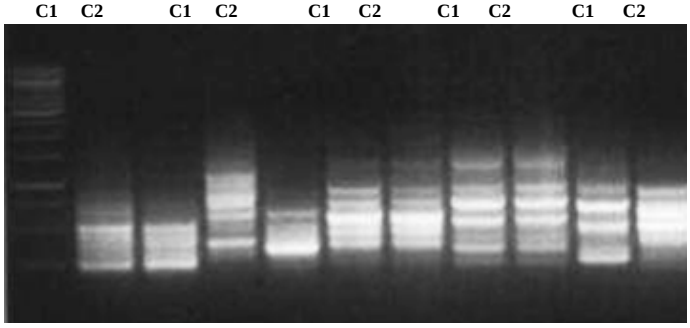

Fig. 1a: RAPD electrophoretic profile of *Conyza dioscoridis* [C1] and *Conyza bonariensis* [C2] using five decamer primers (OPB-05, OPB-07, OPB-14, OPB-15 and OPB-16).

The high percentage of similarity coefficient (88.89 %) between the two species indicates that they are closely related (El-zaeem *et al.,* 2006). The pattern obtained on using each of OPB-14, OPB-15, OPB-19, OPB-20 and OPG-12 was almost identical for the two species; this was supported by their respective similarity coefficients (100%). These primers could be, thus, used as indicators for obtaining genetic markers as well as in the identification of different *Conyza* species and the lowest degree of similarity (44.45%) was recorded using primer OPB-07 which could be used for differentiation between the two selected species as can be seen in (Table 2).

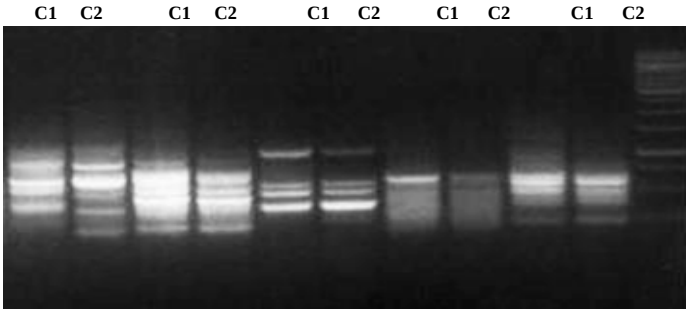

Fig. 1: The RAPD electrophoretic profile of *Conyza dioscoridis* [C1] and *Conyza bonariensis* [C2] using five ecamer primers (OPB-18, OPB-19, OPB-20, OPG-12 and OPO-17).

Determination of Pharmacopoeial Constants

Results obtained were expressed as percentages (calculated on dry weight basis of the plant material) and recorded in Table 3; a noticeable variability was observed among the two species. The total ash, acid insoluble ash, water soluble ash and crude fiber values obtained for *C. bonariensis* (17, 5, 10 and 3.5%, respectively) exceeded those for *C. dioscoridis* (10.5, 4.4, 5 and 1.5%); meanwhile, the moisture content was higher in the latter (10 *vs* 7.5 %).

Yield and Phytochemical Screening of Ethanol Extractives

The percentage yield of ethanol 70% extractives was higher for *C. dioscoridis* (EECD, 4.93%) than for *C. dioscoridis* (EECB, 3.89 %). Preliminary phytochemical screening of EECD and EECB revealed the presence of flavonoids, steroids, terpenoids and tannins in both extracts. Alkaloids were detected in traces; while the occurrence of saponins, anthraquinones and cardenolides could not be recognized.

Total Phenolic, Tannin and Flavonoid Contents

Results revealed that EECD contains a higher amount of all the aforementioned types of constituents when compared to EECB (1.17 *vs.* 0.96 mg/g, total phenolics; 2.43 *vs.* 1.83 mg/g, tannins and 0.62 *vs.* 0.29mg/g, flavonoids).

Median Lethal Doses of the Extracts

The acute toxicity study of EECD and EECB confirmed that both could be considered as safe (LD_{50} upto 0.5g/kg b.wt.) (Spearman and Karber, 1978).

Anti-inflammatory Activity of the Extracts

Results of evaluation of the anti-inflammatory activity (Table 4), revealed that the inflammation inhibitory effect produced by EECD (dose, 50mg/kg b.wt.; % inhibition, 74.2%) is obviously better than that exerted by EECB at the same dose level (50mg/kg; 59.0%), and was very close to that of the standard anti-inflammatory drug Indomethacin (30mg/kg; 78.5%).

Table. 1: Molecular size in base pairs of amplified DNA fragments produced by ten decamer primers in *C. dioscoridis* (L.) Desf. (C_1) and *C. bonariensis* (L.) Cronquist. (C_2).

Band size in bp*	Primer codes																			
	OPB-05		OPB-07		OPB-14		OPB-15		OPB-16		OPB-18		OPB-19		OPB-20		OPG-12		OPO-17	
	C_1	C_2	C_1	C_2	C_1	C_2	C_1	C_2	C_1	C_2	C_1	C_2	C_1	C_2	C_1	C_2	C_1	C_2	C_1	C_2
233	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
246	-	-	-	-	-	-	+	+	+	+	-	-	+	+	-	-	-	-	-	-
259	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
273	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
303	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
319	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
336	-	-	-	-	+	+	-	-	-	+	-	-	+	+	-	-	-	-	-	-
354	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	+
373	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
414	-	-	-	-	+	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-
436	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
459	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
483	+	+	-	-	-	-	+	+	-	-	-	-	-	-	+	+	-	-	+	+
536	-	-	-	-	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	-
565	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
595	-	-	-	-	-	-	-	-	+	+	-	-	-	-	+	+	-	-	+	+
627	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
660	-	-	-	-	+	+	+	+	-	-	+	+	-	-	-	-	-	-	-	-
733	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
772	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	+
856	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
902	-	-	+	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-
950	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
1111	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
1232	-	-	-	-	-	-	+	+	-	-	+	+	-	-	-	-	-	-	-	-
1367	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sum	**4**	**3**	**6**	**3**	**6**	**6**	**6**	**6**	**4**	**5**	**5**	**6**	**6**	**6**	**5**	**5**	**2**	**2**	**6**	**5**

* Approximate band size; (+) and (-): presence and absence of bands.

Table. 2: The total number of RAPD- PCR fragments, distribution of monomorphic and polymorphic bands, percentage of polymorphic fragments and similarity coefficient generated by ten decamer arbitary primers in *C. dioscoridis* (L.) Desf. (C_1) and *C. bonariensis* (L.) Cronquist. (C_2).

Primer codes	RAPD fragment		Monomorphic fragments	Polymorphic fragments	% of Polymorphic Fragments	Similarity coefficient
	C_1	C_2				
OPB-05	4	3	2	3	42.85	57.15
OPB-07	6	3	2	5	55.55	44.45
OPB-14	6	6	6	-	0.00	100.00
OPB-15	6	6	6	-	0.00	100.00
OPB-16	4	5	4	1	11.11	88.89
OPB-18	5	6	5	1	9.09	90.91
OPB-19	6	6	6	-	0.00	100.00
OPB-20	5	5	5	-	0.00	100.00
OPG-12	2	2	2	-	0.00	100.00
OPO-17	7	6	5	1	7.69	92.31
Total	51	48	43	11	11.11	88.89

Table. 3: Pharmacopoeial constants of *Conyza dioscoridis* and *Conyza bonariensis*

Pharmacopeial constant	Percentage g/100g	
	Conyza dioscoridis	*Conyza bonariensis*
Total ash	10.5	17.0
Acid-insoluble ash	4.4	5.0
Water-soluble ash	5.0	10.0
Crude fiber content	1.5	3.5
Moisture content	10.0	7.5

Table. 4: Anti-inflammatory effect of ethanol extracts of *Conyza dioscoridis* (EECD) and *Conyza bonariensis* (EECB) on carrageenan-induced rat paw œdema.

Animal groups[1]	Dose mg/kg b.wt.	Weight of rat hind paw ± S.E.			% Inhibition
		Right	Left	Difference	
Control	1ml saline	1.44 ± 0.22	0.60 ± 0.15	0.84	…….
EECD	50	1.17 ± 0.12	0.93± 0.15	0.24*	74.2 % *
EECB	50	1.17 ± 0.14	0.93± 0.15	0.24*	59.0%
Indomethacin	30	1.16 ± 0.09	0.98 ± 0.07	0.18*	78.5 %

[1]Male albino rats (n=6); ±S.E.: Mean standard error; ∗: *Significantly different from the normal control group at P <0.05*

Table. 5: Antimicrobial activity of ethanol extracts of *Conyza dioscoridis* (EECD) and *Conyza bonariensis* (EECB) on different selected microbial strains.

Microorganism	MIC (µg/ml)	
	EECD	EECB
Bacillus subtilis	400*	800
Escherichia coli	800	800
Mycobacterium phlei	200**	800
Listeria innocua "LMG 2710"	400*	800
Enterococcus faecalis	>800	>800
Staphylococcus aureus "Non-pathogenic LMG 3242"	>800	>800
Staphylococcus aureus "Pathogenic LMG 3240"	>800	>800
Staphylococcus aureus "Lab. Strain"	800	>800
Candida albicans	400*	>800

** Significant activity ; * Moderate activity

Table. 6: Results of Brine Shrimp Lethality Assay on ethanol extracts of *Conyza dioscoridis* (EECD) and Conyza *bonariensis* (EECB).

Tested solution	Concentration (µg/ml)	Number of dead nauplii after 24 hrs	% Mortality at the tested concentration
Negative control: artificial sea water	-	1	10
Positive control: DMSO (v/v)	-	2	30
EECD (w/v)	100	2	30
EECB (w/v)	100	4	40

Table. 7: Cytotoxic activity (IC_{50}) of total 70% ethanol extracts of *Conyza dioscoridis* (EECD) and *Conyza bonariensis* (EECB) against MCF7, HCT116 and HELA human cell lines.

IC_{50}	MCF7	HCT116	HELA
EECD	2.97	34.6	18
EECB	17.5	21	5.4

Antimicrobial Activity of the Extracts

Results of the antimicrobial activity of EECD and EECB revealed that the minimum inhibitory concentrations (MICs) of each extract were determined for a set of nine microbial strains (see material and methods). Results presented in Table 5 showed that, EECD was broadly more active than EECB. The highest activity of EECD was observed against *Mycobacterium phlei* (MIC, 200µg/ml) followed by moderate activities (MIC, 400µg/ml) against two other bacterial strains (*Bacillus subtilis* and *Listeria innocua* "LMG 2710") in addition to the yeast *Candida albicans*.

Evaluation of the cytotoxic potential of the Extracts

By Brine Shrimp Lethality Test (BSLT)

The brine shrimp lethality activity of EECD and EECB was tested at concentration level 100 µg/ml. Results displayed in Table 6 revealed only 30 and 40% mortality respectively; among the nauplii after 24hrs of incubation, in EECD and EECB. This effect was higher than that of the negative control which showed 10% mortality under the same operating conditions and the effect of EECD was equal to that of the positive control which showed up to 30% mortality also under the same operating conditions.

By Sulphorhodamine-B (SRB) Assay

Throughout the *in-vitro* assessment of the antiproliferative activity of EECD and EECB against the three human cell lines (MCF7; HCT116 and HELA) by SRB assay, the half maximal inhibitory concentration (IC_{50}) of the tested samples was taken as a measure of effectiveness. In this respect, EECD appeared more active against MCF7 cell line than EECB (IC_{50}: 2.97 *vs.* 17.5); whereas, the effect of EECB was more significant on HCT116 and HELA ($IC_{50:}$ 21 and 5.4 *vs.* 34.6 and 18, respectively). (Table 7, Fig. (2a & b)).

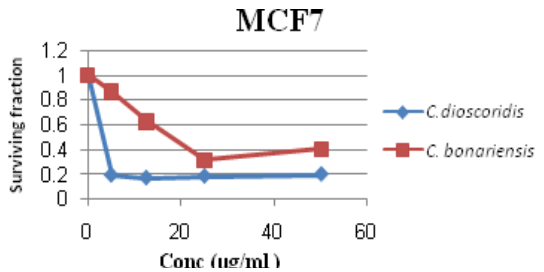

Fig. 2a:

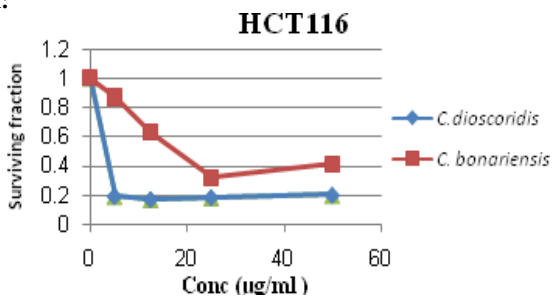

Fig. 2b:

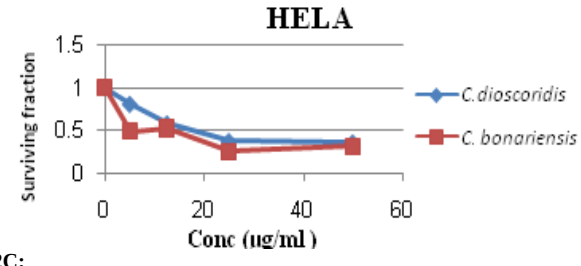

Fig. 2C:

Fig. 2: Survival curves of total ethanol 70% extracts of *Conyza dioscoridis* and *Conyza bonariensis* against MCF7 (2a); HCT116 (2b) and HELA (2c).

The use of herbs as medicines predates written human history. Ethnobotany is recognized as an effective way for discovery of new drug candidates. Natural products derived from plants and microorganisms are used in pharmaceutical formulations as powdered drugs, crude extracts, processed extracts or pure isolates.

Despite the apparent similarity in gross composition detected during the preliminary phytochemical screening of the two closely related species under investigation *viz.*, *Conyza dioscoridis* (L.) Desf. and *Conyza bonariensis* (L.) Cronquist, yet obvious variation was observed in their bioactivity. This could be attributed to qualitative and/or quantitative variability among the individual components within the phytochemical groups detected.
The genotypic identification was carried out using the RAPD technique as a tool for taxonomic characterization of the species. The high similarity coefficient observed, 88.89%, (Fig.1a & 1b, Tables 1 and 2) indicates that the two species are closely related. Five primers (OPB-14, OPB-15, OPB-19, OPB-20 and OPG-12) with similarity coefficients 100% could be considered as genetic markers. The OPB-07 primer was found the most effective in generating polymorphic bands with the least similarity coefficient, 44.45% and thus could be used to differentiate between the two species. From the previous findings, it can be concluded that the most relevant fragment resulting from the successful combination of template and primer was that produced by OPB-07 RAPD primers. Such primer could be used to discriminate between the two *Conyza* species depending on its low value of similarity coefficients and high level of polymorphism. However, the other estimated RAPD-primers, which produce high values of similarity coefficient and low levels of polymorphism, could be used in the identification of different *Conyza* species and accessions.

Pharmacopoeial constants are useful criteria for authentication and detection of adulteration in plant material. This was obvious in the current study were the determined values were dissimilar in the two closely related species under investigation. The relatively high total ash value in *C. bonariensis* indicates a higher content of carbonates, phosphates, silicates and/or silica. The higher percentage weight loss on drying (moisture content) observed in *C. dioscoridis* may increase its susceptibility to bacterial, fungal or yeast growth (Kokate *et al.*, 2006; EP 2005). The better anti-inflammatory activity of EECD relative to EECB (Table 4) may be attributed to its higher phenolic contents: flavonoids, phenolic acids and tannins, which were established by spectrophotometric determination. In fact, phenolics have been reported to ameliorate acute and chronic inflammatory conditions by their modulatory action on free radicals (Muthuraman *et al.*, 2011). The higher growth inhibitory activity observed for EECD over EECB on the majority of the tested microbial strains may be, as well, correlated to the phenolic composition (Raccash, 1984). As a matter of fact, the growth inhibitory mechanism exerted by phenolic compounds was declared to be through either affecting the function and composition of the cellular membrane, the synthesis of DNA, RNA protein and lipid or the function of the mitochondrion (Pérez *et al.*, 1992). Moreover, these results are in accordance with those formerly reported for *C. dioscoridis* during screening of the antimicrobial activity of certain Saudi Arabian desert plants using the well diffusion method (Zain *et al.*, 2012). The present study, being performed by means of different technique, thus confirms the significant effect of the plant extract against certain microbial strains such as *Bacillus subtilis* and *Candida albicans*. Moreover, the results obtained are in agreement with those reported concerning *Staphylococcus* spp. against which the plant extract was almost inactive or exerted a very low activity (Zain *et al.*, 2012).

Brine shrimp method was performed using dilute solutions of the extracts (100µg/ml) at such concentration the extracts were found non toxic to the nauplii, as indicated by the low % of lethality recorded after 24 hrs; however, higher concentrations should be tried. Although, brine shrimp lethality is considered as a predictive method for testing cytotoxicity; yet, it is generally useful for identification of strong anticancer activity and its capacity is limited in distinguishing between strong to moderate and weak potency anticancer compounds and it does not correlate with all cellular systems (Meyer *et al.*, 1982).

The cytotoxic effect of EECD was found remarkable against MCF7 (IC_{50}= 2.97 µg/ml) being more prominent than that of EECB (IC_{50}= 17.5 µg/ml); however, both could be considered as active. The effect of EECB was, on the other hand, more significant on HELA ($IC_{50:}$ 5.4 *vs.*18 µg/ml). The lowest activity was recorded when using HCT116 as cellular model. According to the United States National Cancer Institute plant screening program, a plant extract is considered to have active cytotoxic effect when its IC_{50} value does not exceed 20µg/ml, after an incubation period ranging between 48 and 72 hrs (Lee and Houghton, 2005). The cytotoxic capacity of the extract could correlate as well, with its phenolic content that is also effective in many pathogenic conditions as inflammation and diabetes (Ramirez-Cisneros *et al.*, 2012).

Moreover, the qualitative variation among the phenolic constituents of the extracts may be responsible for their effect on different cell lines. Some compounds seem to have specificity for overexpressing HCT116 and HELA cancer cell lines rather than for breast cancer cell lines (Mekawy *et al.*, 2009). Establishment of the structure-activity relationships requires the study of compounds with varying anticancer activity against different cell lines. In conclusion, this comparative report on *Conyza dioscoridis* and *C. bonariensis* provides helpful genetic and physico-chemical criteria for differentiation between these two closely related species besides throwing light on their bioactive potential as anti-inflammatory, antimicrobial and cytotoxic. Further studies are still necessary for isolation and identification of the bioactive components. However, the extracts could be suggested as useful ingredient in anti-inflammatory and antimicrobial herbal formulations after being subjected to intensive quality control procedures to ensure their purity, safety and efficacy.

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