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Studying the Mutagenicity of Red Florets Safflower IL 111 Using Ames Test

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Abstract: In this study, mutagenic potential of red florets of Iranian Safflowers IL 111 was evaluated by Ames test using *Salmonella typhimurium* TA98 and TA100 strains. Florets collected and dried in two conditions, dried on shade and dried on the bolls by sun. Extractions have been done with 70% hydro-ethanolic solution and also boiling water. Solvents were then evaporated from extracts by freeze drier. Different dilutions of extracts were prepared in either distilled water or DMSO to be evaluated in the presence and absence of S9 bioactivation. Test and control plates were prepared according to the plate incorporation method and incubated for 48 h at 37°C. Then the number of reverted colonies was counted to determine the Mutation Ratio (MR) for each test concentration. The MR for test samples was far less than positive controls to be considered mutagen. There was also no significant difference in MR of extracts before and after S9 bioactivation at studied concentrations. Therefore, it can be concluded that Safflower extracts have no mutagenic activities under different preparatory and assay conditions using Ames mutagenicity assay.

Key words: Safflower, red florets, extracts, food additive, Ames test, mutagenicity

Introduction

Safflower, *Carthamus tinctorius* L., is a member of the family of Compositae or Asteraceae, cultivated mainly for its seeds, which are used as edible oil and as bird seed. Traditionally, the crop was grown for its flowers, used for coloring and flavoring foods and making dyes, especially before cheaper aniline dyes became available and also in medicines. Addition of safflower florets to foods is a widespread and ancient tradition. True saffron is perhaps the world's most costly spice and safflower is a common adulterant or substitute. Rice, soup, sauces, bread and pickles take on a yellow to bright orange color from the florets. Health concerns regarding synthetic food colorings may increase demand for safflower-derived food coloring. Cosmetic rouge can be made from carthamin dye mixed with French chalk and the Japanese cosmetic (beni) (Weiss, 1983) and lipsticks include safflower coloring (Smith, 1996). Many prescriptions for invigorating blood circulation, especially those for treatment of heart disease, include safflower along with other herbs and have been used in treatment of many diseases (Guishen, 1985). Cardiovascular disease treatment is the main use of safflower because it can invigorate the circulation. In 83% of patients with coronary disease, blood cholesterol levels have been reduced after 6 weeks of treatment with safflower (Guimiao *et al*., 1985). Treatment

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of cerebral thrombosis with safflower improved and lowered blood pressure in over 90% of patients (Guimiao *et al*., 1985; Damao, 1987). Herbal decoctions including safflower were also effective in treatment of cerebral embolism (Zuolin, 1992).

Safflower petals have immense medicinal and therapeutic properties as revealed by Chinese researchers. Petals of safflower from India were analyzed for carthamin (red pigment) (0.83%), oil (5.0%), protein (1.9%), ash (10.4%), fiber (12.2%) and fatty acid compositions. The petal oil was shown, for the first time, to contain some short chain fatty acids (10:0, 12:0 and 14:0), gamma linolenic acid along with fatty acids such as palmitic, stearic, oleic and alpha linolenic acids. Similarly the petals were rich in Ca (530 mg), Mg (287 mg) and Fe (7.3 mg/100 g) along with lower levels of Cu, Zn and Mn. The organic and inorganic constituents together may provide the nutritional and medicinal values to the petals (Nagaraj *et al*., 2001).

It is known that human cancers may arise following environmental exposure to the toxic substances. Chemicals that pose a mutagenic risk for humans would not be identified by screening of pure compounds because environmental mixture may contain mutagenic and carcinogenic substances that might not be distinguished in mutagenicity testing. Moreover, chemicals in complex mixture need not act additively in their mutagenicity; rather there may be a synergism between different mutagens and non-mutagens interacting with each other (Sugimura, 1980). Despite current use of safflower petals in different food industries, there is no convincing data on mutagenicity of this herb. The aim of this study was to evaluate the mutagenicity of Iranian safflower IL 111 (Iran Local) using the Salmonella/microsome mutagenicity test (Ames *et al*., 1975; McCann *et al*., 1975; Maron *et al*., 1983) that has been sufficiently developed and validated to be considered for widespread use in such assays.

Materials and Methods

Plant Materials

There are many cultivars of Iranian Safflower, but IL 111 with red florets was used for this study. Florets were collected from farms of Seed and Plant Improvement Institute of Ministry of Jahad and Agriculture in Karaj, a city near Tehran. Florets were identified by Mr. Omidi, A.H. the head of center of Research and Development of Safflower of Iran. One sample of florets dried in shade by fan and another sample dried under the sun light above the bolls. This was done to be able to compare the effect of two drying methods (fan vs. sun light) on samples constituents. Then samples were stored in food grade plastics under vacuum conditions and kept in refrigerator until use.

Preparation of Extracts

The dried florets powdered by coffee blender. The ethanolic extracts were prepared by maceration of 20 g of plant material with 200 mL of 70% ethanolic solution for 3 days at room temperature and this procedure were repeated twice. The corresponding extracts were filtered and dried by Labconco freeze dryer 5 (Labconco Co, USA) and then kept in refrigerator. Boiled extract was prepared by macerating 10 g of powdered florets in 100 mL of distilled water for 1 h and extracted by boiling for 90 min. After filtering and freeze drying, the extract was kept at -20°C.

Bacterial Srains

The Ames tester strains, *Salmonella typhimurium* TA98 and TA100 were maintained as frozen stocks and grown as described by Maron and Ames (Maron *et al*., 1983). They were checked for genetic markers and then frozen working cultures were prepared as described by Mortelmans and Zieger (2000). The media for Ames test were essentially the same as described by Maron and Ames(1983). The S9 fraction mixture purchased from Oriental Yeast Co., LTD, Tokyo-Japan and prepared freshly for each experiment as described by Maron and Ames (1983).

Antibacterial Activity of Extracts on Ames Strains

Samples that have been dissolved in dimethyl sulfoxide (DMSO) were used to determine their antibacterial activities on test strains using well diffusion assay. Nineteen milliliter of molten nutrient agar was inoculated with 0.5 mL of overnight culture of test strains on nutrient broth No. 2. The overnight cultures with OD_{560} equal to 0.1-0.2 that correspond to 2×10^8 cfu mL^{-1} were used for assay (McCann, 1975). The inoculated agar was poured into petri dishes and allowed to molt. Four wells were created in agar plates using a sterile pipette pasteur and 50 μL of different concentrations of samples (12.5, 6.25, 3.12, 1.56, 0.78 and 0.39 mg/50 μL) and also DMSO as control were added to each well. The plates were then incubated at 37°C for 24 h and the zones of inhibition were recorded.

Ames Mutagenicity Test

The procedure was basically the same as described by Maron and Ames (1983). Briefly, in this study 50 μL of different concentrations of extracts (390-6250 μg of either hydro-ethanolic or boiled water extracts/plate) were incubated for 20 min at 37°C with 100 μL of overnight culture of TA 98 and TA 100 ($1-2\times10^8$ cell mL^{-1}) in presence and absence of 500 μL of S9 metabolic activation mixture. Then mixture was added to 2 mL of molten top agar (supplemented with histidine and biotin) in the test tube, mixed well and then immediately poured on top of GM agar plates (minimal medium). The negative controls were 50 μL of DMSO and sterile distilled water. The positive controls were 5 μg sodium azide and 1.2 μg of 4-oxide-1-nitroquinoline (4NQO) per plate in absence of metabolic activation and 1 μg of 2-aminoanthracene (2-AA) per plate in presence of S9 metabolic activation. All tests were carried out in triplicate and incubated for 48 h at 37°C to determine the number of reverted colonies. The criteria for positive mutagenic activity was more than doubling of the number of reverted colonies per test plates in comparison to the negative controls in at least one strain either with or without metabolic activation and also a concentration-dependent increase in the number of reverted colonies. The experiments for each concentration were done two times in 3 plates each time.

Results

Antibacterial Activity of Extracts on Test Strains

The results of antibacterial activity of different extracts concentrations against TA 98 and TA 100 indicated that only concentration of 12.5 mg/50 μL has antibacterial activity that was omitted for subsequent Ames test (Table 1).

Table 1: Antimicrobial activity of Hydro-ethanolic extract of safflower against TA98 and TA100 by Well-diffusion method

Sample	Concentration (mg/50 μL/well)	Inhibition zone (mm) TA98	TA100
IL 111[1], dried by fan	12.50	14	0
	6.25	0	0
	3.12	0	0
	1.56	0	0
	0.78	0	0
	0.39	0	0
IL 111, dried by sun	12.50	10	10
	6.25	0	0
	3.12	0	0
	1.56	0	0
	0.78	0	0
	0.39	0	0
Negative control: DMSO	50 μL/well	0	0

1: Iran Local 111 variety of Safflower with red florets

Table 2: Results of Ames test for hydro-ethanolic extracts of safflower dissolved in distilled water

		TA98				TA100			
		-S9[3]		S9		-S9		S9	
Sample	Concentration (µg/plate)	Mean±SD[4]	MR[5]	Mean±SD	MR	Mean±SD	MR	Mean±SD	MR
IL 111[1],	6250	33±5.5	(0.8)	54±6.6	(1.2)	58±6.4	(0.5)	235±15	(1.2)
dried by fan	3120	38±6	(1.0)	69±13	(1.6)	76±2.5	(0.6)	206±24	(1.1)
	780	34±5.3	(0.8)	53±3.5	(1.2)	101±7.5	(0.8)	182±13	(0.9)
	390	37±4.9	(0.9)	33±5.3	(0.8)	93±9.3	(0.7)	174±11	(0.9)
IL 111,	6250	25±4.6	(0.6)	61±7.9	(1.4)	63±10	(0.5)	163±13	(0.8)
dried by sun	3120	28±4.5	(0.7)	48±6.1	(1.1)	62±9.6	(0.5)	196±25	(1.0)
	780	36±3.6	(0.9)	43±6.1	(1.0)	85±4.5	(0.7)	208±10	(1.1)
	390	33±1.5	(0.8)	34±3.5	(0.8)	89±16	(0.7)	179±13	(0.9)
Negative control: Distilled water		39±3.6	(1.0)	44±4	(1.0)	125±8.7	(1.0)	195±5.5	(1.0)
Positive controls[2]		500±20	(12.8)	623±30	(14.3)	917±76.4	(7.4)	1107±82	(5.68)

1) Iran Local 111 variety of Safflower with red florets, 2) Without S9: 4-NitroQuinoline n-Oxide for TA98, Sodium Azide for TA100, With S9: 2-AminoAntheracene for both strains, 3) S9: liver microsomal fraction, 4) Mean and standard deviation of 3 plates in 2 independent assays, 5) MR: Mutation Ratio of revertant colonies for test sample over the negative control

Table 3: Results of Ames test for hydro-ethanolic extracts of safflower dissolved in DMSO

		TA98				TA100			
		-S9[3]		S9		-S9		S9	
Sample	Concentration (µg/plate)	Mean±SD[4]	MR[5]	Mean±SD	MR	Mean±SD	MR	Mean±SD	MR
IL 111[1],	6250	38±5	(1.3)	39±6.8	(1.3)	88±2.1	(1.0)	259±16	(1.1)
dried by fan	3120	36±11	(1.3)	37±4.4	(1.3)	103±15	(1.2)	250±9.9	(1.0)
	780	38±6	(1.4)	36±1	(1.2)	113±7.6	(1.3)	245±31	(1.0)
	390	38±6.1	(1.3)	28±5.1	(0.9)	114±8.2	(1.3)	222±26	(0.9)
IL 111,	6250	37±2.1	(1.3)	44±2.5	(1.5)	49±12	(0.6)	198±9.3	(0.8)
dried by sun	3120	38±5	(1.3)	31±3.5	(1.0)	73±16	(0.8)	260±7.9	(1.1)
	780	34±6	(1.2)	34±1.7	(1.2)	96±22	(1.1)	239±21	(1.0)
	390	33±5	(1.2)	31±2.3	(1.0)	103±23	(1.2)	217±34	(0.9)
Negative control: DMSO		28±3.5	(1.0)	29±5	(1.0)	89±18	(1.0)	245±5	(1.0)
Positive controls[2]		500±20	(17.8)	487±95	(16.6)	839±59.9	(9.39)	853±120	(3.48)

Abbreviations: Same as in Table 2

Table 4: Results of Ames test for boiled-water extracts of safflower

		TA98				TA100			
		-S9[3]		S9		-S9		S9	
Sample	Concentration (µg/plate)	Mean±SD[4]	MR[5]	Mean±SD	MR	Mean± SD	MR	Mean±SD	MR
IL 111[1],	6250	48±4	(1.2)	48±3.5	(1.0)	345±31.2	(1.7)	370±36	(1.4)
dried by fan	3120	42±7.8	(1.1)	49±9.5	(1.1)	300±36.7	(1.5)	255±40.9	(1.0)
	780	34±6.0	(0.9)	43±5.5	(1.0)	245±40.5	(1.2)	285±64.2	(1.1)
	390	28±3.5	(0.8)	43±4	(1.0)	208±30.2	(1.0)	259±37	(1.0)
IL 111,	6250	48±7.6	(1.2)	52±3	(1.1)	375±22.9	(1.8)	382±24.7	(1.5)
dried by sun	3120	45±12.1	(1.2)	42±7.6	(0.9)	365±18.9	(1.8)	320±65.8	(1.3)
	780	36±5.3	(1.0)	47±7.6	(1.1)	272±8	(1.4)	351±44.1	(1.4)
	390	30±3.5	(0.8)	47±5	(1.1)	276±45.1	(1.4)	283±49.7	(1.1)
Negative control: Distilled water		37±6.5	(1.0)	44±3.5	(1.0)	201±18	(1.0)	253±9.5	(1.0)
Positive controls[2]		500±20	(13.4)	488±156	(11.0)	700±122	(3.5)	804±106	(3.2)

Abbreviations: Same as in Table 2

Mutagenicity of Test Extracts

The results of mutagenicity of extracts on TA98 and TA100 were summarized in Table 2-4. The Mutation Ratio (MR) that is the number of reverted colonies of extract concentration over the negative control was used to determine mutagenicity of extracts. None of the hydro-ethanolic extracts

concentrations dissolved in either distilled water or DMSO were mutagen on TA98 and TA100, with and without S9 bioactivation when compared to distilled water and DMSO as negative controls, respectively (Table 2 and 3). Table 4 shows results of Ames test for boiled water extracts concentrations on TA98 and TA100, with and without S9 bioactivation.

Discussion

Applications of herbal products as medicines or food additives can potentially expose public to adverse or toxic effects of plants components. Mutagenicity and carcinogenicity of chemical and natural compounds have been an important concern in marketing of these products. It has been determined that certain plants contain both carcinogen and anticarcinogen components and therefore, care should be taken to prevent such life threatening toxic effects to happen on human beings. There are different methods to identify the safety of plants being used in traditional medicine in different countries (Aeschbacher *et al*., 1983; Elgorashi *et al*., 2003; Marques *et al*., 2003; Park *et al*., 2004). Safflower is one of the plants used traditionally for many applications including herbal medicine, coloring agent and also bird seeds. Its florets are commercially used as a substitute of Saffron that is quite expensive. Therefore, it is important to elucidate its potential mutagenic effects for safety reasons. We could find only 2 reports in the literature that evaluated the mutagenicity of Safflower cultivar, Carthami flos, available in Japan and China (Morimoto *et al*., 1982; Yin *et al*., 1991). In this study, we decided to evaluate mutagenicity one of the common cultivar of Safflower in Iran, IL 111 with red florets, using scientifically based methodology for collection, identification, drying, storage and extraction. It is known that hydro-alcoholic extraction method is better than boiling water method for capability to more completely extract different plant materials (Samuelsson *et al*., 1999). Therefore, similar to Morimoto *et al*., we used both extraction methods to compare the results with only boiling method used by Yin *et al*. (1991). Similar to both previous studies (Morimoto *et al*., 1982; Yin *et al*., 1991), we also used two tester strains, TA 98 and TA 100, in the presence and absence of S9 metabolic activation to thoroughly investigate mutagenic potential of Safflower. The recommended maximum test concentration for soluble non-cytotoxic substances is 5000 or 10000 μg/plate (Mortelmans *et al*., 2000). Therefore, we used a wide range of concentrations from 390 to 6250 μg/plate which had no antibacterial effects on both tester strains. Despite differences in cultivars as well as the collection, drying, storage and extraction methods in other studies (Morimoto *et al*., 1982; Yin *et al*., 1991) with our study, none of the assays showed mutagenic activity for safflower florets using Ames test. Unfortunately, there is an important clear mistake in published papers of Yin *et al*. (1999) and Nobakht *et al*. (2000) regarding the positive mutagenicity of Carthami flos showed by Morimoto *et al*., However, similar to our findings and Yin *et al*. (1999). Morimoto *et al*. (1982) did not find mutagenic activity for Carthami flos (Koka as Japanese name for plant) in their study (Table 2-4). This clear mistake could be due to improper reading of the report of Morimoto *et al*. (1982) which lead to put an unnecessary ban on usage of Safflower in Iran and possibly other countries with bad economic impact for agriculture industry. Since the results of Ames test can not completely show the safety of natural and chemical compounds, Yin *et al*. (1991) also reported the results of chromosomal aberration and micronucleus assays for different Chinese plants including Safflower. They reported that water extract of *Carthamus tictorius* L. (Safflower), only at very high concentrations can exert intermediate level of chromosomal aberration (4 g kg^{-1}) and micronucleus formation (2-4 g kg^{-1}). Therefore, it is a good idea to test the hydro-ethanolic extracts of Safflower florets for their potential to exert chromosomal aberration and micronucleus formation in comparison to water extracts. In conclusion, present results similar to previous reports indicate no mutagenic effects of IL 111 safflower extracts using Ames test. In addition, due to availability of other cultivars of safflower, we are

currently studying the mutagenicity of other common cultivars available in Iran and in particular the LRV 5151 with yellow florets.

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