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THE EFFECTIVENESS OF LEMON GRASS AND SAGE AQUEOUS EXTRACTS ON ACRYLAMIDE GENOTOXICITY

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ABSTRACT

Acrylamide (ACR) is commonly used in biochemistry and biotechnology laboratories. It may also occur in our daily life; in deep-fried foods; baked goods; tap water and smoking which is also a major ACR producer. In this work, the ameliorative role of lemon grass and sage (1:1) aqueous extract against the toxic potential of acrylamide was investigated after 48 hours of simultaneous treatments on albino rats. The 2, 2-diphenyl-1-picrylhydrazyl assay was carried out to evaluate the antioxidant potential of the plant mixture which gave more activity than each plant alone; perhaps due to synergistic effect between the two plants. The results of acridine orange/ethidium bromide fluorescent staining and total genomic DNA electrophoresis of peripheral blood leukocytes revealed that high dose of plant mixture treatments has a significant amelioration against ACR toxicity. On the other hand, low dose of plant mixture did not give that potential in micronucleus test of leukocytes, total genomic DNA electrophoresis of liver tissues and acridine orange/ethidium bromide fluorescent staining of bone marrow tissues which indicated that low dose was better as ameliorative agent.

Keywords: DNA, MN test, acrylamide, lemon grass, sage, protection.

INTRODUCTION

Numerous harmful chemicals are widely used and produced by human manufacturing processes of which acrylamide (ACR) plays an important role in industry and chemical laboratories. It is a monomer of polyacrylamide whose products are used in scientific research such as sodium dodecyl sulfate polyacrylamide gel electrophoresis, the manufacture of paper, water treatments, and as a soil stabilizer. Acrylamide can also be produced in fried or baked goods as a result of the reaction between asparagine and sugars (fructose, glucose, etc.) or reactive carbonyls. The moderate levels of ACR (5-50 ppb) were noted in heated protein-rich foods, while higher contents (150-4000 ppb) were reported in carbohydrate-rich foods. In addition, the environmental pathways such as the breakdown of glyphosate herbicides, and smoking are also acrylamide producers (Tareke *et al*., 2002). Consuming acrylamide contaminated well drinking water was noted to have sub-acute toxic effects. Acrylamid toxicity to humans includes symptoms such as foot weakness, fatigue, skin irritation, and dysfunction of either peripheral or central nervous system (World Health Organization, 2003). Acrylamide decreases protein synthesis and body weight relative to various concentrations (Yousef and El-Demerdash, 2006). It can also decrease the oxidative defense system in the cell (Zamorano-Ponce *et al*., 2006), and can release reactive oxygen species (Lima *et al*., 2007). Acrylamide is a carcinogenic agent since it induced skin neoplasia in SENCAR mice in presence of the tumor promoter TPA, (Bull *et al*., 1984b). It also induces lung tumors in SWISS-ICR mice (Bull *et al*., 1984a), thyroid tumors in male rats, and mammary and central nervous system tumors in female rats (Dearfield *et al*., 1988).
Since ancient times crude extracts of aromatic plants have been used for different purposes of food, drugs and perfumery. Sage (*Salvia officinalis*, Lamiaceae) is widely used all over the world for its medicinal properties and for culinary purposes. As a herbal tea, sage is a popular beverage served widely in coffee houses and traditionally used to treat sore throats. The plant is a rich source of di- and tri-terpenoids, phenolic acids, and flavonoids. The main antioxidative effect of sage has been reported to be related to the presence of carnosic acid and rosmarinic acid (Başkan *et al*., 2007).

Twelve phenolic compounds were identified in sage including gallic acid, 3-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid, caffeic acid, rosmarinic acid, hesperetin, epirosmanol, hispidulin, genkwanin, carnosol, carnosic acid, and methyl carnosate (Santos-Gomes *et al*., 2002). Large number of diterpenoids and phenolic acids has been investigated in sage extracts (Lima *et al*., 2007).

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Sage is also a source of some potent antioxidants (Lu and Foo, 2000), and sage extract was reported to have antimutagenic activity (Trninic *et al*., 1996). It has also been shown that sage methanol extracts have an antimutagenic potential against UV induced-mutations in *E. coli* strains (Filipic and Baricevic, 1997).

Lemongrass (*Cymbopogon citrates*, Poaceae) leaves extract shows many biological activities and did not induce any toxic effects (Souza-Formigoni *et al.,* 1986).
Isoorientin, isoscoparin, swertiajaponin, isoorientin 2' '-O-rhamnoside, orientin, chlorogenic acid, and caffeic acid were identified in *Cymbopogon citratus* extracts (Cheel *et al*., 2005). It was also reported that caffeic acid has anti-inflammatory, antimutagenic, and antitumoral effects (Karekar *et al*., 2000). Moreover, *Cymbopogon citratus* extract exhibits antimutagenic activity in various models (Vinitketkumnuen *et al*., 1994). *Cymbopogon citratus* has also been shown to prevent rat colon carcinogenesis initiated with azoxymethane and the formation of azoxymethane-DNA adducts in the colon (Suaeyun *et al*., 1997). *Cymbopogon citratus* extracts exhibited antioxidant effect in the DPPH and superoxide anion assay, the extract also has free radical scavenging activities in human erythrocytes (Cheel *et al*., 2005).

MATERIALS AND METHODS

(I) Animals

The present study was carried out using adult female Albino rats *(Rattus norvegicus)* weighing about 150±10 g. Experimental animals were healthy, normal, and were obtained from the animal house of the National Organization for Drug Control and Research (NODCAR, Giza, Egypt). They were housed in plastic boxes with steel-wire tops. Food and water were provided *ad libitum*. Animals were divided randomly into four groups:

1) Normal control,
2) ACR orally (50 mg/kg b.w.) as a single dose in the first day of the experiment,
3) ACR+ medicinal plants mix at low dose (LD) in drinking water (1.5mg/ml).
4) ACR+ medicinal plants mix at high dose (HD) in drinking water (3mg/ml).

Animals were sacrificed and dissected after 48hours.

(II) Chemicals

a) Acrylamide (2-propenamide, ACR) was obtained from Sigma pharmaceuticals.
b) Ascorbic acid (Vitamin C), Sigma pharmaceuticals (assay: 99.1%), was prepared fresh in methanol as a standard stock solution. All other chemicals used in this work were of high analytical grade.

c) 2,2-diphenyl-1-picrylhydrazyl free radicals (DPPH), Sigma pharmaceuticals, was freshly prepared as a 0.3 mM stock solution in methanol.

(III) Medicinal plants

Lemon grass (*Cymbopogon citratus*) and Sage (*Salvia officinalis*) were freshly obtained from Applied Research Center of Medicinal Plant (ARCMP), Kafr El Gabal, Giza, Egypt.

Preparation of both aqueous extracts

Fresh leaves were collected and minced into fine pieces. 200g of leaves were extracted with 40X (w/v) of hot distilled water at 55°C for 6 hours. The extract was then filtered using Whatman filter paper (No. 1). The filtrate was lyophilized by a freeze-dryer (Sniffers scientific, Holland), and then it was stored at -20°C until use (Kumazawa *et al*., 2002).

Determination of free radical scavenging potential of *Cymbopogon citrates* and *Salvia officinalis* extracts by DPPH assay

The 2, 2-diphenyl-1-picrylhydrazyl free radical scavenging assay was done to determine the antioxidant activity of both plants leaves aqueous extract. The scavenging reaction cause the changes in color from violet to yellow which evaluated colorimetrically at 517 nm using a UV-VIS spectrophotometer (Hewlett Packard , USA) (Blois, 1958). The % antioxidant activities (AA %) was calculated according to the following equation:

$$AA\% = 100 - [100 \times (Abs_{sample} - Abs_{blank}) / Abs_{control}]$$

The test was carried out three times independently.

Peripheral leukocytes isolation

Leukocytes were isolated from treated and control rats heparinized peripheral blood by incubation with 8ml erythrocyte lysing buffer (0.015M NH_4C1, 1mM $NaHCO_3$, 0.1 mM EDTA). Then, they were centrifuged for 5 min at 1000 rpm at 4°C using (Sigma 3K 30, Germany). Centrifugation was repeated till a white pellet appeared (Punareewattana *et al*., 2001).

Preparation of femoral bone marrow

The distal and proximal epiphyses of femoral bones of treated and untreated rats were removed, and then bone marrow was washed out using normal saline and collected in Falcon tube. The suspension was then centrifuged for 10 minutes at 2000 rpm at 4°C using (Sigma 3K 30, Germany). The supernatant was decanted and the pellet was re-suspended again in appropriate amount of normal saline to be ready for testing.

Dual acridine orange/ethidium bromide fluorescent staining for peripheral leukocytes and bone marrow tissues

Ethidium bromide/acridine orange staining was carried out to detect the morphological evidence of apoptosis, 10µl of cells were incubated for 1 min with 10µl (10 µg/ml) of a solution of ethidium bromide/acridine orange 1:1 ratio of (100 µg/ml) in PBS and wash immediately. Stained cells were visualized under (Olympus fluorescence microscopes,

Japan) and the images were digitally photographed. The apoptotic cells, with their shrunken, nuclear fragmentation, bright orange fluorescent and condensed chromatin were scored and the percentage of apoptotic cells were calculated in 500 counted cells per rat (1000x).

Analysis of micronuclei (MN)

The micronucleus assay from rats peripheral leukocytes was performed as the following, cells were spread on a clean glass slide, air dried, then fixed with 95% methanol (5min.) and stained with May-Grünwald Giemsa stain (5%), 500 cells of each treated animal were examined under (Olympus, Japan) light microscope (1000x).

Detection of total genomic DNA fragmentation by agarose gel electrophoresis

DNA extraction and detection of fragmentation were carried out according to "salting out extraction method" of Aljanabi and Martinez (1997) with some modifications by Hassab El-Nabi and Elhassaneen (2008). Cells were lysed in lysing buffer (50 mM NaCl, 1 mM Na_2 EDTA, 0.5% sodium dodecyl sulfate, pH 8.3) overnight at 37°C then; 4M NaCl was added to the samples. Centrifuge the mixture at 5,000 rpm for 10 minutes. The supernatant was transferred to a new tube then DNA was precipitated by 1 ml cold isopropanol by centrifugation for 5 min at 10.000 rpm. Wash the pellets with 1 ml 70% ethyl alcohol. Resuspend the pellets in of TE buffer (10 mM Tris, 1mM EDTA, pH 8). Incubate for 30 - 60 min with loading mix (0.1% RNase + loading buffer), and then loaded directly into the gel-wells. All centrifugation steps were done at 4°C using (Sigma 3K 30, Germany). The extracted DNA samples were estimated spectrophotometrically for purity at optical density (OD) 260/280 using (Hewlett Packard 8453, USA) spectrophotometer (Surzycki, 2000).

Gels were prepared using 1.8% normal melting electrophoretic grade agarose in 1X Tris borate EDTA buffer (89 mM Tris, 89 mM boric acid, 2mM EDTA, pH 8.3) for 1 h at 50 volts. The apoptotic bands of DNA fragmentation appeared and located at 180 bp and its multiples 360, 540 and 720 bp against thirteen bands of DNA marker (100–3000 bp). The intensity of DNA apoptotic bands were measured by (Biogene software, France) as a maximum optical density values.

Statistical analysis

Results were expressed as the Mean ± Standard Deviation (M ± SD). For the comparison of significance between groups, Student's *t*-test, for normally distributed data (McClave and Dietrich, 1991) was used to calculate the significance of differences observed between mean values of treated and control groups at a level of significance of ($P < 0.005$).

RESULTS

Evaluation of free radical scavenging activity

As shown in the (Table 1 and Fig. 1) the results obtained from AA% of the (1:1) mixture show an increase in about three concentration points than each plant individually, this might be due to synergistic effect between each others.

Table 1. Free radical scavenging activity of plants using stable DPPH radicals. The percentage data was presented as a mean of three replicates.

Conc. (µg/ml)	Antioxidant activity (AA %)			
	Standard	Sage	Lemon grass	Mix.
5	56.30	11.44	14.00	13.91
10	94.50	17.69	11.10	17.82
20	95.10	29.78	22.40	45.77
40	96.60	56.57	27.40	53.45
60	97.80	75.16	45.80	77.50
80	97.30	92.33	60.00	81.79
100	97.00	92.33	92.50	90.90

Evaluation of peripheral blood leukocytes dual acridine orange/ethidium bromide fluorescent staining

Cytological studies using ethidium bromide/acridine orange staining method has revealed typical apoptotic morphology with condensed nuclei, changes in staining intensity, and formation of apoptotic bodies in ACR - treated group (Fig. 2) and the obvious improvement was observed in plant-treated groups when compared to untreated rats Table 2.

Evaluation of bone marrow cells dual acridine orange/ethidium bromide fluorescent staining

Table 2 represents the result of bone marrow tissue fluorescent staining (Fig. 3) which proved the ameliorative effect of extracts mixture against ACR toxicity. Both doses were nearly to be equal with a slightly increase in normal cells percentage at low dose of medicinal plant extracts mixture.

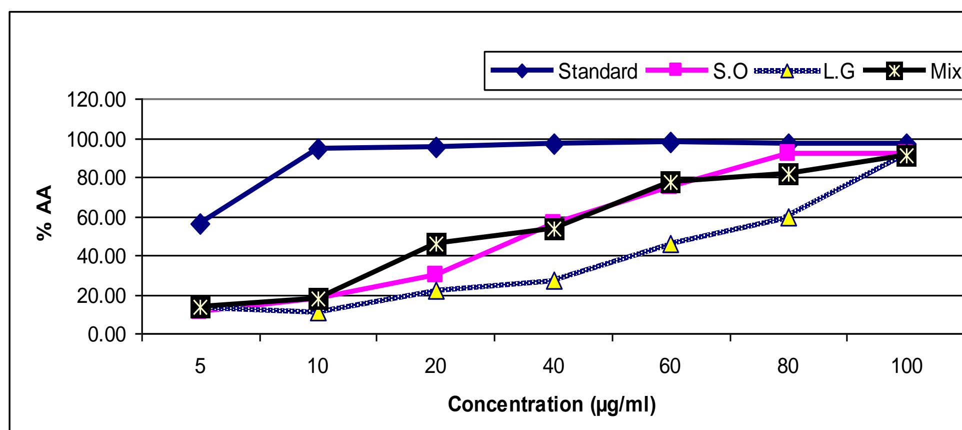

Fig.1. Diagram shows the absorbance curves against serial concentrations of plants using ascorbic acid as a standard. S.O: Sage (*Salvia officinalis*) and L.G: Lemon grass (*Cymbopogon citratus*).

Table 2. Peripheral blood leukocytes and bone marrow cells dual acridine orange/ethidium bromide fluorescent staining of treated and control groups, *: statistically significant ($p < 0.005$ *t*-test) compared to the ACR and #: statistically significant ($p < 0.005$ *t*-test) compared to the control. The percentage data was presented as (M ± SD) of three independent replicates.

	% Apoptotic cells	
	Bone marrow cells	**Peripheral blood leukocytes**
Control	7.33±1.5	4.8±0.76
ACR 50 mg/Kg	76.67±1.5$^{\#}$	48.8±1.04$^{\#}$
ACR+Low dose	50.33±2.5$^{*\#}$	32.0±1.00$^{*\#}$
ACR+High dose	35.0±2.6$^{*\#}$	34.3±1.52$^{*\#}$

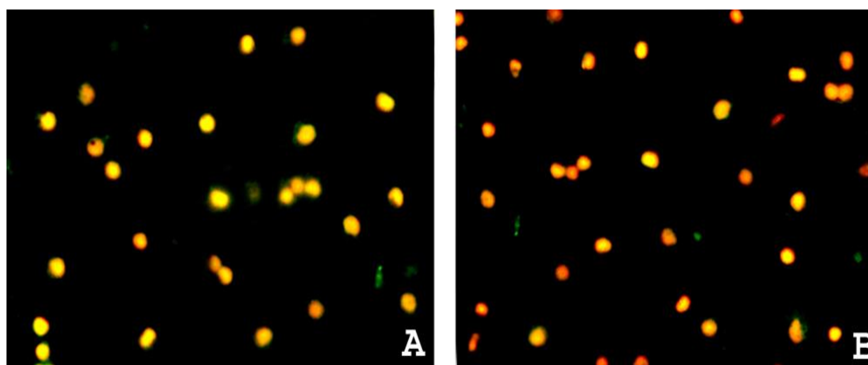

Fig. 2. Peripheral blood leukocytes dual acridine orange/ethidium bromide fluorescent staining of treated and control groups. Where (A): control cells, (B): apoptotic cells. (1000x).

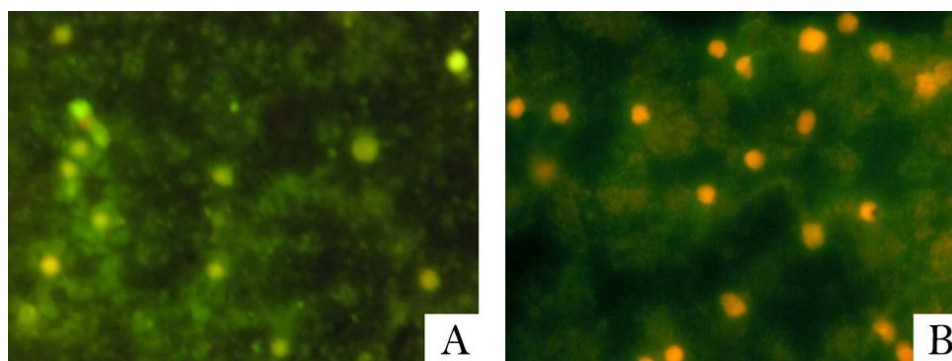

Fig. 3. Bone marrow cells dual acridine orange/ethidium bromide fluorescent staining of treated and control groups. Where (A): control cells, (B): apoptotic cells (1000x).

Analysis of peripheral blood leukocytes micronuclei:
The results were presented in Table 3, we found that the percentage of micronucleated leukocytes was increased significantly with ACR treatment than that of untreated records. The simultaneous low dose of extract treatment significantly protected the cells and reduced the percentage of micronucleated cells from (2.87±0.06) to (1.83±0.15). While high dose of plant extracts failed to exhibit the ameliorative effect.

Table 3. Peripheral blood leukocytes micronuclei investigation of treated and control groups, *: statistically significant ($p < 0.005$ t-test) compared to the ACR and #: statistically significant ($p < 0.005$ t-test) compared to the control. The percentage data was presented as (M ± SD) of three independent replicates.

	% micronucleated peripheral blood leukocytes
Control	0.38±0.08
ACR 50 mg/Kg	$2.87±0.06^{\#}$
ACR+Low dose	$1.83±0.15^{*\#}$
ACR+High dose	$3.53±0.42^{*\#}$

Genomic DNA extraction and damage detection in liver tissues
The results of liver tissues total genomic DNA extraction (Fig. 4A) showed the significant DNA protection as a result of treatment with low dose of plants mixture against the DNA damage induced by ACR dosing on liver tissues. The DNA damage was observed as sever apoptotic DNA fragmentation with laddering pattern in ACR group (lane: 2). While low dose of plant extract (lane: 3) gave good improvement in DNA damage. High dose gave very little improvement which can be evaluated by decreasing of apoptotic bands intensity (Table 4).

Genomic DNA extraction and damage detection in peripheral blood leukocytes
Figure 4B and Table 4 show the results of peripheral blood leukocytes total genomic DNA extraction which revealed the occurrence of DNA protection due to the treatment with low dose of plants mixture (lane: 3) against the DNA damage induced by ACR dosing on leukocytes (lane: 2). The DNA damage was observed as sever necrotic DNA fragmentation with smear pattern in ACR group (OD: 95.1±4.9). While low dose of plant extract gave good improvement in DNA damage (OD: 50.5±5.7). High dose gave very little improvement by decreasing of released DNA intensity (OD: 75.7±5.1).

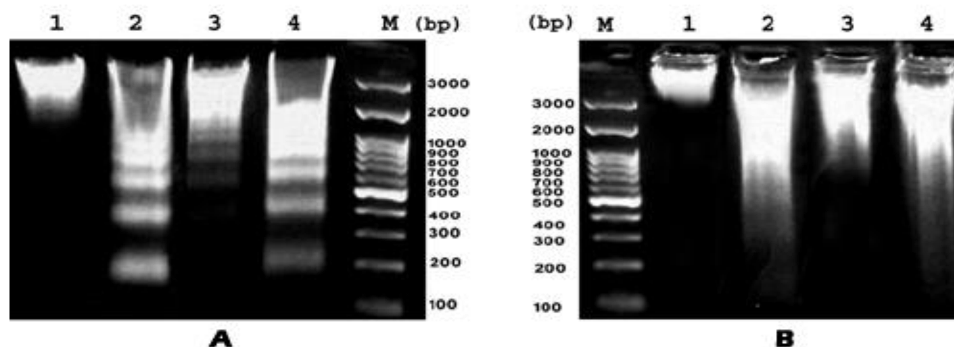

Fig. 4. Digital photo of total genomic DNA of liver (A) and peripheral blood leukocytes (B) showing the ameliorative effect of plant mixture against the damage induced by ACR, where 1: control, 2: ACR, 3: ACR+ extract low dose, 4: ACR+ extract high dose and M: 100-3000 bp marker.

Table 4. Total genomic DNA extraction from peripheral blood leukocytes and liver tissues of treated and control groups, *: statistically significant (p < 0.005 t-test) compared to the ACR and #: statistically significant (p < 0.005 t-test) compared to the control. The percentage data was presented as (M ± SD) of three independent replicates.

	Total DNA intensity	
	Liver tissues	**Peripheral blood leukocytes**
Control	31.5±3.6	22.3±2.4
ACR 50 mg/Kg	102.8±6.3#	95.1±4.9#
ACR+Low dose	56.4±3.2*	50.5±5.7*
ACR+High dose	94.9±5.0*	75.7±5.1*

DISCUSSION

ACR itself can be cytotoxic and genotoxic by decreasing the oxidative defense system in the cells (Zamorano-Ponce *et al*., 2006). Moreover, it released the reactive oxygen species (ROS) (Jiang *et al*., 2007). ACR administration increases the oxidative stress (Teodor *et al*., 2011). In this work, DPPH test results proved the antioxidant potential of our extracts. The extracts may exert their antioxidant activities by scavenging ROS, by enhancing the cellular antioxidant enzymes as SOD, CAT and GPx and increase glutathione in the cells (Zhang *et al*., 2012). Our results of total genomic DNA, peripheral blood leukocytes micronuclei assay and cytological studies by AO/EB of bone marrow and peripheral blood leukocytes supports the concept that biological effect of lemon grass and sage leaves aqueous extracts against the harmful action of ACR could be probably due to the antioxidant properties of these crude extracts due to their active constituents (Santos-Gomes *et al*., 2002 and Başkan *et al*., 2007). The compounds in the crude extracts could be acts as scavengers of the free radical and ROS generated by the ACR toxicity or as a preventer of DNA adducts formation induced by ACR and its metabolites (Manière *et al*., 2005; Koyama *et al*., 2006).

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