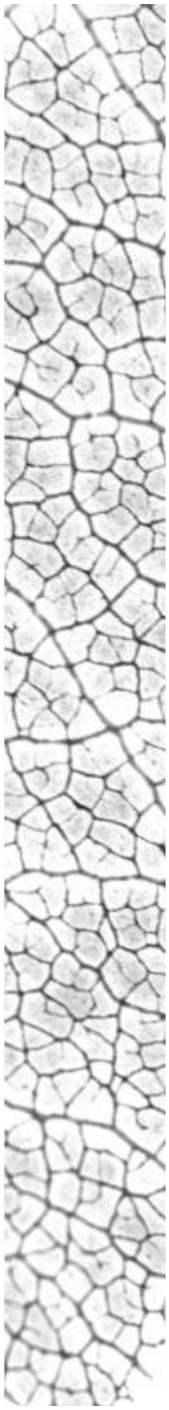

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The role of Moringa leaf extract to reduce the negative effect of some food additives on gene expression of CYP11A1 gene in male albino rats

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Abstract

This study aimed to investigate the effect of *Moringa oleifera* leaf extract on the gene expression of the Cyp11A1 gene in male white rats treated with some food additives (Carmoisine, Sodium benzoate, Monosodium glutamate).The study included 40 male rats divided into eight groups and each group consist of 5 animals dosed for 60 days. The groups were divided as follows: Negative control group (C_1) given drinking water only, Positive control group(C_2) given Moringa leaf extract in dose(200mg/kg/B.W),The first treatment group (T_1) given orally Carmoisine in the dose (250mg/kg/B.W),The second treatment group (T_2) given orally monosodium glutamate in the dose (15mg/kg/B.W),The third group (T_3) given orally Sodium benzoate in the dose (50mg/kg/B.W),The fourth group (T_4) given orally Carmoisine in the dose(250mg/kg/B.W) then given Moringa leaf extract in the dose(200mg/kg/B.W) Concurrently, The fifth group (T_5) given orally monosodium glutamate in the dose (15mg/kg/B.W) then given Moringa leaf extract in the dose(200mg/kg/B.W) Concurrently, and The sixth group (T_6) given orally Sodium benzoate in the dose (50mg/kg/B.W) then given Moringa leaf extract in the dose(200mg/kg/B.W) Concurrently. The results showed a significant decrease ($P < 0.05$) in the level of gene expression of the CYP11A1 gene in animal groups (T_1, T_2, T_3) which were given food additives (Carmoisine, Monosodium Glutamate, Sodium Benzoate) compared with the negative and positive control groups. No significant differences between the three groups mentioned. As for the groups (T_4, T_5, T_6), no significant differences appeared between the mentioned groups, but showed a significant increase ($P < 0.05$) compared to the groups (T_1, T_2, T_3), and no significant differences appeared compared to the negative control group (C_1), but showed significant differences compared to the positive control group (C_2). The positive control group (C_2), which included animals that were given Moringa leaf extract, showed significant differences compared to the negative control group (C_1).From the current study, we conclude that the Moringa leaf extract plays an important positive role in reducing the harmful effects of some food additives.

Keywords: Moringa, gene expression, Food additives, CYP11A1 gene

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INTRODUCTION

Moringa oleifera Lam is a fast-growing plant that has many names such as, the Yusr tree, the Drumstick tree, the Horseradish tree, and the Miracle Tree (Sanjayand Dwived, 2015 and Udoji Itodo et al., 2018). It grows in tropical and semi-tropical regions, its origin is from India, but at present, its cultivation spread in the Middle East and Africa and Asian countries. It has multiple uses because of its nutritional, medical, and industrial importance (Leone *et al*., 2015; Saini et al, 2016). Because Moringa leaves contain Flavonoids, Glycosides, Vitamins, Saponins, Tannins, and Carotenoids, they are used as a source of antioxidants, cancer, asthma treatment, pneumonia, bronchitis, skin and eye diseases (Charoensin, 2014; Upadhyay et al. 2015; Daba, 2016; Lamou et al. 2016).Moringa contains Flavonoids such as Quercetin, Rutin, and Luteoline, Phytosterols such as Stigmasterol, Sitosterol, and Campesterol which reduce cholesterol and blood sugar, arteriosclerosis, high blood pressure, andprotect the body from heart disease (Kumar and Pandey, 2013; Gopalakrishnan et al. 2016). Also, Moringa is used as a nutrient because it contains high levels of carbohydrates, proteins, vitamins, and minerals such as magnesium, potassium, iron, zinc and phosphorous and can be used to treat malnutrition, especially among

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infants and pregnant women (El Sohaimy et al. 2015; Nalamwar et al. 2017).Moringa seed oil can be used in biofuels and this is because its oil contains high concentrations of unsaturated fatty acids and meets all major specifications of the biodiesel standards of the United States of America, Germany, and Europe (Mofijur et al. 2014; Padhi, 2016). Moringa also pulls pollutants out of the water, such as heavy metals and pesticides (Ali et al. 2010; Hendrawati et al. 2016).

In recent years, after the development in the food industry, it's marketing, and its increasing consumption, it has become necessary to add a lot of materials that can preserve food from spoilage, increase its nutritional value, improve its physical characteristics, such as color, taste, smell, and texture, and make it easy to cook and increase the consumer's attractiveness. These materials are called Food Additive, Which are diverse and plentiful as Food dyes, Preservatives, Sweeteners, Anti-caking agents, and Flavor enhancers (Bawazir, 2016; Dar et al. 2017).Food additives are added to many foods such as dairy products, fish, juices, jams, ice cream and sweets, in addition to being used in the manufacture of medicines, soap, shampoo, toothpaste, cosmetics, and others (Carocho et al. 2014; Masoneand Chanforan, 2015).

A lot of industrial food additives have become controversial due to their toxic or carcinogenic effects, and therefore many of them have been prevented from using. The effects of food additives may be immediate or may be harmful at continuous exposure for long periods. The immediate effects include headache and changing energy levels, mental focus, behavior, and response. Immunostaining. Many studies have shown the harmful effects of these additives, especially when used for long periods, as they cause liver, kidney, and spleen diseases, allergic diseases, asthma, neuropathy, diseases of the digestive system, indigestion, growth retardation, anemia, influencing immunity, increased risk of cancer and others (Soltan et al. 2012; Al-Shinnawy and El-Kattan, 2013; Elbanna et al. 2017; Dar et al. 2017; Aminand Al-Shehri, 2018).Therefore, this present study was designed to know the effect of Moringa leaf extract in reducing the harmful effects caused by some food additives (Carmoisine, Sodium Benzoate, and Monosodium Glutamate) on the gene expression of the CYP11A1 gene.

MATERIALS AND METHODS

Experimental animals

This study was conducted in the animal house of the Department of Life Sciences / College of Education / University of Al-Qadisiyah. Forty adult male Albino rats (12-14 week old, 180-200 gm weight) were maintained under standard conditions (12 hours light and 12 hours dark at 25Cº).The animals were housed in sanitized polypropylene cages (50x35x15) cm with stainless steel grill top, under sterile conditions.

Experiments Design

Forty adult male Albino rats divided randomly into eight groups, each group contains 5 animals and treated for 60 days as follows: Negative control group (C_1) given drinking water only, Positive control group(C_2) given Moringa leaf extract in dose(200mg/kg/B.W),The first treatment group (T_1) given orally Carmoisine in the dose (250mg/kg/B.W),The second treatment group (T_2)given orally monosodium glutamate in the dose (15mg/kg/B.W),The third group (T_3) given orally Sodium benzoate in the dose (50mg/kg/B.W),The fourth group (T_4) given orally Carmoisine in the dose(250mg/kg/B.W) then given Moringa leaf extract in the dose(200mg/kg/B.W). Concurrently, The fifth group (T_5) given orally monosodium glutamate in the dose (15mg/kg/B.W) then given Moringa leaf extract in the dose (200mg/kg/B.W) Concurrently, and The sixth group (T_6) given orally Sodium benzoate in the dose (50mg/kg/B.W) then given Moringa leaf extract in the dose(200mg/kg/B.W) Concurrently.

Preparing alcoholic extract for plant

The extract was prepared according to the method Harborne, (1984) by taking 20 gm of dry leaf powder of Moringa plant in Thimbles then, put it in the Soxhlet Extractor and added 200 ml of 95% ethyl alcohol and extracted at a temperature of 50 °C for 24 hours. Then the extract was concentrated in a rotary evaporator at a temperature of 40°C - 45°C. Then it was filtered using Watman filter paper after that, it was put in clean glass containers and dried in the electric oven at a temperature of 45 ° C. The dry matter was collected and placed in clean, sterile, sealed glass containers and kept until use (Afolabi *et al*., 2013; Alsudani *et al*., 2019).

Preparation of Carmoisine

The Carmoisine dye powder produced by the Indian company RohaDyechem was used at a concentration of 250 mg/kg of body weight and 1 ml orally was administered to each animal (Fijer and Al-Mashhedy, 2016).

Preparation of monosodium glutamate

MSG was used at a concentration of 15 mg/kg of body weight and the animals were dosed through 1 ml orally per each animal (El- Imam and Abd El- Salam, 2019).

Preparation of sodium benzoate

Sodium benzoate was used at a concentration of 50 mg/kg of body weight and the animals were dosed through 1 ml orally per each animal (Hadi and Mahdi, 2019).

Sacrifice animals

All animals were sacrificed at the end of the experiment and samples of testis were taken, made flashing by put them in liquid nitrogen (-196 Cº), and

Table 1. Primers sequences of CYP11A1 and GAPDH genes

qPCR master mix		Volume
cDNA template		2.5µL
Primers(10pmol)	Forward primer	1.25 µL
	Reverse primer	1.25 µL
2x green star master mix		25
DEPC water		20 µL
Total		50 µL

Table 2. qPCR reactions mix of genes

Primer	Sequence (5'-3')	Amplicon
CYP11A1 gene	TACACAGACGCATCAAGCAG	71bp
	AAAGCGGAATAGGTCATCGC	
GAPDH	GCTGCCTTCTCTTGTGACAAAG	121bp
	TGACTGTGCCGTTGAACTTG	

Table 3. The program of qPCR

qPCR step	Initial Denaturation	Denaturation	Annealing \ Extension Detection (scan)	Melting
Temperature	95 °C	95 °C	60 °C	60-95°C
Time	3 min	20 sec	30 sec	30 sec
Repeat cycle	1	45		1

store in (-20Cº), to study gene expression for CYP11A1 which responsible for testosterone hormone production.

Quantitative Reverse Transcription Real-Time PCR

Quantitative Reveres Transcription Real-Time PCR technique was performed for quantification of relative gene expression analysis for the CYP11A1 (Testosterone) gene. These genes were normalized by using the housekeeping gene (GAPDH).Total RNA was extracted using the Trizol kit equipped by the Korean company Pioneer. Then the detection of the extracted RNA through the use of a special device, Nanodrop spectrophotometer, by determining the concentration of ng \ µl RNA and measuring the RNA purity through an absorbance reading of (260/280 nm). Then the acid extract was treated. RNA using Dnase I treatment to get rid of DNA remains in the recovery process. The complementary cDNA DNA was then synthesized from extracted RNA samples using the Accupower Rockscript RT Premix kit and equipped by the Korean company Pioneer. The qPCR examination of the cDNA samples for the trial groups was performed using the Accupower 2x Green Star qPCR kit equipped by the Korean company Pioneer.The primers used are designed using the special sequence of genes from the NCBI Gene Bank and using the Primer3 plus prefix design program as per **Table 1**.

To perform this examination, this contains the green cyber pigment that interacts with the enlarged genes in the Real-Time PCR device as **Table 2**.

1- Prepare the qPCR reaction mix for the CYP11A1 target gene

2- Prepare the qPCR reaction mix GFDH genes

Table 4. The effect of the alcoholic extract of Moringa leaf on the gene expression of CYP11A1 Gene for male rats treated with some food additives

Gene Expression of CYP11A1 Gene	Stander groups
5.293±3.164 b	C1
6.457±1.867 a	C2
0.684±0.214 c	T1
0.760±0.328 c	T2
1.698 ±0.194c	T3
4.201±0.730 b	T4
4.119±0.446 b	T5
4.613±0.377 b	T6
1.114	L.S.D

Then these components were added to the special qPCR tubes, then all tubes were put into a vortex centrifuge (Exispin) at a 3000rpm speed for three minutes. And then the plate was transferred to a Miniopticon Real-Time PCR device and qPCR Thermocycler conditions were applied to all genes according to the way the tool works, as in **Table 3**.

We analyze data from a polymerase chain reaction in quantitative real-time using a method Livak and schmittgen, (2001) Which relies on the extraction of Relative Quantitive and Absolute Quantitive through the process of correcting the target gene with control samples so that the results are biologically meaning Each of the target samples are corrected with the control sample to produce a specific level of relative expression as in the following equations:

1- ΔCT (test) = CT (target, test) – CT (ref, test)

2- ΔCT (control) = CT (target,control) – CT (ref, control)

3- $\Delta\Delta$CT= ΔCT (test) – ΔCT (control)

4- Gene expression Ratio =$2^{-CT\ \Delta\Delta C}$

RESULTS AND DISCUSSION

Estimation of gene expression of CYP11A1 Gene

The results of the current study showed in **Table 4** and **Fig. 1** a significant decrease ($P < 0.05$) in the level of gene expression of CYP11A1 gene in animal groups (T_1, T_2,T_3) treatment with food additives (Carmoisine, Monosodium Glutamate, Sodium Benzoate) compared with the positive and negative control groups. No significant differences emerged between the three groups mentioned, and the reason for this may be due to the effect of free radicals formed as a result of treatment with food additives and a decrease in antioxidants as the accumulation of free radicals in cells leads to oxidation of fats, proteins, and DNA, which leads to cellular damage and genetic instability (Owusu-Ansah and Banerjee, 2009). Also, the imbalance between oxidants and antioxidants causes oxidative stress leading to reduced gene expression and in turn reduces the production of Testosterone Hormone (Shen et al. 2014).Also, oxidative stress and increased free radicals affect mitochondrial DNA damage and sperm nuclei (Agarwal et al. 2003).The results of our study

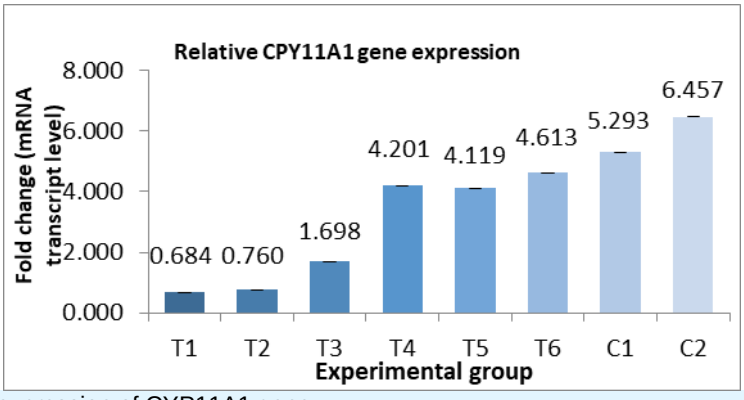

Fig. 1. The level of gene expression of CYP11A1 gene

agree with (Raposa et al. 2016), as they mentioned that some food additives (Tartrazine, Azorubine, Sodium Benzoate, and Potassium Sorbate) can contribute to activating the inflammatory pathways that lead to cancer by the effect of these food additives on some genetic expressions of liver NFKB, GADD45α and MAPK8 Gene. Also, some food dyes especially the azo dyes contain fixed aromatic rings that do not degrade and have toxicity, and their metabolites are of genetic toxicity and cause mutations and sometimes they are carcinogenic (De Campose and Maria, 2013). Exposure to some food additives such as Monosodium Glutamate at a concentration (250, 500,1000,2000 mg/ml) (Ataseven et al. 2016), and Sodium benzoate at a concentration (0.5,1.0,1.5 mg/ml) (Patel and Ramani, 2017), have an effect on damage DNA in human peripheral lymphocytes. Treating rats with Monosodium Glutamate at a concentration of 8 g / kg of body weight for 90 days would damage the testicular DNA (Ismail, 2012). Also, chemicals may cause endocrine disruption and altered gene expression either through direct interaction with receptors or changing enzymes involved in the synthesis of steroid hormones (Hilscherova et al. 2004).

As for the groups (T_4, T_5, T_6), there were no significant differences between the groups mentioned, but showed a significant increase ($P < 0.05$) compared to the groups (T_1, T_2, T_3), and no significant differences were observed compared to the negative control group (C_1). However, significant differences were observed compared to the positive control group (C_2). Also, the positive control group (C_2) of the doses with Moringa leaf extracts showed significant differences compared to the negative control group (C_1). This may be attributed to the fact that Moringa contains many phytochemicals, essential antioxidant nutrients, minerals, vitamins (A, E, C, B), proteins and amino acids that have effects in improving fertility (Vongsak et al. 2014; Liang et al. 2019). Also, Moringa contains flavonoids, which have a protective role for tissues and reproductive organs by increasing antioxidants, preserving the integrity of cell membranes, stimulating mitochondrial enzymes and can protect biopolymers from the influence of oxygenic free radicals that cause DNA damage (Izawa et al. 2007; Turgut et al. 2008).

It is the most important flavonoids in Moringa plant Quercetin, Luteolin and Rutin (Khudaer et al. 2016). Quercetin improves Leydig cellsfunction and facilitates the transfer of cholesterol from the outside to the inside of the mitochondrial membrane to form steroids This process is regulated by the CYP11A1 gene, converting Cholesterol to Pregnenolone, which is the first step in the formation of steroids (Thompson et al. 2004). In addition to that, Quercetin is effective in maintaining the normal level of steroids in the Leydig cells of rats subject to oxidative stress by Atrazine (Abarikwu et al. 2012).

Moringa also contains active compounds such as phenolic compounds such as (Chlorogenic acid and Coumaric acid) which have a role in the chemical prevention of colon cancer and rectal cancer (Cuellar-Nunezl et al. 2018).While Barakat et al. (2015) mentioned that Moringa leaves have improved the rate of maturation of sheep oocytes because they contain a high level of calcium and can be activated for mRNA expression and synthesis essential proteins for the maturational process. The use of Moringa seed powder as a food additive in the Japanese quail food (Coturnix japonica) with a concentration of (0.2%, 0.3%) has significantly improved the level of gene expressions of some ovarian genes (FSHR, ESR2, STAR and PRLR) (Abou-Elkhair et al. 2020). Also, treatment with selenium nanoparticles conjugated with Moringa leaf extract (MOLE-SeNPs conjugate) improved the level of gene expression (HSD17B3 and CYP11A1) which decreased as a result of treatment with Melamine(Mansour et al. 2020).

The numbers represent the mean ± standard error. Different letters show significant differences at the level of significance ($P<0.05$) between groups.

CONCLUSION

We conclude from the results of our current study that using an alcoholic extract of Moringa leaves at a concentration of 200 mg / kg of body weight for two

months has a role in improving the gene expression of CYP11A1 gene of rats treated with some food additives (Carmoisine, Monosodium Glutamate, Sodium Benzoate), because Moringa leaves contain many phytochemical compounds that act as antioxidants such as flavonoids, vitamins, Minerals, amino acids, phytosterols and others.

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