

Comparative study of antioxidant effects of different alcoholic and oil extracts of dry dates and leaves (*Phoenix dactylifera* L) palm against CCL4 induced oxidative stress in rats

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Abstract

A study was performed to investigate the protective effect of different extracts of alcoholic and oil of leave and dry date of (*Phoenix dactylifera*) against oxidative stress induced by CCL4 at a dose of 100 mg/kg given orally daily to five treated groups (CCL4 control (+ve), alcoholic extract of leave (150 mg/kg), alcoholic extract of dry date (100 mg/kg), oil extract of leave and date (250 mg/kg), also two control dosed with vehicles (distal water and corn oil) were used. At the end of two months experiment blood were collected and oxidant and antioxidant parameter were studied in serum of all animal groups including Malon dialdehyde (MDA) and reduce glutathione (GSH). Furthermore, the possibility of genomic polymorphism in glutathione-S- transference enzyme (GSTs) isomers (GSTT1 and GSTM1) were studied in all animal groups blood by using conventional PCR technique to understand their involvement in oxidative toxicity induced by CCL4 alone and with different palm date and leave extracts. The biochemical results recorded a significant increase in serum MDA with significant decrease in reduce glutathione (GSH) only in CCL4 treated group compared to other extract treated one or control groups indicating lipid peroxidation effect induced by CCL4 and improvement effect of such effect by all used date palm extracts. Also polymorphism of GSTs isomers were recorded only in CCL4 group genomic result manifested by absence of GSTM1 isomer that indicate reduction in GSTs enzyme function and its involvement as one of the main antioxidant enzyme while the appearance of both GSTT1 and GSTM1 in genomic results of different extract treated groups were indicative of antagonistic ability of different date palm extract to oppose oxidative effect that cause improvement in oxidant / antioxidant balance nearly as that of control groups.

Keywords: *Phoenix dactylifera*, leave, dry date, oxidative stress, antioxidant, polymorphism

Introduction

Phoenix dactylifera

Date palm tree, *Phoenix dactylifera* L., is an important plantation crop for many countries extending from North Africa to the Middle East including many states of the Arabian Gulf countries especially in Iraq. (Trabzuni, *et al.*, 2014) and multipurpose tree providing, fiber, carbohydrates, minerals and vitamins besides having certain medicinal properties. Dates are loaded of various nutrients with medicinal importance for ailment of certain diseases. Because of its high nutritional value and its long life the date palm has been mentioned as the 'tree of life'. Whole part of dates are useful, also the byproducts arising from date processing can be used for different purposes. (Parvin *et al.* 2015). The leaves of *Phoenix dactylifera* show antidiabetic activity and antilipademic activity. (Gangwar *et al.*, 2014). Oxidative stress is an imbalance between oxidant production and

antioxidant enzymatic and nonenzymatic antioxidant reduce the reactive oxygen species (ROS) induced by oxidation. Superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), oxidized glutathione (GSSG) and glutathione S-transferase (GST) are the enzymatic antioxidant of concern. *P. dactylifera* has antioxidant potential via its phenolics, flavonoids and small molecules such as vitamin C, vitamin E and GSH. The most widely accepted mechanism of CCL4 induced hepatotoxicity is the formation of free radicals which is a rate limiting process in tissue peroxidative damage. This free radical and related reactive species may cause oxidative stress, which produces major interconnected changes of cellular metabolism, increases the serum marker enzymes, DNA fragmentation, and destruction of the cells by lipid peroxidation. (Al-kreathy *et al.* 2014).

These are attributed to the rich contents of antioxidant in date fruit such as the coumaric acid and ferulic acid. Moreover, it contains flavonoids, sterols, procyanidins, carotenoids, anthocyanins, sugar (glucose,

sucrose and fructose), dietary fibers, less protein and fats, vitamins such as riboflavin, biotin, thiamine, ascorbic and folic acid, and minerals for example calcium, iron, copper, cobalt magnesium, fluorine, manganese, phosphorus, potassium, sodium, boron, sulfur, zinc and selenium within the date palm itself. (Bukhat *et al.*, 2012) .

Materials and Methods

Powder of phoenix dactylifera leave and dry date paste were prepared and yield were calculated.

Alcoholic Extraction

Done according to Harbone and Mabray, (1975) method by using methanol 70% and magnetic stirrer for 24 hours then concentrated under reduced pressure at 40°C, 90 rpm using a rotary evaporator.

Leave oil extraction

Extraction of leave oil was done by using hexane and soxhlet apparatus heated at 45°C for period 5- 6 hours under reflex (10– 12 cycle / h) after that the extraction solvent was evaporated using a Rotary vacuum evaporator at a temperature 40 °C with 90 rpm according to Charef, *et al*, (2008) method.

Dry Fruit oil extraction

According to soxhlet extraction method by Luque, *et al*, (2010) that was done by hexane to which added methanol as modified method achieved by the researcher, this increase the yield of oil extraction by 2 fold. Extraction was done under electric thermal at temperature 45 C for 6 hrs during 10-12 cycles. The extract is concentrated by Rotary vacuum evaporator for removing of solvent at temperature 40 C with 90 rpm.

Experimental Design

Forty nine adult males of Sprague dawley albino rats weighing (200 - 225 g) aged between 3-5 months were used in the present study. They have free access to standard laboratory feed and water, with a 8-16 hrs dark / light cycles. The animals were left in optimum conditions for two week for acclimatization in animal house of al kufa veterinary medicine college. The animal were randomly divided to seven groups treated daily orally for two months in which five groups treated with different phoenix dactylifera extracts plus CCL4 induced oxidative stress compared with two control groups according to the following design:

Group I: Control: (-ve) distal water orally administered

Group II: control: (-ve) corn oil orally administered at 0.1ml/100g B.W.

Group III: control (+ve) CCL4 100 mg/kg B.W.

Group IV(fruit alcoholic extract group) : CCL4 (100 mg/kg) +fruit alcoholic extract (100 mg/kg B.W).

Group V(leave alcoholic extract group):CCL4 (100 mg/kg) + leave alcoholic extract (150 mg/kg B.W).

Group VI (fruit oil extract group) : CCL4(100 mg/kg) +fruit oil (250mg/kg BW).

Group VII (leave oil extract group) : CCL4(100 mg/kg) + leave oil (250 mg/kg B.W.)

At the end of two month treatment, cardiac blood from all animal groups were collected to determine the level of reduced glutathione and malone dialdehyde as parameters of non enzymatic oxidative stress as well polymorphism for GSTs enzyme isomers GSTT1 and GSTM1 as enzymatic parameter indices.

Parameters (Antioxidant assays)

1. Determination of serum Malone dialdehyd (MDA)

Assay Principle: The Malondialdehyde ELISA kit employs the quantitative competitive immunoassay mmunoassay technique, utilizing a microtiter plate that is has been pre:coated with a monoclonal anti-MDA antibody. The assay samples and standards are incubated together with MDA-HRP conjugate for one hour..A competition for limited antibody binding sites on the plate occurs between MDA-HRP conjugate and MDA in the samples and standards. After the incubation period, the wells are decanted and washed five times. The wells are then incubated with a substrate for HRE enzyme. The product of the enzyme-substrate reaction forms a blue colored complex. Finally, a stop solution is added to terminate the reaction, which will then turn the solution yellow. The intensity of color is measured spectrophotometrically at 450 nm in a microplate reader. The intensity of the color is inversely proportional to the MDA concentration in the sample or standard. This was done according to method mentioned by United States Biological (Malondialdehyde Bioassay ELISA kit Rat)

2-Determination of serum Reduce glutathione(GSH)

The same principle as (MDA) analysis except the use of specific glutathione conjugate and its substrate enzyme. The reduce glutathoine concentration in the serum sample or standard was estimated according to method mentioned by United States Biological (Reduce glutathione Bioassay ELISA kit Rat)

Genotoxicity (molecular genetics study)

Genomic DNA was extracted from cardiac blood after collection of blood at the end of experiment. Genomic DNA concentration of all groups were estimated by using conventional multiplex PCR and agarose gel electrophoresis according to the kit procedure of KAPA Express. A sample of cardiac blood was collected in a test tube containing anticoagulant (EDTA) . (KAPA BOISYSTEMS).

Estimate of GSTs polymorphism

The multiplex PCR technique was used to detect the polymorphisms of GSTs isomers (GSTT1 and GSTM1) DNA genes of rat groups using procedure according to KAPA2G Fast Multiplex PCR kit .

Primers

In this study, two set of primers sequence was made map according NCBI web has been used, the first primer was

used in GSTT1 which its sequence was Forward 5'- ATG ATC CAG TGA CGT CAG AAG C - 3' and Reverse 5'- GCC CAA ACT CAA AGG GAA AAG - 3', these primers could amplify a nucleotide sequence of 180 base pairs region of GSTM1 gene . While the primer that used in GSTM1 the following sequencing Forward 5'- CAG GGA TGG TCT TCA AGA AC- 3' and Reverse 5'-TGT CTT CTC CCA GGG TCT CTG T-3', these primers could amplify a nucleotide sequence of 330 base pairs region of GSTM1 gene as listed in table (1)

Table -1 Sequence of primer for GSTT1 and GSTM1 gene

primer	Primer Sequence 5' → 3'	Product size
GSTT ₁ forward	5'- ATG ATC CAG TGA CGT CAG AAG C - 3'	
GSTT ₁ reverse	5'- GCC CAA ACT CAA AGG GAA AAG - 3'	180 bp
GSTM1 forward	5'- CAG GGA TGG TCT TCA AGA AC- 3'	
GSTM1 reverse	5'-TGT CTT CTC CCA GGG TCT CTG T-3'	330 bp

Table- 2 Level concentration of Malondialdehyde (MDA) (μmol/L) and Reduce glutathione (GSH) (μmol/L) in serum rat groups treated with palm date and leave oil and alcoholic extract compared to control once

Group N=7	Mean ± SE MDA	Mean ± SE GSH
date alcoholic extract 100 mg /kg + CCL4 100 mg/kg	1.4340 ± 0.117 B	1.0373 ± 0.48 A
leaf alcoholic extract 150 mg /kg + CCL4 100 mg/kg	1.4645 ± 0.153 B	1.0775 ± 0.49 A
date oil extract 250 mg /kg + CCL4 100 mg/kg	1.5018 ± 0.101 B	1.0958 ± 0.86 A
leaf oil extract 250 mg / kg + CCL4 100 mg/kg	1.5418 ± 0.085 B	1.2130 ± 0.51 A
CCL4 100 mg / kg	1.823 ± 0.118 A	0.175 ± 0.90 B
Oil at dose 0.1 ml / 100 g BW	0.7960 ± 0.116 C	1.0445 ± 0.62 A
D.W. at dose 0.1 ml / 100 g BW	0.2945 ± 0.215 D	1.2820 ± 0.2 A

LSD = 0.279, LSD = 0.711

Different capital letters denote significant result between groups (p ≤ 0.05)

To determine homozygote deletion in GSTT1 and GSTM1 by Multiplex PCR method, six pairs of GSTM1, three pair of GSTT1. For the GSTM1, The sequence of designed forward primers was purchase from Integrated DNA Technologies (IDT) USA.

PCR products were analyzed in agarose gel at 2%, stained with ethidium bromide, and the null genotype (both alleles with a deletion) for the GSTT1 and GSTM1 genes was identified by the absence of amplification fragments of 180 base pairs (bp) and 333 bp respectively which was evidence of a successful amplification reaction.

Statistical analysis

Data were analyzed statistically using the Microsoft program (SPSS) statistical analysis of data was performed on the bases of One Way of Variance (ANOVA) using a significant level of (P<0.05). Specific group differences were determined using least significant differences (LSD) as described by (snedecor, and Cochran .,1973).

Results

Antioxidant effect

A -Determination of serum Malone dialdehyd serum

Result listed in table (2) showed significant increase in level of serum Malone dialdehyde in group treated CCL4 compared with those in the control groups (distal water and oil) while the groups treated by CCL4 + methanol and oil extracts of dry date and leave were showed less increase in level of serum Malondialdehyde but significant difference (P<0.05) from group treated with CCL4 or control groups while serum level of reduce glutathione was only significantly decline (P< 0.05) in CCL4 group compared with the normal levels in serum GSH in different treated extracts groups and control one.

Genotoxicity (molecular genetics study)

1- Polymerase chain reaction (PCR)

PCR result recoded in the following lanes 1, DNA ladder while lanes 2 & 3, there were normal genotype of GSTT1 and GSTM1 respectively for groups of methanol extract of dry date administered orally at dose 100 mg/kg and leaf alcoholic extract at dose 150 mg/kg with CCL4. Also Lanes 4&5 showed normal of genotype of GSTT1 and GSTM1 in the groups of oil extracts of date and leaf at dose 250 mg/kg, while group dosed orally with CCL4 showed polymorphism of GSTM1 isomer gene since it absent in lane while the GSTT1 isomer gene was present. The Lane 7 &8 showed normal GSTT1 and GSTM1 isomer genes respectively in control groups with distal water and oil. Figure (1).

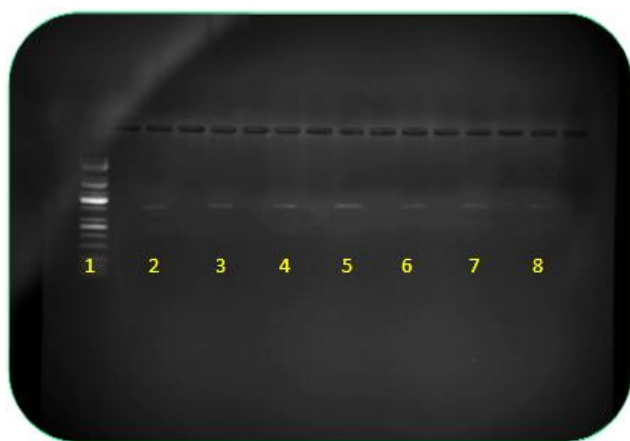


Figure -1 Whole genomic DNA of all groups.(1) DNA ladder;(2) alcoholic dry date; (3) alcoholic leaf; (4) oil dry date; (5)oil leaf;(6) CCL4 (7) control oil; (8) contro, distal water



Figure -2 Electrophoresis product appearance lane: (1) DNA ladder lane, (2) alcoholic dry date; (3) alcoholic leaf; (4) oil dry date; (5)oil leaf groups with CCL4, normal GSTT1 and GSTM1, lane (6) polymorphism GSTM1 in group treated with CCL4, lane (7,8) normal GSTT1 and GSTM1 of control group ones

Discussion

Oxidative stress usually occur in biological systems when there is an overproduction of ROS/RNS on one side and a

deficiency of enzymatic and non-enzymatic antioxidants on the other. In other words, oxidative stress results from the metabolic reactions that use oxygen and represents a disturbance in the equilibrium status of pro-oxidant/antioxidant reactions in living organisms. (Kohen *et al* 2002).

Oxidative stress is highly correlated with a wide variety of inflammatory and metabolic disease states, including obesity. It is highly correlated with cumulative damage in the body done by free radicals inadequately neutralized by antioxidants. It has been shown that free radicals may adversely affect cell survival because of membrane damage through the oxidative damage of lipid, protein and irreversible DNA modification. Lipid peroxidation such as thiobarbituric acid reactive substances and hydroperoxides levels as well as markers of protein oxidation such as carbonyl proteins are markers of oxidative damage of ROS. (Noeman, *et al*. 2011).

Antioxidants are substances capable of counteracting the damaging effects of oxidation in body tissues. Antioxidants are divided into two classes based on mechanism of action: chain-breaking antioxidants, such as Vitamin E and beta-carotene, "break the chain" of free radical formation by donating an electron to stabilize an existing free radical and preventive antioxidants are enzymes that scavenge initiating radicals before they start an oxidation chain. (Kohen and Nyska., 2002)

The phase II glutathione S-transferases (GSTs) GSTT1, GSTM1 and GSP1 catalyse glutathione-mediated reduction of exogenous and endogenous electrophiles. These GSTs have broad and overlapping substrate specificities and it has been hypothesized that allelic variants associated with less effective detoxification of potential carcinogens may confer an increased susceptibility to cancer . (Spurdle , 2001).

Two genes in particular - GSTT1 and GSTM1 - that code phase II enzymes belonging to the glutathione S-transferases (GSTs) family seem relevant for susceptibility to head and neck squamous cell carcinoma; they detoxify carcinogenic tobacco smoke reactive metabolites. The GSTM1 gene is polymorphic in humans, including a null-activity allele (GSTM1) due to a major genetic deletion, and two functional alleles (GSTM1A and GSTM1B). The GSTT1 gene is also polymorphic in humans, and may have a deletion null genotype. Individuals that have one of these null genotypes in homozygosis may be grouped into the negative conjugating phenotype, where there is complete loss of enzyme activity, while those that have at least one functional allele are grouped in the positive conjugating phenotype. Thus, individual gene variability in the metabolic activation and detoxification process appears to be crucial to head and neck cancer susceptibility. (Biselli *et al.*, 2006).

The GSTT1 protein catalyzes the conjugation of reduced glutathione to electrophilic moieties of xenobiotics, drugs and endogenous compounds such as peroxidized lipids. The conjugated products often after

further metabolism are more soluble, allowing them to be more readily eliminated from the body. The related glutathione s-transferase mu gene, GSTM1 also has a null variant. Since GSTM1 is also involved in drug and xenobiotic detoxification various studies have examined the effects of deletion of GSTM1 null alone and in combination with GSTT1 null for both disease risk and drug response. (Thorn *et al.*, 2012) .

The present PCR genomic results was highly indicative of the sensitivity of GST enzyme isomers to the subchronic oxidative stress of CCL4 treatment specially the GSTM1 that was clearly absent in CCL4 group which clearly indicative of homozygous null allele (polymorphism) in this GST isomer while GSTT1 was more resistant to CCL4 oxidative impact. The absence of GSTM1 isomer cause decline in GST antioxidative action as reported by Thorn, *et al.*, (2012) who suggested that GSTT1 null variant may be protective against certain cancers including bladder cancer, because GSTT1 dependent conjugation of xenobiotics such as the industrial chemical trichloroethylene produces compounds with increased toxicity. The related glutathione S transferase mu gene, GSTM1 also has a null variant. Since GSTM1 is also involved in drug and xenobiotic detoxification various studies have examined the effects of deletion of GSTM1 null alone and in combination with GSTT1 null for both disease risk and drug response . and reported by Yang, *et al.* (2015) have the association of GSTM1 deletion polymorphism with increased lung cancer risk in Chinese population.

This explain the present biochemical results that showed significant increase in lipid peroxidative parameter (MDA) in serum of CCL4 group, also with significant decrease in its serum glutathione level as another sign of increase of the oxidative damage possibly due to the decrease in the GST function and reduction in its involvement in the action of GSH possibly because of the loss of GSTM1 isomer.

In the present study, the elevations in the levels of end products of lipid peroxidation in the liver of rat treated group with CCL4 was observed. The increase in malondialdehyde (MDA) levels in serum and the decrease in serum level of GSH in CCL4 treated group only suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals. This was attributed to the palm leaf and fruit active component that reported by many authors to opposes antioxidant effect like reported by Fahmy, and Hamdi.,(2011) and Hismiogullari,*et al.*, (2014).

Conclusion

CCL4 induce polymorphism in GSTs enzyme isomers since it cause absence of GSTM1 isomer while treatment with palm date and leaf extracts reverse such effect indicating sensitivity of such isomer to the oxidant and antioxidant effect of both CCL4 and extracts, this was

supported by biochemical results that indicated improvement in oxidant/ antioxidant parameters balance recorded only in different extract treated groups + CCL4 same as that of control one.

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