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Molecular and pharmacological investigation of *Stipagrostis scoparia* growing in North Sinai

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ABSTRACT

Although many plants of Poaceae family attracted several investigators regarding their pharmacological aspects, there is almost no report on the pharmacology of *Stipagrostis scoparia*. Accordingly, it was necessary to carry out pharmacological study on the wild Egyptian plant which grows in North Sinai and selectively discriminate the plant using Random Amplified Polymorohic DNA technique. RAPD DNA analysis of the plant was performed using ten decamer primers. It showed distinguishable bands and generated 72 fragment patterns. The analysis of RAPD data can select the use of primers Z-02, C-17 and Z-08 for the selective discrimination of *Stipagrostis scoparia* growing in Egypt. Concerning the biological studies, the methanolic extract of *Stipagostis scoparia* exhibited significant analgesic, anti-inflammatory and anti-oxidant activities after *in-vivo* assays. Results obtained on assessing the long-term anti-hyperglycemic effect revealed a significant reduction in blood glucose level in Alloxan-diabetic rats treated with the methanolic extract. Prolonged administration of the methanolic extract significantly decreased the level of liver enzymes in carbon tetrachloride-intoxicated rats. According to the acute toxicity studies it was found that the methanolic extract of *Stipagrostis scoparia* is safe up to 7 g/kg b.wt.
Key words: *Stipagrostis scoparia;* RAPD analysis; analgesic; anti-inflammatory; anti-oxidant.

INTRODUCTION

The genus *Stipagrostis* contains species that occur in xerophytic open habitats that include desert and semi-desert and also dunes. There are 50 species occurring in Africa, Southwest Asia and Northwest India. *Stipagrostis scoparia* (Trin. & Rupr.) de Winter is a North African perennial grass which appears to be dominant in North Sinai dune-fields; it is used among Bedouins for grazing purposes of their camels (Arab Millennium Ecosystem Assessment, 2006; Raafat *et al.*, 2008). For selective discrimination of S*tipagrostis scoparia* growing in Egypt Random Amplified Polymorohic DNA (RAPD) analysis was performed. Plant genotype, or DNA fingerprinting of plants is a technology that has matured and is poised for very widespread practical application. Plant genotype analysis has application in the identification of plants in commerce, plant breeding and research. Commercial applications include the protection of plant breeder's rights and patents, quality control in plant production and processing and labeling of plant-derived foods and other products (Henry, 2001).

The simplicity and applicability of the RAPD technique have captivated many scientists' interests. Perhaps the main reason for the success of RAPD analysis is the gain of a large number of genetic markers that require small amounts of DNA without the requirement for cloning, sequencing or any other form of the molecular characterization of the genome of the species in question (Bardakci, 2001).

The grasses of Poaceae are "ecologically the most dominant and economically by far the most important family in the world" (Heywood, 1978). Some of the grass species have been proved to show strong antioxidant properties and have been effective in the treatment of inflammations (Rice-Evans *et al.*, 1996; Adom and Liu, 2002; Jana *et al.*, 2009). The fresh or dried rhizomes of *P. communis* (*Rhizoma Phragmites*) are used in diabetic complications in the traditional Chinese Medicine (TCM) (Li *et al.*, 2004). The herb is also used to treat breast cancer, leukaemia and diabetes in TCM (Rice-Evans *et al.*, 1996). There is almost no report on the pharmacology of *Stipagrostis scoparia*. Accordingly, it was necessary to carry out pharmacological study on the wild Egyptian plant which grows in North Sinai.

MATERIALS AND METHODS

Materials for DNA profiling

Extraction buffer n: 2% N-cetyl-N, N, N-trimethyl ammonium bromide (CTAB), 0.1 M Tris-HCI (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). Washing buffer 1: 76% ethanol, 0.2 M Na-acetate. Washing buffer 2: 76% ethanol, 10 mM NH_4-acetate. TE-buffer: 10 mM Tris-HCl (pH=8.0), 1 mM EDTA. 10X incubation buffer: 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 0.01% (w/v) gelatin. Taq DNA polymerase (Perkin-Elmer/Cetus, USA; Advanced Biotechnologies, UK). RNAse: Boehringer Mannheim. DNTP's mix: Pharmacia, Sweeden. Ten primers: purchased from Operon Technologies Inc. (Almeda, California, USA), were used for Random Amplified Polymorphic DNA (RAPD) analysis, with the following sequences: D-01 5'-ACCGCGAAGG-3', C-17 5'-TTCCCCCCAG-3', M-03 5'-GGGGGATGAG-3', M-16 5'-GTAACCAGCC-3', O-09 5'-TCCCACGCAA-3', B-16 5'-TTTGCCCGGA-3', Z-02 5'-CCTACGGGGA-3', Z-04 5'-AGGCTGTGCT-3', Z-08 5'-GGGTGGGTAA-3' and Z-13 5'-GACTAAGCCC-3'.

Materials for evaluation of the biological effects

Experimental models

Albino mice (20-25 g), used for determination of LD_{50} and analgesic activity; adult male albino rats of Sprague Dawley Strain (130-150 g), utilized for assessment of the different pharmacological effects,

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were obtained from the animal house colony at the National Research Center (Dokki, Giza, Egypt).

Drugs and kits

Alloxan: (Sigma, USA) solution (10 mg/0.1 ml). Carrageenan: (Sigma, USA). Indomethacin: Egyptian International Pharmaceutical Industries Co, (EIPICO, under license of Merck & Co. INC-RAHAWY N.J., USA). Silymarin: Sedico Pharmaceutical Co., 6 October City, Egypt. Vitamin E: (dl-*α*-tocopheryl acetate): Pharco Pharmaceutical Co. Metformin: (Cidophage®): Chemical Industries Development Co. (CID Co.), Giza, Egypt. Biomerieux kit: used for the assessment of blood glucose level. Biodiagnostic kit: Wak-Chemie Medical Germany (for measuring the antioxidant activity). Transaminase Kits (Bio-Merieux Co., France) biochemical kits for assessment of serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) enzymes.

Plant material

The plant was collected from El Arish and kindly authenticated by Prof. Dr. Abd Elraof A. Professor of Botany, Faculty of Science, Suez Canal University. A voucher specimen (# SAA-153) was deposited in the herbarium section of Pharmacognosy Department, Faculty of Pharmacy, Suez Canal University, Egypt. For DNA profiling, the whole fresh plant was collected during the year 2010, freeze-dried and ground to fine powder under liquid nitrogen. For biological screening, the extract was prepared after extraction of the whole plant by maceration with 90% methanol till complete exhaustion, filtered, evaporated till dryness.

DNA fingerprintig of *Stipagrostis scoparia*

DNA extraction

The plant powder was mixed with buffer n and incubated for 10 min at 65ºC. It was mixed about 2-3 times during incubation by inverting tube. The mixture was extracted with chloroform: isoamylalcohol (24: 1) and centrifuged at 10.000 rpm for 10 min. The DNA in the aqueous phase was precipitated by the addition of 3 M sodium acetate and isopropanol. The precipitates were washed in 70% ethanol and dissolved in a small volume of TE buffer. Polymerase Chain Reaction (PCR) amplifications were tested with RAPD primers from commercially available kits D-01, C-17, M-03, M-16, O-09, Z-02, Z-04, Z-08, Z-13, and B-16. The amplification reaction was carried out in 25 µl reaction volume containing 10X incubation buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 µM primer F, 1 µM primer R, 1 U *Taq* DNA polymerase and 25ng template DNA.

Thermocyling Profile and Detection of the PCR Products

PCR amplification was performed in a Perkin-Elmer/GeneAmp® PCR System 9700 (*PE* Applied Biosystems) programmed to fulfill 40 cycles after an initial denaturation cycle for 5 min at 94ºC. Each cycle consisted of a denaturation step at 94ºC for 1 min, an annealing step at 36ºC for 1 min, and an elongation step at 72ºC for 1.5 min. The primer extension segment was extended to 7 min at 72ºC in the final cycle.

Electrophoresis and visualization of PCR product

The amplification products were resolved by electrophoresis in a 1.5 % agarose gel containing ethidium bromide (0.5 µg/ml). PCR products were visualized on UV light and photographed using a Polaroid camera.

Biological studies

Acute toxicity studies (Determination of median lethal dose, LD_{50})

Determination of the LD_{50} of the methanolic extract of *S. scoparia* was estimated according to the Karber method, (1931). Preliminary experiments were done to determine the minimal dose that kills all animals (LD_{100}) and the maximal dose that fails to kill any animal. Several doses at equal logarithmic intervals were chosen in between these two doses, each dose was injected in a group of six animals by subcutaneous injection. The mice were then observed for 24 hours and symptoms of toxicity and mortality rates in each group were recorded and the LD_{50} was calculated.

Using the carrageenan-induced rat paw oedema test as described by Winter *et al*., (1962), Twenty-four male albino animals divided in to four groups (each of six animals) were used. They were administered one single oral dose of the tested extract at a dose of (100 mg/kg b.wt.) and the reference drug, Indomethacin at a dose of (20 mg/Kg. b.wt.). The negative control group received saline. One hour later all the animals had a subplanter injection of 0.1 ml of 1% carrageenan solution in saline, in the right hind paw and 0.1% of saline in the left hind paw. Four hours after drugs administration, the rats were sacrificed. Both hind paws were excised and weighed separately.

The percentage of oedema produced and that of oedema inhibition due to drug administration were, respectively calculated as follows:

$$\text{Oedema} = (\text{Wt. of right paw} - \text{Wt. of left paw}) \times 100/\text{Wt. of left paw}$$

$$\%\ \text{Oedema inhibition} = (Mc - Mt) \times 100/Mc$$

Where, Mc is the mean oedema in control rats and Mt is the mean oedema in drug-treated animals.

Analgesic activity

Swiss male albino mice (20-25 g) were used. Animals were acclimatized to the laboratory conditions for at least one hour before testing. The analgesic activity was estimated using acetic acid induced writhing test, the extract was administrated orally at a dose of (100 mg/kg b.wt.) and the reference drug, indomethacin at a dose of (20 mg/Kg. b.wt.). Thirty minutes later 0.6% acetic acid was injected intraperitonial (0.2% ml / mouse) each mouse was then placed in an individual clear plastic observation

chamber and the total number of writhes/minute was counted for each mouse (koster *et al.*, 1959).

Antioxidant activity

Diabetes mellitus was induced to male albino rats of the Sprague Dawely Strain (130-140 g) by intraperitoneal injection of alloxan (150 mg/kg b.wt.) as described by (Eliasson and Samet, 1969). Hyperglycaemia was assessed after 72 hours by measuring blood glucose (Trinder, 1969) using 36 diabetic rats divided into 6 groups each of 6 animals. They were administered one single dose of the tested samples and the reference drug in specific doses but the negative control and positive control groups received saline. Blood samples were collected after 7 days for estimation of blood glutathione level using biodiagnostic glutathione kit for assessment of antioxidant activity. The restoration of blood glutathione levels (reduced due to induction of diabetes) was taken as a measure of antioxidant activity. The percentage change observed after dose administration was, in each case, calculated according to the following equation:

$$\% \text{ of change} = (G–G0) \times 100/G0.$$

Where, G0 represents the glutathione level in diabetic animals, prior administration of the samples and G that measured after.

Hepatoprotective activity

Liver damage in rats was induced according to the method of (Klassen and plaa, 1969) by intraperitoneal injection of 5 ml/kg of 25 % carbon tetrachloride (CCl_4) in liquid paraffin. Seventy-two hours after administration of CCl_4, blood samples were withdrawn to be used for the biochemical study.

Adult male albino rats of Sprange Dawely Strains (130-140 g) were randomly divided into 5 groups each of 10 animals. The methanolic extract of the plant (100 mg/kg b.wt.), as well as, the standard drug (Silymarin, 25 mg/kg b.wt.), were separately administered daily for one week before and one week after liver damage. A group of animals were kept untreated (receiving only saline) and served as a negative control.

Biochemical studies were carried out; followed by an overnight fast; whole blood was obtained from the retro-orbital venous plexus through the eye canthus of anaesthetized rats. The blood samples were collected at zero time, after one week of receiving the tested drug, 72 hours after induction of liver damage then after a week of treatment with the tested samples and standard. Serum was isolated by centrifugation and divided for analysis of aspartate amino-transferase (AST), alanine amino-transferase (ALT) (Thefweld *et al.*, 1974) and alkaline phosphatase (ALP) enzymes (Kind and King, 1954).

Hypoglycemic activity

Male albino rats of the Sprague Dawely Strain (130-140 g) were injected intra-peritoneal with alloxan (150 mg/kg body weight) to induce diabetes mellitus (Eliiasson and Samet, 1969). Hyperglycemia was assessed after 72 hours by measuring blood glucose (Trinder, 1969) and after 2 weeks and 4 weeks intervals. They were administered one single dose of the tested samples and the reference drug in specific doses but the negative control and positive control groups received saline. At the end of each study period, blood samples were collected from the retro-orbital venous plexus through the eye canthus of aneasthetized rats after an overnight fast. Serum was isolated by centrifugation and the blood glucose level was measured (Trinder, 1969).

The percentage of change in blood glucose level from initial glycemia was, in each case, calculated according to the following equation:

$$\% \text{ of change} = (G0–Gt) \times 100/G0$$

RESULTS

DNA fingerprintig of *Stipagrostis scoparia*

The banding profile produced by the ten decamer primers used in RAPD analysis of *Stipagrostis scoparia* which grows in North Sinai of Egypt is illustrated in fig. (1). The RAPD electrophoretic profile of DNA sample amplified with the ten decamer primers showed distinguishable bands and generated 72 fragment patterns. RAPD fragments showed 10 bands by Z-02, 9 bands by C-17 and Z-08, 8 bands by Z-04, 7 bands by D-01 and M-16, 6 bands by Z-13 and B-16 and 5 bands by M-03 and O-09. The analysis of RAPD data can thus select the use of primers Z-02 (10 bands) and C-17 and Z-08 (9 bands) for the selective discrimination of *Stipagrostis scoparia* growing in Egypt.

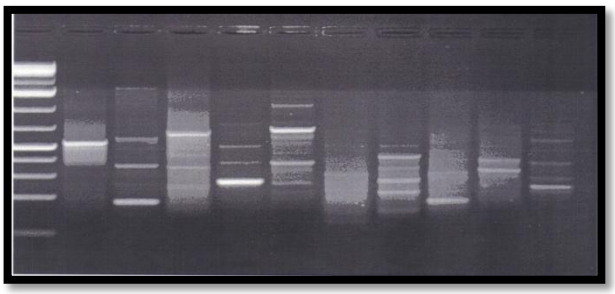

Figure (1): The obtained RAPD-PCR product for *Stipagrostis scoparia* using ten decamer primers.

Biological studies

Acute toxicity studies (Determination of median lethal doses, LD_{50})

It was found that the methanolic extract of *Stipagrostis scoparia* is safe up to 7 g/kg b.wt.

Anti-inflammatory activity

The anti-inflammatory activity (table 1) of the extract of *Stipagrostis scoparia* was evaluated on carrageenan-induced rat hind paw oedema model. The extract (100 mg/kg) has been found to possess significant anti-inflammatory activity on the tested experimental model.

Table (1): acute anti-inflammatory activity of the methanolic extract of *Stipagrostis scoparia* plant and indomethacin drug in male albino rats (n=6).

Group	Dose in mg/kg.b.wt	% Oedema		Potency[1]
		Mea± S.E.	% of Change	—
Control	I ml Saline	60.3±1.7	—	—
S. scoparia	100	32.4±1.1*	46.3	0.73
Indomethacin	20	21.8±0.6*	63.8	1

[1]Potency calculated as compared to the standard anti-inflammatory drug indomethacin,*Significantly different from control group at P<0.01, S.E. = Standard Error, % of change is calculated as regards to the control group.

Analgesic activity

The analgesic activity (table 2) of the extract of *Stipagrostis scoparia* was estimated using acetic acid induced writhing test. The extract (100 mg/kg) has been found to possess significant analgesic activity on the tested experimental model.

Table (2): Effect of methanolic extract of *Stipagrostis scoparia* plant on number of abdominal constrictions and indomethacin in mice (n=6).

Group	Dose mg/kg b.wt.	Number of abd. constrictions	% inhibition	Potency[1]
Control	1 ml saline	47.2±1.4	—	—
S. scoparia	100	29.3±0.7*	37.9	0.63
Indomethacin	20	18.7±0.4*	60.4	1

[1]Potency calculated as compared to the standard anti-analgesic drug Indomethacin, *Significantly different from control group at P<0.01, S.E. = Standard Error,‘% of inhibition is calculated as regards to the control group.

Antioxidant activity

The antioxidant activity of the extract of *Stipagrostis scoparia* was estimated using the restoration of blood glutathione levels (reduced due to induction of diabetes) as a measure of antioxidant activity. The extract (100 mg/kg) has been found to possess significant antioxidant activity on the tested experimental model. Results obtained for the methanolic extract are recorded in (table 3)

Table (3): Antioxidant activity of the methanolic extract of *Stipagrostis scoparia* plant and vitamin E drug in male albino rats (n=6).

Group	Blood glutathione (mg %)	% change from control	Potency[1]
Control (1 ml saline)	36.4±1.2	———	———
Daibetic	22.1±0.4*	———	———
Diabetic + Vitamin E (7.5m/kg)	35.8±1.1	61.9	1
Diabetic + extract (100mg/kg)	35.3±0.9	59.7	0.96

[1]Potency calculated as compared to the standard anti-oxidant drug vitamin E, * Statistically significant different from negative control group at p < 0.10, % of inhibition is calculated as regards to the positive control group.

Hepatoprotective activity

The data obtained were analyzed using student's t- test where means of the treated groups were compared to that of the control group for each variable. Results obtained for the methanolic extract are recorded in (table 4)

Table (4): Effect of the methanolic extract (100 mg/kg) of *Stipagrostis scoparia* plant and silymarin drug on serum enzymes level (AST, ALT and ALP) in liver damaged rats (n=6).

Group	AST (u/L)			ALT (u/L)			ALP (KAU)		
	Zero	72h	7d	Zero	72h	7d	Zero	72h	7d
Control	41.8±1.3	131.8±4.6*	161.4±0.9*•	36.8±1.2	141.4±4.2*	156.8± 0.9*•	7.1± 0.1	41.6± 1.8*	56.7± 2.1*•
extract	43.2±1.7	81.7±1.8*	63.8±1.9*•	33.5±1.1	76.2±2.1*	59.1± 2.1*•	7.3± 0.1	29.8± 0.9*	24.3±0. 5*•
Silymarin	38.6±1.4	56.4±1.9*	34.9±1.1•	39.1±1.3	51.7±1.4*	38.2± 1.1•	7.2± 0.1	19.3± 0.4*	7.5± 0.1•

***** Statistically significant from zero time at p < 0.01,•Statistically significant from 72h after ccl4 at p <0.01.

Hypoglycemic activity

A strong significant hypoglycimic effect in alloxan induced diabetes mellitus rats was observed for the methanolic extract in a dose of 100 mg/kg. The results are listed in table (5). Nuclear DNA markers usage was a useful tool to explore the origin, diversity, and parentage of many plants as in *Spartina anglica* (Poaceae), Such primers are used mainly to discriminate the plant (Ayres and Strong, 2001). In this study *Stipagrostis scoparia* was subjected to RAPD assay; this was performed using ten different primers and it can be concluded that the most relevant fragment resulting from the successful combination of template and primer was that produced by Z-02, C-17 and Z-08 RAPD primers.

Secondary metabolites as phenolic compounds and flavonoids commonly occur in both cultivated and wild Poaceae (Sánchez–Moreiras *et al.*, 2004) and this content of the phenolic compounds may be the cause of its antioxidant activities as in *Saccharum officinarum* (Poaceae) leave extracts (Ghiware *et al.*, 2012). The hypoglycemic and the hepatoprotective activities of *Stipagrostis scoparia* could be attributed to its constituents of antioxidants as the antioxidant compounds have been reported to beneficially improve pancreatic β-cell function by preventing or delaying β-cell dysfunction due to glucose toxicity as in the aqueous extract of the leaves of *Bambusa arundinacea* (Joshi *et al.*, 2009) and improve the liver function as in the methanolic extract of *Hordeum vulgare* Linn. (Poaceae) seeds (Shah *et al.*, 2009). Flavonoids which are some of the constituents of Poaceae were found to have anti inflammatory properties (Trease and Evans, 1989; Parmer and Gosh, 1978). It contributed to the anti inflammatory activity of the ethanolic extract of *Setaria megaphylla* (Poaceae) Leave (Okokon, et al., 2006) so the flavonoidal content of *Stipagrostis scoparia* may be the main cause of the anti-inflammatory activity exhibited by the methanolic extract.

The analgesic activity may be contributed to the content of the essential oil as in *Cymbopogon winterianus* (Poaceae) (Quintans-Ju´ nior *et al.*, 2008).This study opens the gate towards mining for the secondary metabolites responsible for the mentioned interesting activities within the tissues of *Stipagrostis scoparia*.

Table (5): Effect of the methanolic extract of *S. scoparia* plant and metformin drug on blood glucose level in male albino rats.

Group Time	Diab. non treated	Diab. treated with methanolic extract (100 mg/kg)		Diab. treated with metformin (100 mg/kg)	
	Mean±S.E	M±S.E	% of change	M± S.E	% of change
Zero	251.6±6.4	263.8±9.2	-	265.7±8.9	-
2 weeks	262.3±6.9	186.4±6.3*	28.9	136.2±4.2*	48.1
4 weeks	265.2±5.7	132.3±4.7*	50.1	85.6±2.3*	67.7

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