Original Research Article

Comparative studies on the effect of environmental pollution on secondary metabolite contents and genotoxicity of two plants in Asir area, Saudi Arabia

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Abstract

Purpose: To investigate the phytochemical contents and genotoxic effects of Ficus carica and Schinus molle grown in healthy and polluted environments in Asir area, Saudi Arabia.
Methods: Extracts of the aerial parts of the plants were screened for phytochemical constituents. Genotoxicity tests were carried out in mice using Comet assay, micronucleus test and chromosomal analysis.
Results: Extracts of the two plants grown in polluted soil showed elevations in phytochemical and heavy metal contents, when compared with extracts from non-polluted sites. In genotoxicity tests, F. carica and S. molle extracts produced significant increases in the number of micro-nucleated cells in mice, compared to control. Cytotoxicity tests showed that extracts from plants grown in non-polluted environments did not decrease polychromatic erythrocytes (PCE) to normochromatic erythrocytes (NCE) ratio in bone marrow cells, relative to control. Mice in the vehicle control group showed some aberrant metaphases and a few aberrations per hundred metaphases.
Conclusion: Pollution has significant effects on phenolic compounds, total flavonoids, and genotoxic potential of the two plants.

Keywords: Ficus carica, Schinus molle, Pollution, Genotoxicity, Total phenolic compounds, Total flavonoids

INTRODUCTION

Accumulation of heavy metals in the environment causes different types of health problems to humans. Man-made pollution are of various types, such as air pollution, water pollution, and thermal pollution. Lead in petrol, and emissions of CO, NO_2 and SO_2 contribute significantly to atmospheric pollution in cities [1]. Air quality is now under monitoring by many organizations specializing in this point [2]. It is important to monitor the response of living organisms

because some compounds formed in these responses may be used as bio-indicators.

The flavonoid and other phenolics in plants varies between tissues, organs and developmental stages, and is affected by environmental indicator such as UV, temperature, nutrient and water availabilities, and CO_2 concentration [3]. *Ficus* comprises one of the largest genera of angiosperms [4], of which *F. carica* is an important member. It is normally deciduous and commonly referred to as "fig". The edible part of the fruit is fleshy and hollow [5]. The plant has been traditionally used for its medicinal purposes due to its metabolic, cardiovascular, respiratory, antispasmodic, and anti-inflammatory benefits [6]. *Ficus carica* possesses antibacterial, antiviral, antioxidant, anticancer, anti-mutagenic, anti-inflammatory, antipyretic, antidiabetic, and fertility-enhancing properties [7,8]. It also exerted hormonal, immunological, dermatological, hypolipidemic, antispasmodic, antidiarrheal, as well as hepatoprotective and nephroprotective effects [7,8].

The essential oil of *S. molle* contains α-phellandrene, limonene, β-phellandrene, elemol and α-eudesmol, and it possesses antibacterial activity [9]. In traditional medicine, *S. molle* has been used for treating a variety of wounds and infections due to its antibacterial and antiseptic properties [10].

In this study, the effect of heavy metals on secondary metabolites and biological activities of these plants were investigated.

EXPERIMENTAL

Plant samples were collected from polluted and non-polluted sites in Asir area, KSA in the month of April, 2017, and were identified by Dr. Omran Naser from Ecophysiology Unit, Desert Research Center, Egypt. Voucher specimens (nos. 46 DRC 2017 and 47 DRC 2017 for *Ficus carica and Schinus molle,* respectively) were kept at the Herbarium of Desert Research Center.

Phytochemical screening

Extracts of the aerial parts of the plants were screened for phytochemical contents according to a previously described procedure [11].

Total phenolic compounds

Total phenolics were determined by using the method of [12], 200 µl of sample and 0.5 mL of Folin reagent add to 10 mL of distilled H_2O.

Three min. later, we added 1 mL of Na_2CO_3 and the volume was adjusted to 25 mL. Samples were remained for 1 h in the dark and we measure the absorbance against a blank at 725 nm.

Total flavonoids

Total flavonoids were determined colorimetrically [13], one mL of the extract was mixed in 5 mL of distilled H_2O, 0.3 mL of a 5% $NaNO_2$ was added, 6 min. later 0.6 mL of a 10% $AlCl_3$ $6H_2O$ was added. After 5 min, 2 mL of 1 M NaOH was added, and the total was adjusted to 10 mL with distilled H_2O. The absorbance at 510 nm against the blank. Quercetin was used in calibration curve.

Heavy metal analysis

Samples were digested according the method described earlier [14]. The digests were used to determine the heavy metal contents by aspirating directly to inductively coupled plasma (ICP) for the elements Co, B, Al, Cr, Cu, Mo Fe, Mn, Ni, Zn and Sr.

Genotoxicity assays

Animals

Animals used in this study were male Swiss albino mice, 10 - 12 weeks old, 20 - 25g in weight and were acquired from the Animal unit, Prince Sattam Bin Abdulaziz University (PSAU), Al kharj. The animals were fed with standard mice diet and water *ad libitum*. The animals were maintained under standard conditions of humidity, temperature (25 ± 2 °C), and light (12-h light/12-h dark). All the animal experiments were conducted in accordance with NIH guidelines and approved by the Ethics Committee, PSAU.

Study design

A total of sixty male mice were randomly divided equally into six groups (10 per group). Group 1 served as vehicle control and was treated with saline (0.1ml normal saline, i.p.). Group 2 was given a single i.p. injection of cyclophosphamide (50 mg/kg) on day 13 of the experiment and chosen as positive control. Groups 3 and 4, were given 500 mg/kg *F. carica* non pol. and *F. carica* pol. orally for fourteen consecutive days respectively. Similarly, Groups 5 and 6, were given 500 mg/kg b.w. *S. molle* non pol. and *S. molle* pol. orally for fourteen consecutive days respectively. All the animals were sacrificed on the 15th day of the study protocol.

Comet assay

Comet assay was used to study the DNA damage. Immediately after the mice sacrifice, both femurs were exposed, cut just above the knee and the bone marrow collected and kept on ice to allow the cells to settle before use. Single cell suspensions from each animal were prepared with 20 m EDTA and 10 % DMSO, and comet assay slides prepared within 1 h after sacrifice. Prior to the assay, cell suspension was gently shaken on ice for 15 – 30 s until the clumps settled. A supernatant portion was diluted with aliquot of 0.5 % agarose dissolved in Dulbecco's phosphate buffer and layered onto pre-coated microscope slides with 1 % normal melting agarose. Low-melting point agarose 0.5 % (w/v) was layered on the gel-embedded cells.

After 1 h incubation in cold lysing solution, slides (one per sample) were rinsed with neutralization buffer and the remaining slides immersed in chilled lysing solution overnight in dark under refrigeration. The next day, the slides were rinsed in neutralization solution, randomly positioned in an electrophoresis unit (submarine-type), and treated with cold alkali solution for 20 min to allow for DNA unwinding. Then, electrophoresed at 1 – 9 °C for 20 min at 25 V, with a current of approximately 300 mA. After electrophoresis, the slides were neutralized with 0.4 M Trizma base (pH 7.5) for 5 min and then dehydrated by immersion in ice-cold 100 % ethanol for ≥ 5 min. The slides were air-dried and stored at room temperature at a relative humidity of <60 %. After staining the slides with SYBR® Gold (Molecular Probes, Invitrogen, Carlsbad, CA, USA), 100 cells were scored per sample using Comet Assay IV image analysis software.

Micronucleus test (MN test)

PCEs and NCEs were scored. The bone marrow from each sacrificed mice were aspirated into a syringe filled with a small amount of fetal bovine serum (FBS). The bone marrow cells were transferred to centrifuge tube containing 2-5 ml fetal calf serum, centrifuged at 1100 rpm for 10 min. The cells were suspended again and a drop of the bone marrow was spread on glass slide.
Two smears were prepared from each animal, air-dried and fixed in alcohol (methanol). Slides are air dried and smears stained with May-Grunewald/Giemsa. From one mice, 1,000 PCEs and 1,000 NCEs were examined for micro-nucleated erythrocytes (MNPCE and MNNCE) using a Nikon microscope. In addition, marrow suppression was evaluated by recording the number of PCEs in 1,000 NCEs per animal. PCE: NCE ratio was calculated as in Eq 1.

$$PCE\ (\%) = \{PCE/(PCE + NCE)\}100 \dots\dots\dots (1)$$

Chromosomal analysis

Colchicine was injected in mice (4 mg/kg) intraperitoneally 90 minutes before sacrifice. Bone marrow cells were obtained from the femur bones of mice by flushing with 1 % sodium citrate solution and then fixed in acetic-acid: methanol (1:3) followed by refrigeration for 30 minutes. Slides were prepared, stained with 5 % Giemsa stain after 24hours and assessed by observing under a high resolution microscope. Fifty metaphase plates per mouse were recorded for structural and numerical aberrations in marrow cells. Cells were classified into five types: cells with gaps only, cells with breaks, acentric fragments, centric rings and polyploidy. The mitotic index was evaluated as the number of dividing cells in a 1,000 cell population.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Data were analyzed by ANOVA using SPSS ver. 14.0. At significant value of $p < 0.05$.

RESULTS

Heavy metals and micronutrients

The levels of calcium and magnesium were higher in *F. carica* grown in polluted environment, relative to that grown in normal soil. In *S. molle,* the levels of macronutrients (especially calcium and magnesium) were also higher in samples obtained from polluted growth environments, relative to samples from unpolluted environment. These results are shown in Table 1, which also depicts the levels of micronutrients in the two sample extracts from polluted and unpolluted growth environments. The results show that there was slight difference in the level of cadmium between samples from plants grown in polluted and non-polluted soils, but these values were within the permissible limit recommended by WHO. Lead concentrations in the two plants were also within the permissible limits recommended by WHO, that is 10 mg/kg. However, nickel levels in extracts of plants obtained from polluted sites were above the EPA acceptable limit, which might predispose to kidney damage and liver disorders [15]. Chromium concentrations in *F. carica* and *S. molle* from polluted sites ranged from 5.23 to 6.88 ppm. The permissible limit for chromium by WHO is 1.5 mg/kg. Chromium levels between 5 and 30 mg/ kg can lead to reduction in the yield

of plants [16]. The copper concentrations were in the range of 3.19 to 4.30 ppm in *F. carica* and *S. mole* grown in non-polluted soil, which are within the permissible limit.

However, there were higher levels of copper in the polluted plants, especially in *S. molle* with copper levels up to 109 ppm. The permissible limit for copper by WHO is 10 mg/kg. High levels of copper have been associated with metal fumes, fever, and hair and skin discolorations [17]. Zinc has very important roles in DNA synthesis, normal growth, and brain development. However, at high levels, zinc is neurotoxic [18]. There was a slight difference in the levels of zinc in *F. carica* from polluted and non-polluted sites, but the difference in zinc levels between polluted and non-polluted samples of *S. mole* was very high.

High concentration of iron causes tissue damage in humans [19]. The results achieved in this study showed that iron levels in *F. carica* from polluted and non-polluted sites ranged from 310 to 2576 ppm, whereas in *S. mole* samples (from polluted and non-polluted sites), iron concentration ranged between 239 and 619 ppm. The WHO recommended level of iron in medicinal plants is 20 mg/kg. Thus, the levels of iron were very high, especially in extracts of plants from polluted sites. Manganese deficiency produces severe skeletal and reproductive abnormalities. High concentration of Mn causes hazardous effects on the lung and brain [20]. All the *F. carica* and *S. molle* samples contained low amounts of manganese which were within the WHO permissible level in medicinal plants (200 mg/kg).

Total phenolics and flavonoids

Table 2 indicates the amounts of total phenolics and flavonoids in plant extracts. The total phenolic compounds in *F. carica* from polluted site (53.44 mg/g) were higher than the corresponding values in *F. carica* from non-polluted site (45.23 mg/g). In addition, total phenolic compounds in *S. molle* grown in polluted soil (65.25 mg/g) were higher than the corresponding level for *S. molle* from non-polluted sites (61 mg/g). For total flavonoids, the level in *F. carica* from polluted site (14.76 mg QE/g) was higher than that from non-polluted site (13. 08 mg QE/g). A similar pattern was seen in *S. molle*, where the total flavonoids in the polluted sample (20 mg QE/g) was higher than that in the unpolluted sample (18.66 mg QE/g).

Genotoxicity

Comet assay results (Table 3) showed no significant induction of DNA strand breaks was observed in any of the treated groups. In *F. carica* and *S. molle* grown in polluted soil, there were significant increases in DNA damage. Clastogenic/aneugenic damage was estimated by micronuclei formation in the bone marrow.

Table 2: Phytochemical composition of the two plants

Plant extract	Total phenolic compounds (mg/g)	Total flavonoids (mg QE/g)
Ficus non-pol	45.23±1.42 a	13. 08±1.11 a
Ficus pol.	53.44±2.12 b	14.76±1.08 b
Schinus non-pol.	61±2.23 a	18.66±2.15 a
Schinus pol.	65.25±3.17 b	20±1.29 b

Values are expressed as mean ± SD. Means within columns with different letters are significantly different ($p < 0.05$). Pol. = polluted

Table 1: Total heavy metals contents of the two plants studied (ppm)

Sample	Al	Ca	Cd	Co	Cr	Cu	Fe	Mg	Mn	Ni	Pb	Zn
Ficus non-pol.	1665	21855	0.045	1.815	0.615	4.3	310.4	4542	41.34	0.44	1.865	19.72
Ficus pol.	2306	27690	0.115	0.29	5.23	19.4	2576.5	6550	66.3	3.365	2.185	25.84
Schinus non-pol.	179.15	11375	0.11	0.345	0.595	3.19	239.2	1225.5	17.85	0.465	1.94	23.95
Schinus pol.	393.15	13300	0.17	0.525	6.885	109.5	619.5	1912.5	40.45	4.44	5.19	55.45

Pol. = polluted, Non pol. = Not polluted

Table 3: DNA damage in mice after the treatment as described in Experimental (n = 10)

Treatment group	Head DNA	Tail DNA	Tail moment	Migration
Normal	94.54 ± 1.29	5.46 ± 0.87	0.49 ± 0.21	1.67 ± 0.44
Cyclophosphamide	71.65 ± 2.36	28.35 ± 1.56	8.61 ± 0.89	16.52 ± 1.39
Ficus non-pol.	91.36*# ± 2.65	8.64*# ± 1.34	0.39*# ± 0.13	2.98*# ± 0.54
Ficus pol.	87.58*# ± 2.39	12.42* ± 0.93	0.59* ± 0.23	2.39*# ± 0.43
Schinus non-pol.	89.22*# ± 2.54	10.78*# ± 1.29	11.25* ± 0.69	15.54 ± 1.38
Schinus pol.	82.63*# ± 2.68	17.37* ± 1.92	0.95* ± 0.29	1.85*# ± 0.56

*, # significant differences from the control and positive control groups, respectively ($p < 0.05$). (Pol. = polluted)

There was no significant change in the micronucleus frequency in the bone marrow cells, and in PCE/NCE ratio (Table 4). As expected, exposure to *F. carica* and *S. molle* grown on polluted soil induced significant increase in the number of micro-nucleated cells comparing with control.

Table 4: Frequencies of MNPCEs and PCEs in bone marrow of mice treated as described in Methods. Male mice (n = 10)

Treatment group	MNPCE (%)	PCE (%)
Normal	0.49 ± 0.26	50.36 ± 7.22
Cyclophosphamide	1.95 ± 0.72	41.26 ± 4.31
Ficus non-pol.	0.51*# ± 0.39	48.49*# ± 4.98
Ficus pol.	0.63*# ± 0.77	43.29*# ± 6.35
Schinus non-pol.	0.71*# ± 0.69	47.58*# ± 5.62
Schinus pol.	0.79*# ± 0.59	44.73*# ± 2.67

*, # Denote significant differences from the control and positive control groups, respectively ($p < 0.05$). Pol. = polluted

Bone marrow cell cytotoxicity was measured by PCE/NCE ratio quantification and it revealed that extracts of the unpolluted plants did not change/decrease PCE/NCE ratio, when compared to the control. The vehicle control group of mice showed a few aberrant metaphases and a few aberrations per hundred metaphases. Cyclophophamide-treated positive control group showed many instances of aberrant metaphase and a statistically significant number of chromosomal aberrations, relative to the vehicle control group. The incidence of chromosomal aberrations in other treatment groups, whereas the positive control group contained a total of approximately 50 % abnormalities (Table 5).

DISCUSSION

The use of herbal medicines and herb-based nutrition has increased substantially in the last decades, so much so that 65 - 80 % of people globally use herbal medicines as treatment options for many diseases [21]. Toxic agents in high levels can occur in herbal medicines when they are collected from high pollution areas, such as areas close to roadways, industrial areas, oil refineries or metal mining sites [22]. The results obtained in the present study revealed that pollution led to elevation in total phenolics and total flavonoids, when compared with samples of plants from non-polluted sites. This suggests that elevations in total phenolics and total flavonoids can be considered as stress defense mechanisms in plants against pollution. Toxic agents can gradually build up in plants because they provide the first and vast available surface for bioaccumulation of these pollutants at

concentrations substantially higher than the permissible values considered safe for human use [23,24].

The exposure of humans to high levels of toxic agents constitutes a significant health risk [25]. Only a few studies on plants grown in polluted areas have been done to determine the toxic effects of pollutants [23,24]. In this study, the *in vivo* genotoxic potential of *F. carica* and *S. molle* grown in normal and polluted areas were investigated and compared.

Comet assay results indicated that *F. carica* and *S. molle* collected from polluted areas not only induced significant DNA damage in the bone marrow cells, but also increased the number of micro-nucleated cells in the mice exposed to them. These results imply that *F. carica* and *S. molle* collected from polluted areas were genotoxic to bone marrow cells, since there was no genotoxicity in cells from the group treated with *F. carica* and *S. molle* collected from control, non-polluted areas.

The genotoxicity produced by metals generally occurs through DNA repair system inhibition and oxidative stress, which results in genetic instability and DNA damage [26]. The oxidative damage leads to the generation of reactive oxygen species, which can cause direct DNA damage with the formation of breaks in single or double-stranded DNA [27,28]. Interestingly, these metals are required for cellular processes in humans. However, increased bioaccumulation and exposure can induced severe health-related effects such as psychological dysfunction, intestinal ulcer, skin eruptions, and cancer.

The reactive oxygen species produced by heavy metals can produce DNA damage, mainly through reaction (Fenton's) mechanism [28]. Studies have shown that Ni and Co, both in soluble and particulate forms, cause lung tumors in animals. In mammalian cells, nickel evoked chromosomal aberrations, chromatid exchange DNA-protein cross links and DNA breaks. There are reports of metallic cobalt and cobalt dust causing DNA single strand breaks and micronuclei formation in mammalian cells [26].

CONCLUSION

The findings of this study demonstrate that pollution has significant effects on the genotoxicities of *F. carica* and *S. molle*, and on their contents of total phenolics and flavonoids. These results also show the potentially severe effect of pollution on human health.

Table 5: Frequencies of different types of chromosomal aberrations (CA) and mitotic activity in bone marrow of mice after treatment with *F. carica* and *S. molle.* Male mice (n = 10)

Parameter	Control	Cyclophosphamide	*Ficus (non*-pol.)	*Ficus* (pol.)	*Schinus* (non-pol.)	*Schinus* (pol.)
Normal	91.46 ± 3.39	50.29 ± 3.67	90.35 ± 2.69*#	85.28 ± 3.19*	89.83 ± 0.49*#	81.72 ± 3.68*
Fragments	3.64 ± 0.31	14.23 ± 1.08	3.63 ± 0.32*	4.08 ± 0.43*#	3.26 ± 0.74*	5.62 ± 0.24*#
GAP	2.38 ± 0.29	12.17 ± 0.92	2.22 ± 0.26*#	3.29 ± 0.34*#	2.92 ± 0.43*#	4.26 ± 0.20*
SCE	1.43 ± 0.19	21.19 ± 0.87	2.20 ± 0.18*#	4.21 ± 0.16*	3.03 ± 0.32	4.74 ± 0.25*#
Break	-	-	-	-	-	-
Ring	1.29 ± 0.27	2.11 ± 0.26	1.60 ± 0.22	3.04 ± 0.09*#	2.50 ± 0.26*#	3.66 ± 0.19
TA	8.54 ± 0.39	49.71 ± 3.67	9.65 ± 0.49	14.62 ± 0.68	11.61 ± 0.65	18.28 ± 0.24

SCE: Sister chromatid exchange; TA: total aberration. *,# significant differences from the control and positive control groups ($p < 0.05$)

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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