

## Anti-Oxiflammatory Profile of Date Extracts (*Phoenix sylvestris*)

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**Abstract**— Fruit of date palm (*Phoenix sylvestris*L.) is edible and used as an anti-geriatric, anti-oxidant ethnomedicine. In this study, three different types of date extracts, methanolic, acidic ethanolic and basic ethanolic were evaluated for their putative *in vitro* scavenging effects on reactive oxygen species (ROS), where scavenging of hydroxyl radicals (basic ethanolic>acidic ethanolic>methanolic), superoxide radicals (acidic ethanolic>basic ethanolic>methanolic), DPPH radicals (acidic ethanolic>methanolic>basic ethanolic), nitric oxide (NO) (methanolic>acidic ethanolic>basic ethanolic) and inhibition of lipid peroxidation (basic ethanolic>acidic ethanolic> methanolic) were found to occur in a dose dependent manner. Their flavonoid and phenolic contents proved to be the source of this potent free radical scavenging activity and indicated a direct correlation with their total anti-oxidant capacity. The date extracts have a positive therapeutic effect on bacterial lipopolysaccharide (LPS) - treated human embryonic kidney cell line (HEK) and on murine RAW macrophages. They significantly inhibit intracellular oxidative stress. This reinstatement of cellular homeostasis presumably occurs via mitochondrial pathways.

**Keywords**— Inflammation; anti-oxidant; phenolic compounds; scavenger activities; reactive oxygen nitrogen intermediates.

### INTRODUCTION

One of the most primitive and yet effective mechanisms the mammalian physiology adopts in an exigent situation, which although predominantly non-specific is remarkably fast and conclusive for the next phases of immune response or structural tissue remodeling, is inflammation. Inflammatory response occurs in a phase-wise manner (Crunkhorn and Meacock, 1971). Inflammation is the principal and sometimes critical initiator of most disorders. A cross-talk between the structural and immune cells causes destruction on the one hand and healing or reconstruction on the other. In diseases such as asthma, rheumatoid arthritis, psoriasis, multiple sclerosis, obesity and inflammatory bowel disease (Winter et al., 1962), the first phase of

disease onset, followed by establishment, development, maintenance and exacerbations, leads to a completion of various steps that are characteristic of the particular inflammatory disease. Although specific characteristics of inflammatory response in each disease and their site of occurrence may vary, a universal feature governing this step-wise phenomenon is the complex interplay amongst the various cell subsets of the inflammatory cascade and tissue resident cells, and the network of signaling governing the two (Vinegar et al., 1969; Winter et al., 1962), that affect various target tissues (Cortran, 2010).

Unmet needs in medicine and unknown phenomena prevailing in the mechanism of disease onset remain (Mohan, 2010). In traditional medicine, that has provided solution to prevailing health issues at a global level, medicinal plants continue to provide valuable therapeutic agents. To avoid and mitigate various side effects and complications of modern medicine, and to address unmet needs of diseases, especially in the context of emerging complex etio-pathophysiological pathways, traditional medicine is gaining importance and is now being studied systematically using biotechnological tools, to find the scientific basis of their therapeutic actions.

Phytochemicals from fruits and other edible plant parts have been shown to possess significant antioxidant properties that may be associated with lower incidence and lower mortality rates of degenerative diseases in human. Different biological properties, antioxidant capacities and radical-scavenging activities of various herbal extracts have been widely demonstrated, using *in vitro* techniques and *in vivo* models by different groups of researchers (Amann et al., 1995; Chatpalliwar VA, 2002). The anti-proliferative and anti-inflammatory activities of these herbal extracts have been documented in human oral, breast, colon, cervical, and prostate cancer cell lines as well as in preclinical animal models by attenuating certain inflammatory intermediates, including nitric oxide, NF-kB, and TNF $\alpha$  (Miles and Miles, 1952; Whittle, 1964).

This study was designed to identify anti-inflammatory potential in extracts from date which is a well-known ethno-medicine with high nutritive value. Among its contents, large amounts of several phenolic and non-phenolic compounds and other uncharacterized moieties may contribute to its use as a food supplement, a functional food or a nutraceutical substance with prophylactic and therapeutic functions in oxidative inflammatory diseases. Anti-oxidative and anti-inflammatory activities of three types of date palm extracts, namely methanolic, basic ethanolic and acidic ethanolic, have been assessed, compared and validated, using both murine and human cell lines, in which inflammation has been induced by *E. coli* LPS, a potent pro-inflammatory agent. In addition, intracellular cell organelle specific targeting of this extract has also been assessed in order to seek information regarding its mode of action in modulating the inflammatory cascade in a biological system, which is the

most critical part for any drug discovery programme addressing inflammatory disorders.

## MATERIAL AND METHODS

### Extraction of date

Mesocarp tissue of date fruit was macerated and treated with 50% water-methanol solution at 4°C. This was then divided into two parts: part A was evaporated to dryness, the remaining part was rinsed with water, dried at 50°C, alkaline hydrolyzed with sodium hydroxide (0.1M) and filtered. The filtrate was acidified at pH 2.0, extracted with ethyl acetate and rinsed with 1% sodium bicarbonate and water. The organic layer was evaporated to dryness and then dissolved in water, to give part B. The residue after filtering out part B was alkaline hydrolyzed with 2M sodium hydroxide and filtered. The filtrate was further acidified at pH 2, extracted with ethyl acetate, rinsed with 1% sodium bicarbonate and water, and the organic layer evaporated to dryness (part C) (Fig. 1).

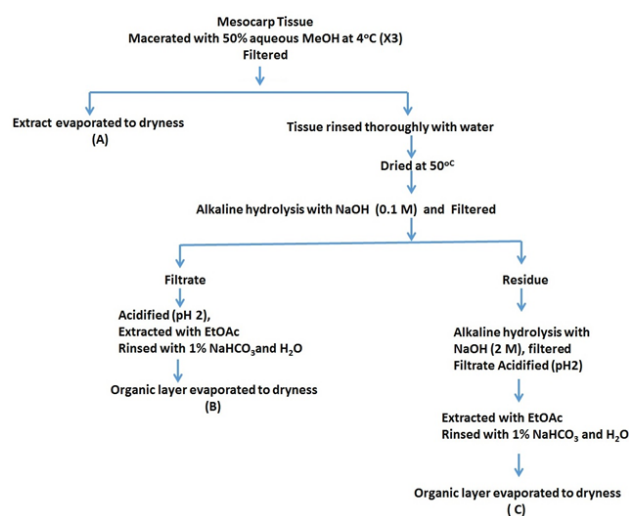


Figure 1. Schematic flow chart for extraction of bioactive compounds from mesocarp tissue of *Phoenix sylvestris* L using various solvent systems. Methanolic extract is represented as A, Acidic ethanolic extract as B & basic ethanolic extract as C.

### Reagents

Chemicals, such as ethylenediamine tetra acetic acid (EDTA), trichloroacetic acid (TCA), butanol, ammonium molybdate, and sodium dodecyl sulphate, benzoic acid, sodium phosphate, DMSO were purchased from E. Merck (India) Limited. 1,1 Diphenyl-2-picrylhydrazyl and malondialdehyde, potassium fer-

ricyanite, thiobarbituric acid (TBA) were procured from Sigma, USA. N-butanol, ferrous sulphate, ferric chloride, Folin's reagent, riboflavin, naphthylethylenediaminedihydrochloride, sulphanimide in phosphoric acid, sodium bicarbonate, sodium hydroxide, and potassium hydroxide were purchased from Sisco Research Laboratories PVT. Ltd India. Nitroblue tetrazolium (NBT), MTT reagent [(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], DMEM media were purchased from Himedia, India. Fetal bovine serum (FBS) was purchased from Gibco. DCFH-DA and DHR 123 were purchased from Invitrogen. All other reagents were of analytical grade.

#### DPPH radical-scavenging activity

The antioxidant activity of the extracts was measured on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical (Braca et al., 2001). Aqueous extract was added to a 0.004% Methanol solution of DPPH on a 96 well ELISA plate. Absorbance at 517 nm was determined after 30 min, and the percent inhibition activity was calculated.

#### Assay of superoxide radical $O_2^{\cdot-}$ scavenging activity

The method used by Martinez et al. (Martinez et al., 2001) for determination of the superoxide dismutase was studied in the riboflavin-light-nitrobluetetrazolium (NBT) system (Beauchamp and Fridovich, 1971). Each 0.1 ml of reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2  $\mu$ M riboflavin, 100  $\mu$ M EDTA, NBT (75  $\mu$ M) and various doses of sample solution. The production of blue formazan was followed by monitoring the increase in absorbance at 560 nm after 15 min of illumination from a fluorescent lamp.

#### Assay of hydroxyl radical (-OH)-scavenging activity

The assay was based on the benzoic acid hydroxylation method (Chung et al., 1997). Hydroxyl radicals were generated by direct addition of iron(II) salts to a reaction mixture containing phosphate buffer. In a 24 well plate, 0.15 ml of sodium benzoate (10 mM) and 0.15 ml of  $FeSO_4 \cdot 7H_2O$  (10 mM) and EDTA (10 mM) were added. Then the sample solution and a phosphate buffer (pH 7.4, 0.1 M) were added to give a total volume of 1.6 ml. Finally, 0.15 ml of an  $H_2O_2$  solution (10 mM) was added. The reaction mixture was then incubated at 37°C for 2 h. After that, the fluorescence was measured at 407nm emission ( $E_m$ ) and excitation ( $E_x$ ) at 305 nm. Measurement of spectrofluorometric

changes has been used to detect the damage by the hydroxyl radical.

#### Lipid peroxidation assay

A modified thiobarbituric acid reactive species (TBARS) assay (Ohkawa et al., 1979) was used to measure the lipid peroxide formed, using egg yolk homogenates as lipid-rich media (Ruberto et al., 2000), where lipid peroxidation was induced by  $FeSO_4$ . Malondialdehyde (MDA), produced by the oxidation of polyunsaturated fatty acids, reacts with two molecules of thiobarbituric acid (TBA), yielding a pinkish red chromogen with an absorbance maximum at 532 nm, which was measured using a 96 well ELISA plate reader. Percentage inhibition of lipid peroxidation by different concentrations of the extract was calculated.

#### Nitric oxide (NO) scavenging activity

Nitric oxide was generated from spontaneous decomposition of the Sodium nitropruside (20mM) in phosphate buffer (pH 7.4) which interacts with oxygen molecule to produce nitrite ions, which can be measured by the Griess reactions. The nitric oxide scavenging activity of the date extracts, collected by three different methods was determined by Shirwaikar *et al* (2006) with a slight modification. Briefly a stock solution of each extract was prepared to contain 1 mg/ml. Different amounts of the stock solution were then transferred to different test tubes and volume was adjusted to 1ml by adding double distilled water. 0.2 ml of sodium nitropruside (20mM) in PBS (pH 7.4) and 1.8 ml of PBS solution was added and incubated at 37°C for 3 h. 1 ml of each solution was taken and diluted with 1 ml of Griess reagent [1% sulfanilamide, 5%  $H_3PO_4$  and 0.1% N-(1-naphthyl)ethylenediamine]. Similarly, a blank was prepared containing the equivalent amount of reagents (only the sodium nitropruside and PBS), but without the extract. The absorbances of these solutions were measured at 540 nm against the corresponding blank solution. Ascorbic acid (100  $\mu$ g/ml) was used as the positive control. The percentage inhibition of nitric oxide was calculated.

#### Determination of total antioxidant capacity

The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo(V) complex at acid pH (Prieto et al., 1999). Each well of a 96 well ELISA plate containing extract and reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molyb-

date) were incubated at 95°C for 90 min. After the mixture had cooled to room temperature, the absorbance of each solution was measured at 695 nm against a blank. The antioxidant capacity was expressed as ascorbic acid equivalent (AAE).

#### Determination of reducing power

The reducing power of date extracts was determined according to the method (Salah et al., 1995) where different concentrations of extracts were mixed with phosphate buffer and potassium ferricyanide. The mixture was incubated at 50°C for 20 min. 2.5 ml of trichloroacetic acid was added to the mixture. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl<sub>3</sub>, and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as positive control.

#### Determination of total flavonoid content

Total flavonoid content was determined using aluminium chloride (AlCl<sub>3</sub>) according to a known method, using Fisetin as a standard. The date extracts (0.1 ml) were added to 0.3 ml distilled water followed by 5% NaNO<sub>2</sub> (0.03 ml). After 5 min at 25°C, AlCl<sub>3</sub> (0.03 ml, 10%) was added. After further 5 min, the reaction mixture was treated with 0.2 ml of 1 mM NaOH. Finally, the reaction mixture was diluted to 1 ml with water and the absorbance was measured at 510 nm. The results were expressed as mg Fisetin/g date extract.

#### Determination of total phenolic content

The total phenolic content of the date extracts was determined using the Folin-Ciocalteu reagent. The reaction mixture contained: 200 µl of diluted extract, 800 µl of freshly prepared diluted FolinCiocalteu reagent and 2 ml of 7.5% sodium carbonate. The final mixture was diluted to 7 ml with deionized water. Mixtures were kept in dark at ambient conditions for 2 h to complete the reaction. The absorbance at 765 nm was measured. Gallic acid was used as standard and the results were expressed as mg gallic acid (GAE)/g of the date extract.

#### UV based spectrophotometric Analysis:

The samples of various extracts was analysed using UV-Vis Spectrophotometer. About 40 mg of the air-dried samples was dissolved in 1 ml methanol, diluted to 1 mg/ml solution in different volumetric flasks and then applied on the spectrophotometer and scanned through the UV and visible region. The herbal func-

tional groups were determined by analyzing the peaks.

#### Cell culture

RAW 267.4 murine macrophage cell line and HEK 293 cell line were obtained from NCCS, Pune, India. The cells were grown in DMEM medium containing 5% inactivated fetal bovine serum, penicillin (100 U/mL), and streptomycin (20 µg/mL) and kept at 37°C in a T-25 tissue culture flasks. Cell were grown to confluence in a humidified atmosphere containing 5% CO<sub>2</sub>.

#### Cell viability using MTT assay

To test the cytotoxicity, 5×10<sup>4</sup> cells/well were seeded in a 96-well plate and incubated for 24 h with different concentrations of the date extract. The cells were washed, and each well was filled with 100 µL of medium and 10 µL of a tetrazolium salt, MTT. The plate was incubated for various time periods, and the absorbance was measured at 540 nm. The percentage of viable cells was calculated using the absorbance of the control cells without extract as 100%. The assay was performed in triplicate twice.

#### Proteomic analyses of extracts

*HPLC analyses:* Crude methanolic A, basic ethanolic B & acidic ethanolic C were filtered by 0.22 µm syringe filter (Milipore, Germany), and then the extracts were analysed with RP-HPLC by using Waters 515 System with C-18 column as stationary phase. The mobile phase was Water (HPLC grade) and 90% acetonitrile. Sample volume was 100 µl. Flow rate was 0.5ml/min.

*TLC analyses:* Methanolic extract (A), Acidic Ethanolic extract (B) and Basic ethanolic extract (C) were applied on silica gel coated TLC plates (Milipore, Germany) by using capillary tubes and developed in a TLC chamber using mobile phase methanol:water:chloroform (2.5:0.2:7.3 v/v). The developed TLC plates were air dried and observed under ultra violet light UV at both 254 nm and 366 nm. TLC plates were sprayed with Libermann's solution, heated at 100–105°C and visualized under daylight. The movement of the analyte was expressed by its retention factor (R<sub>f</sub>).

#### Methods

Taking 0% inhibition in the mixture without plant extract, regression equations were prepared from the concentrations of the three date extracts which were

collected by three different methods and percentage inhibition of free radical formation/prevention in different systems of assay were calculated, viz. DPPH assay, superoxide radical-scavenging assay, hydroxyl radicals scavenging assay, lipid peroxidation assay and nitric oxide radicals scavenging assay. IC<sub>50</sub> values (concentration of sample required to scavenge 50% of available free radicals or to prevent lipid peroxidation by 50%) were calculated from these regression equations. IC<sub>50</sub> value is inversely related to the activity of the extracts.

#### Assay of hydroxyl radical (OH)-scavenging activity

Superoxide radical was measured by the NBT reduction assay. Each well of a 96-wells plate was seeded with RAW 264.7 macrophages suspension containing  $5 \times 10^4$  cells/ml. The treatment of cells proceeded as described previously. After incubation, 40  $\mu$ L of a NBT solution at 1 mg/mL was added to the medium and incubated at 37°C, for 1 h. Then, the incubation medium was removed and cells were lysed with DMSO:2 M NaOH (1:1). The absorbance of reduced NBT, formazan, was measured at 620 nm, in a microplate reader (Multiskan ASCENT Thermo®).

#### NO estimation

In culture, the NO released by the macrophages into the medium is converted to several nitrogen derivatives, from which only nitrite is stable, being easily measured by Griess reagent (1.0% sulphanilamide and 0.1% *N*-(1)-naphthylethylenediamine in 5% phosphoric acid). After incubation, 100  $\mu$ L of culture medium supernatant was mixed with the same volume of Griess reagent, during 10 min, at room temperature. The nitrite produced was determined by measuring the optical density at 540 nm, in a microplate reader (Shimadzu).

#### ROS measurement

Intracellular formation of ROS was assessed by using oxidation sensitive dye DCFH-DA as a substrate (Wijesinghe et al., 2011). RAW macrophage cells were seeded in 24-well black plate at a concentration of  $5 \times 10^4$  cells/ml. Cells were treated with 1  $\mu$ g/ml of *E.coli* LPS and then therapeutically various concentrations of date extracts were added on to it and incubated for 6 h. Negative control cells, i.e. only LPS treated as well as cells without any treatment were incubated for the same time period and then washed in PBS and after the addition of DCF-DA (5  $\mu$ g/mL) incubate for 30 min at 37°C in dark. Non-fluorescent DCFH-DA dye, that

freely penetrate into cells get hydrolyzed by intracellular esterase to 207-dichlorofluorescein (DCFH), and is trapped inside the cells. The formation of 207-dichlorofluorescein (DCF) due to oxidation of DCFH in the presence of ROS was read after 30 min at an excitation wavelength of 485 nm and emission wavelength of 525 nm using a spectrofluorometer. Percent scavenging power of hydroxyl, superoxide and peroxide radicals was computed taking that by Ascorbic acid as 100%.

#### Measurement of mitochondrial membrane potential (MMP)

Mitochondrial membrane potential was monitored by the fluorescent dye, Rhodamine123. It is a cell permeable cationic dye that preferentially enters into mitochondria based on highly negative mitochondrial membrane potential ( $\Psi_m$ ). Depolarization of MMP results in the loss of Rhodamine 123 from the mitochondria and a decrease in intracellular fluorescence intensity. After the addition of 1  $\mu$ g/ml *E.coli* LPS, various concentrations of date extracts were added to RAW macrophages cell line and incubated for 6 h in 37°C. After incubation, the cells were washed twice in cold PBS, then Rhodamine 123 (10  $\mu$ M) was added and incubated for 30 min at 37°C in dark. Fluorescence was measured by spectrofluorometer with an excitation wavelength of 485 nm and emission wavelength of 525 nm.

#### TLC of Khejur Extracts

Methanolic extract (A), Acidic Ethanolic extract (B) and Basic ethanolic extract (C) were applied on silica gel coated TLC plates (Milipore, Germany) by using capillary tubes and developed in a TLC chamber using mobile phase methanol:water:chloroform(2.5: 0.2:7.3 v/v). The developed TLC plates were air dried and observed under ultra violet light UV at both 254 nm and 366 nm. TLC plates were sprayed with Libermann's solution, heated at 100–105°C and visualized under daylight. The movement of the analyte was expressed by its retention factor (*R<sub>f</sub>*).

#### HPLC Analysis

Crude methanolic A, basic ethanolic B & acidic ethanolic C were filtered by 0.22  $\mu$ m syringe filter (Milipore, Germany), and then the extracts were analysed with RP-HPLC by using Waters 515 System with C-18 column as stationary phase. The mobile phase was Water (HPLC grade) and 90% acetonitrile. Sample volume was 100  $\mu$ L. Flow rate was 0.5 mL/min.

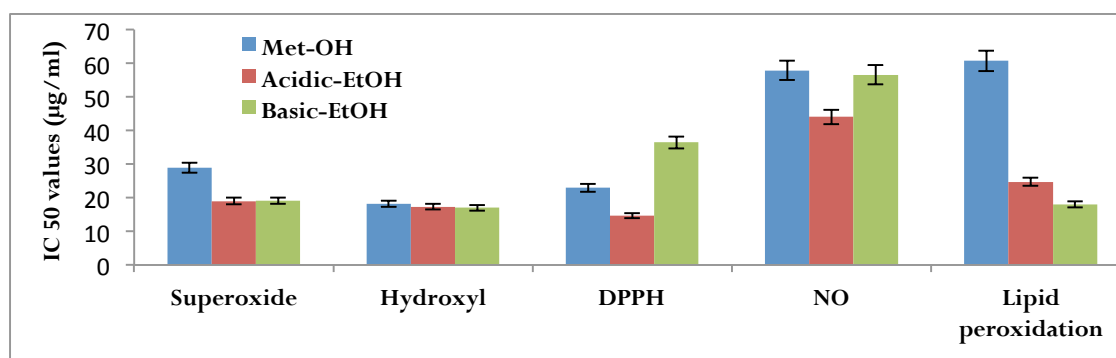
### Statistical analysis

Statistical differences among samples were tested by Student t- test. A P-value less than 0.05 or 0.01 (as applicable vis-à-vis the assay performed) was considered statistically significant.

## RESULTS

Taking 0% inhibition in the mixture without plant extract, regression equations were prepared from the concentrations of the three date extracts which were collected by three different methods and percentage inhibition of free radical formation/prevention in different systems of assay were calculated, viz. DPPH assay, superoxide radical-scavenging assay, hydroxyl radicals scavenging assay, lipid peroxidation assay

Various concentrations of date extracts, collected by three distinct methods, required to scavenge 50% free radical or to prevent lipid peroxidation by 50%. IC<sub>50</sub> value is inversely related to the activity of the extracts. Both acidic and ethanolic date extracts are more active in scavenging the superoxide ions in *in vitro* assay system than the methanolic date extracts, where as there is no significant variation in any of the three extracts in scavenging the hydroxyl radical. The DPPH radical scavenging and nitric oxide scavenging activities are greater in acidic ethanolic extracts in comparison to the methanolic extracts and basic ethanolic extracts respectively. Interestingly prevention of lipid peroxidation activity is highest in basic ethanolic extracts than the acidic ethanolic extracts and methanolic extracts. So, the ability to scavenge various free radicals or to prevent lipid peroxidation is different in all three extracts and it exerts the activity in a dose de-

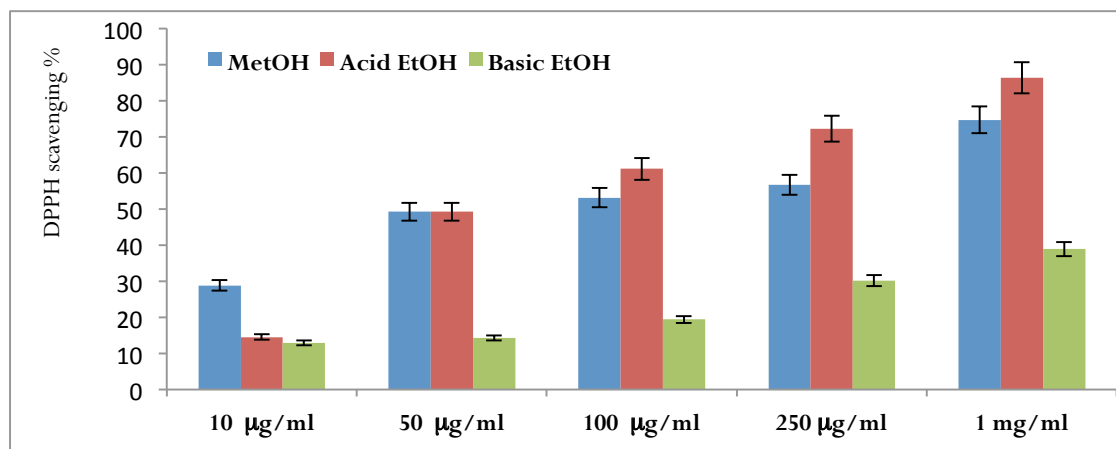


**Figure 2. Comparison of IC<sub>50</sub> values of three different extracts of date palm.** Various concentrations of date palm extracts, collected by three distinct methods, required to scavenge 50% free radical or to prevent lipid peroxidation by 50%. IC<sub>50</sub> value is inversely related to the activity of the extracts where both acidic and ethanolic date palm extracts are more active to scavenge the superoxide ions in a *in vitro* assay system than the methanolic date palm extracts, where as there is no significant variation in all these three extracts to scavenge the hydroxyl radical but for the DPPH radical scavenging activity and Nitric oxide scavenging activity is greater in acidic ethanolic extracts in comparison to the methanolic extracts and basic ethanolic extracts respectively. Interestingly prevention of lipid peroxidation activity is highest in basic ethanolic extracts than the acidic ethanolic extracts and methanolic extracts respectively. So the scavenging power of various free radicals or to prevent the lipid peroxidation activities is different in all these three extracts and it exerts the activity in a dose dependent manner which has been shown in latter.

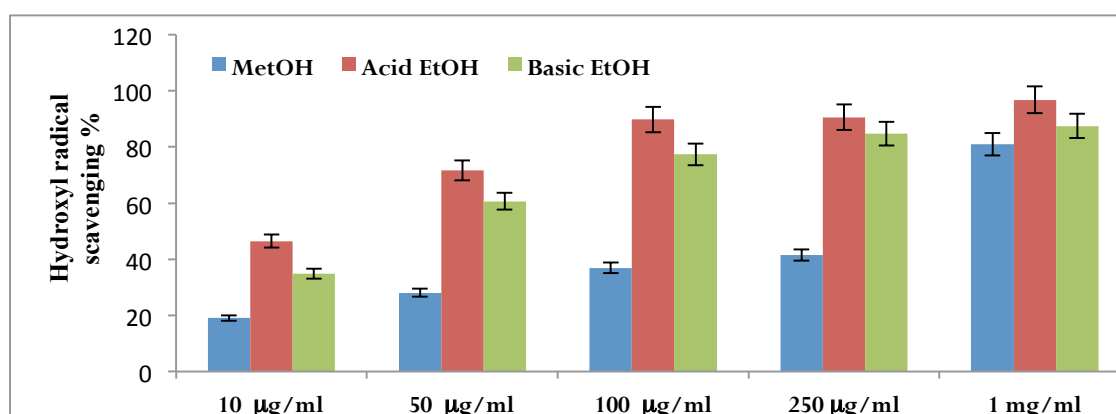
and nitric oxide radicals scavenging assay. IC<sub>50</sub> values (concentration of sample required to scavenge 50% of available free radicals or to prevent lipid peroxidation by 50%) were calculated from these regression equations. IC<sub>50</sub> value is inversely related to the activity of the extracts (Fig. 2).

pendent manner.

*DPPH radical-scavenging activity*



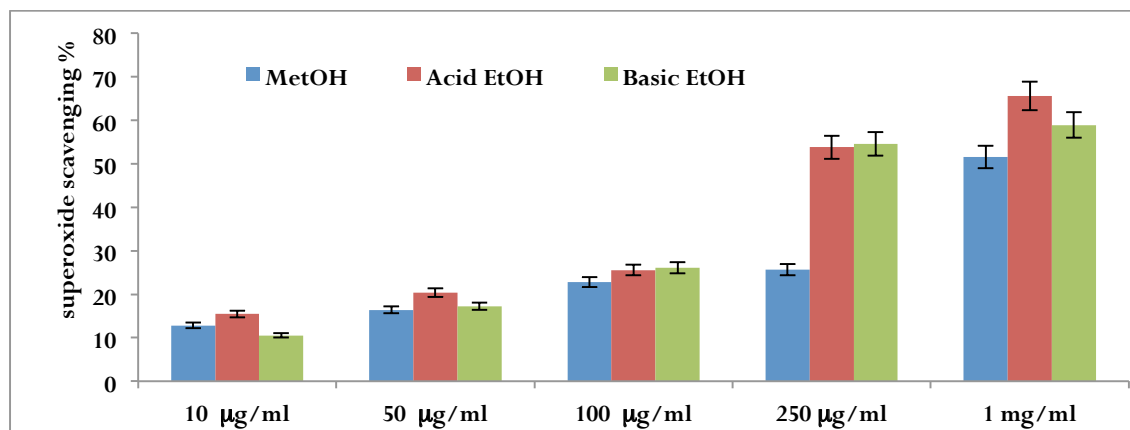
**Figure 3. DPPH radical scavenging activities (DPPH assay shows that, in this system, the radical scavenging activities of the three date palm extracts are in this order: acidic ethanolic extracts>methanolic extract>basic ethanolic extract).** Various concentrations of date palm extracts, collected by three distinct methods, quenched DPPH free radical in a dose-dependent manner [ $r^2=0.909$  ( $p<0.01$ ) for methanolic extract;  $r^2 = 0.933$  ( $p<0.01$ ) for acidic ethanolic extract;  $r^2=0.930$  ( $p<0.01$ ) for basic ethanolic extract]. As per  $IC_{50}$  values acidic ethanolic extract is more potent to scavenge DPPH radicals ( $IC_{50}14.61$  µg/ml) than the methanolic extracts ( $IC_{50} 22.91$  µg/ml) and basic ethanolic extracts ( $IC_{50} 36.44$  µg/ml). Ascorbic acid has been shown as positive control.



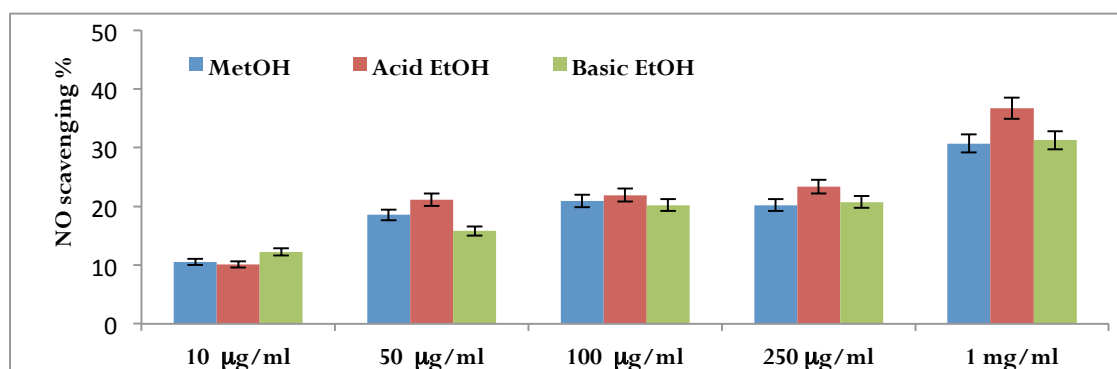
**Figure 4. Hydroxyl radical (OH) scavenging activity (Hydroxyl radical scavenging assay shows that, in this system, the hydroxyl radical-scavenging activities of the three date palm extracts are in this order: basic ethanolic extracts>acidic ethanolic extract>methanolic extract).** Various concentrations of date palm extracts, collected by three distinct methods, scavenge hydroxyl radical in a dose-dependent manner [ $r^2=0.832$  ( $p<0.01$ ) for methanolic extract;  $r^2 = 0.852$  ( $p<0.01$ ) for acidic ethanolic extract;  $r^2=0.882$  ( $p<0.01$ ) for basic ethanolic extract] As per  $IC_{50}$  values basic ethanolic extract is more potent to scavenge hydroxyl radicals ( $IC_{50} 17.00$  µg/ml) than the acidic ethanolic extracts ( $IC_{50} 17.30$  µg/ml) and methanolic extracts ( $IC_{50} 18.20$  µg/ml). Ascorbic acid has been shown as positive control.

Antioxidants, on interaction with DPPH transfer an electron (hydrogen atom) to DPPH, neutralizing its free radical character (Naik et al., 2003). The colour changes from purple to yellow and its absorbance at wavelength 517 decreases. Various concentrations of date extracts, collected by three distinct methods, quenched DPPH free radical in a dose-dependent

manner [ $r^2=0.909$  ( $p<0.01$ ) for methanolic extract;  $r^2 = 0.933$  ( $p<0.001$ ) for acidic ethanolic extract;  $r^2=0.930$  ( $p<0.001$ ) for basic ethanolic extract].  $IC_{50}$  values were 22.91 µg/ml for methanolic extract, 14.61 µg/ml for acidic ethanolic extract and 36.44 µg/ml for basic ethanolic extract. DPPH assay shows that, in this system, the radical-scavenging activities of the three varieties



**Figure 5. Assay of superoxide radical  $O_2^{\cdot -}$  scavenging activity** (Superoxide radical scavenging assay shows that, in this system, the superoxide radical-scavenging activities of the three date palm extracts are in this order: acidic ethanolic extracts>basic ethanolic extract>methanolic extract). Various concentrations of date palm extracts, collected by three distinct methods, scavenge Superoxide radical in a dose-dependent manner [ $r^2=0.809$  ( $p<0.01$ ) for methanolic extract;  $r^2 = 0.908$  ( $p<0.01$ ) for acidic ethanolic extract;  $r^2=0.928$  ( $p<0.01$ ) for basic ethanolic extract] As per  $IC_{50}$  values acidic ethanolic extract is more potent to superoxide radicals ( $IC_{50}$  19.00 µg/ml) than the basic ethanolic extracts ( $IC_{50}$  19.15 µg/ml) and methanolic extracts ( $IC_{50}$  28.88 µg/ml).



**Figure 6. Nitric oxide (NO) scavenging activity** (Nitric oxide scavenging assay shows that, in this system, the NO radical-scavenging activities of the three date palm extracts are in this order: methanolic extracts>acidic ethanolic extract>basic ethanolic extract). Various concentrations of date palm extracts, collected by three distinct methods, scavenge produced NO radicals, Sodium nitropruside used as substrate, in a dose-dependent manner [ $r^2=0.852$  ( $p<0.01$ ) for methanolic extract;  $r^2 = 0.857$  ( $p<0.01$ ) for acidic ethanolic extract;  $r^2=0.900$  ( $p<0.01$ ) for basic ethanolic extract] As per  $IC_{50}$  values methanolic date palm extract is more potent to NO radicals ( $IC_{50}$  5.505 µg/ml) than the acidic ethanolic extracts ( $IC_{50}$  5.53 µg/ml) and basic ethanolic extracts ( $IC_{50}$  5.60 µg/ml). But there is no significant difference in all these three different extracts of date palm to scavenge the Nitric oxide in an in vitro system.

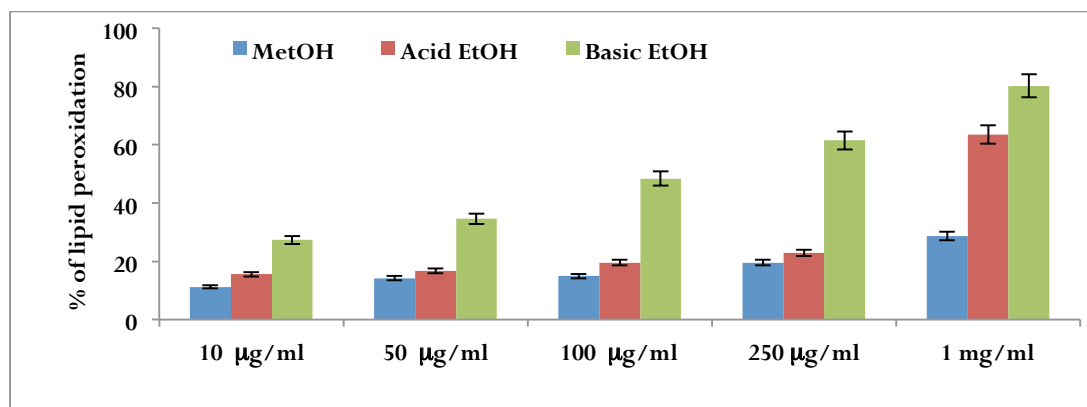
of date extracts are in the order acidic ethanolic extract>methanolic extract>basic ethanolic extract (Fig. 3).

#### Assay of hydroxyl radical (OH)-scavenging activity

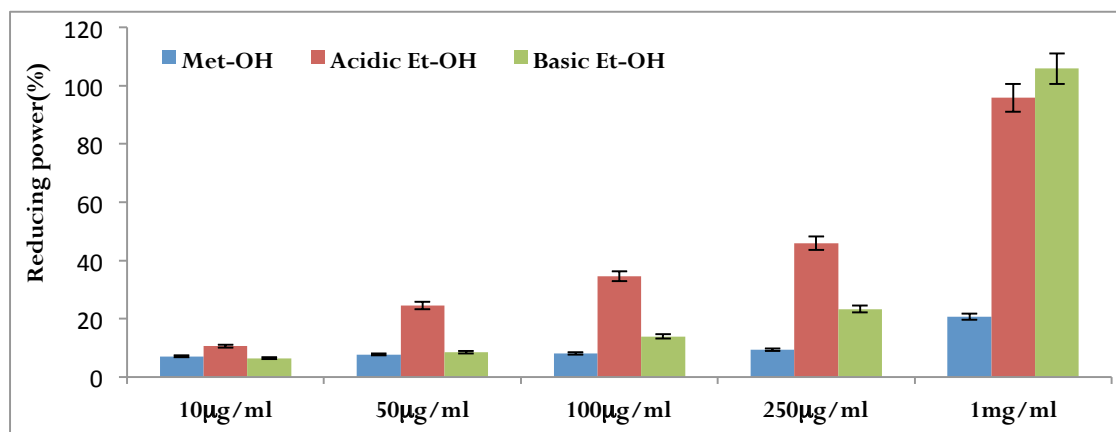
By the addition of iron (II) salts to a phosphate buffer containing reaction mixture, hydroxyl radicals can be generated (Gutteridge, 1983). Benzoate, weakly fluorescent, after monohydroxylation forms highly fluorescent hydroxybenzoates (Gutteridge, 1987). Measurement of this spectrofluorometric change has been used to detect damage by hydroxyl radical. Date ex-

tracts collected by three distinctly separate methods was found to be a powerful scavenger of hydroxyl radicals. There is a linear correlation between concentration of extract and OH-scavenging activity [ $r^2 =0.832$  ( $p<0.01$ ) for methanolic extracts of date;  $r^2=0.835$  ( $p<0.01$ ) for acidic ethanolic extracts of date;  $r^2=0.882$  ( $p<0.01$ ) for basic ethanolic extracts of date].  $IC_{50}$  values are 18.20 µg/ml for methanolic date extracts, 17.30 µg/ml for acidic ethanolic date extracts and 17.00 µg/ml for basic ethanolic extracts. Highest hydroxyl radical-scavenging activity was found in the date extract collected by using basic ethanolic methods. The





**Figure 7. Lipid peroxidation inhibition assay (Lipid peroxidation assay shows that, in this system, to prevent the lipid peroxidation, the three date palm extracts are in this order: basic ethanolic extract>acidic ethanolic extract> methanolic extracts).** Various concentrations of date palm extracts, collected by three distinct methods, inhibit lipid peroxidation, using egg yolk homogenate as a substrate, in a dose-dependent manner [ $r^2=0.870$  ( $p<0.01$ ) for methanolic extract;  $r^2 = 0.635$  ( $p<0.01$ ) for acidic ethanolic extract;  $r^2=0.978$  ( $p<0.01$ ) for basic ethanolic extract]. As per  $IC_{50}$  values basic ethanolic date palm extract is more potent to inhibit the lipid peroxidation ( $IC_{50}$  18.03 µg/ml) than the acidic ethanolic extracts ( $IC_{50}$  24.75 µg/ml) and methanolic date palm extracts ( $IC_{50}$  60.69 µg/ml).



**Figure 8. Determination of reducing power (The reducing power activities are highest in acidic ethanolic extract followed by basic ethanolic and methanolic extracts of date palm).** Various concentrations of date palm extracts, collected by three distinct methods, reduce ferric ions in a dose-dependent manner [ $r^2=0.640$  ( $p<0.01$ ) for methanolic extract;  $r^2 = 0.864$  ( $p<0.01$ ) for acidic ethanolic extract;  $r^2=0.646$  ( $p<0.01$ ) for basic ethanolic extract]. The reducing power activities are highest in acidic ethanolic extract followed by basic ethanolic and methanolic extracts of date palm.

hydroxyl radical scavenging properties of date extracts are: basic ethanolic extracts> acidic ethanolic extracts> methanolic extracts (Fig. 4).

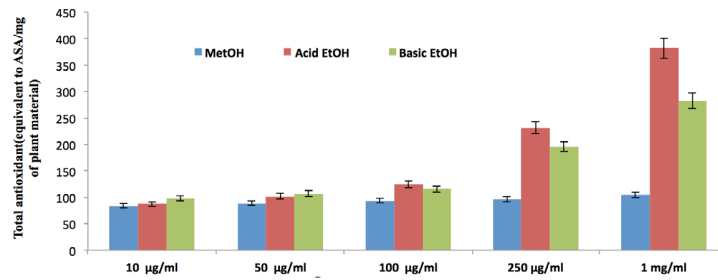
#### Superoxide radical $O_2^{\cdot-}$ scavenging activity

Photochemical reduction of flavins generates  $O_2^{\cdot-}$  which reduces NBT, resulting in the formation of blue formazan (Beauchamp & Fridovich, 1971). Three types of date extracts inhibited the formation of the blue formazan and % inhibition is proportional to the concentration [ $r^2 = 0.809$  ( $p<0.01$ ) for methanolic extract;  $r^2 = 0.908$

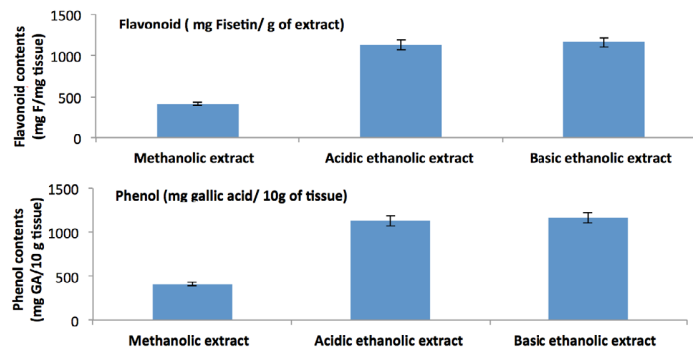
( $p<0.01$ ) for acidic ethanolic extract;  $r^2 = 0.928$  ( $p<0.01$ ) for basic ethanolic extract].  $IC_{50}$  values were 28.88 µg/ml for methanolic extract, 19.00 µg/ml for acidic ethanolic extract and 19.15 for acidic ethanolic extract. The superoxide ion scavenging activities of all these three extracts are: acidic ethanolic extract> basic ethanolic extract> methanolic extracts (Fig. 5).

#### Nitric oxide (NO) scavenging activity

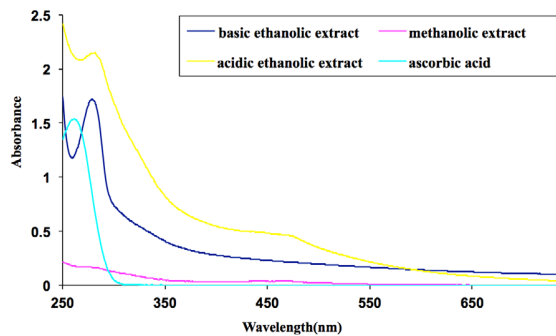
The calculated  $IC_{50}$  values of all three different date extracts suggested that methanolic extract is the most



**Figure 9. Total antioxidant capacity (equivalent to ASA/mg of plant material)** (The phosphomolybdenum method is quantitative method to detect the antioxidant activity, expressed as the number of equivalents of ascorbic acid where acidic ethanolic date palm extract had a higher capacity than the basic ethanolic date palm extracts, followed by methanolic date palm extracts). Determination of the total anti oxidant capacity of three different date palm extracts, based on the basis of the reduction of Mo (VI) to Mo (V). Results were expressed as equivalent to ascorbic acid (ASA)/mg of plant material. (n=5) Acidic ethanolic date palm extract had a higher capacity than the basic ethanolic date palm extracts, followed by methanolic date palm extracts.



**Figure 10. Flavonoid and Phenol contents in three date palm extracts** (Though the basic ethanolic extract contain maximum phenolic contents than other two tested extracts, but the bioactive flavonoid content is highest in acidic ethanolic extracts. Methanolic date palm extract is very poor in containing phenol and flavonoids)/ Total flavonoid content of the date palm extracts was determined using aluminium chloride (AlCl<sub>3</sub>) where as the total Phenolic content was determined using the Folin-Ciocalteu reagent. (n=5) results were expressed as mg of Flavonoid contents in fisetin/g of extracts and mg of gallic acid/ 10 mg of the extract, taking ±SD. Total Flavonoid and Phenol contents show a direct correlation with the total anti oxidant capacity of the tested compounds. Acidic ethanolic extract is rich in both Phenolic and Flavonoid contents, and it can be correlated to it's radical scavenging activities and total antioxidant properties as the data has shown, than the other two tested extracts of date palm.



**Figure 11. UV based spectrophotometric Analysis.** UV-VIS based spectrophotometric analysis reveal that acidic and basic ethanolic extracts contain maximum amounts of phenolic compounds which comes under the UV zone where the methanolic extracts shows a very poor quantities of these compounds in this area under study. Significantly the acidic ethanolic extracts indicates that it contain more phenolic and flavonoid contents than the standard control ascorbic acid solution which has been correlated to the radical scavenging activities, lipid peroxidation inhibitory potential, total phenolic and flavonoid contents and the total anti oxidant properties also.

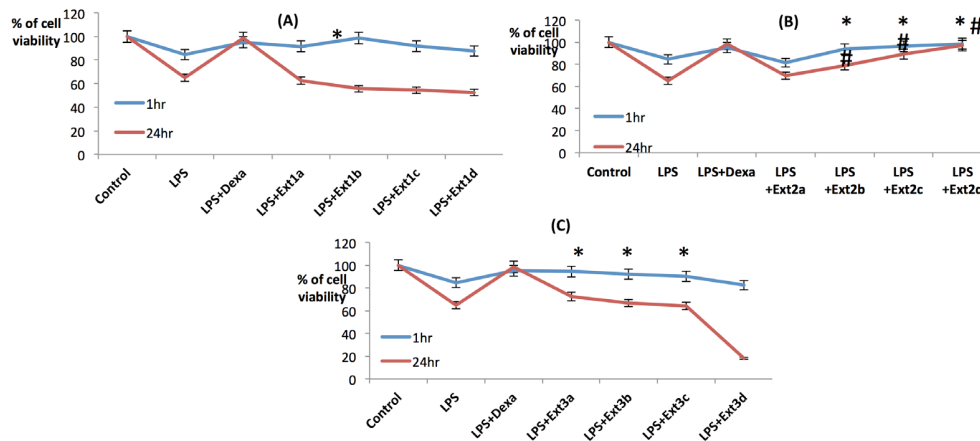
potent extract to scavenge the NO molecules followed by acidic ethanolic extracts and basic ethanolic extracts. But surprisingly there is little or no significant variation of NO radical scavenging properties of all three date extracts (Fig. 6).

### Lipid peroxidation inhibition assay

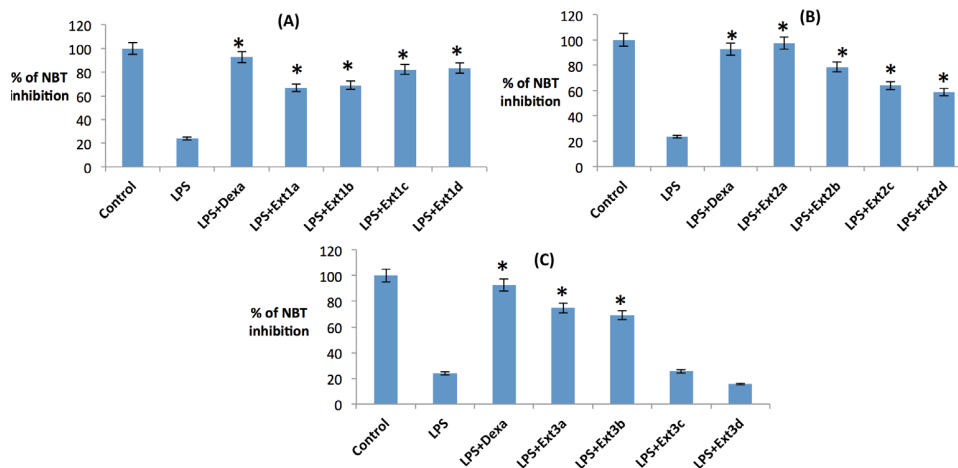
Egg yolk lipids undergo rapid non-enzymatic peroxidation when incubated in the presence of ferrous sulphate. Lipid peroxides are likely involved in numer-

ous pathological events, including inflammation, metabolic disorders and cellular aging (Ames et al., 1993; Wiseman et al., 1996). Date extracts inhibited lipid peroxidation in a concentration-dependent manner [ $r^2 = 0.870$  ( $p < 0.01$ ) for methanolic extract;  $r^2 = 0.635$  ( $p < 0.01$ ) for acidic ethanolic extract;  $r^2 = 0.9596$  ( $p < 0.01$ ) for basic ethanolic extract].  $IC_{50}$  values for the inhibition of lipid peroxidation were 60.69  $\mu\text{g/ml}$  for methanolic

extract, 24.75  $\mu\text{g/ml}$  for acidic ethanolic extract and 18.03  $\mu\text{g/ml}$  for basic ethanolic extract. The results suggested that consumption of date may afford a cytoprotective effect by lowering the lipid peroxidation level (Fig. 7).



**Figure 12. Cell viability determination using MTT assay.** (A) RAW 264.7 Cells were treated with 1 $\mu\text{g/ml}$  of E.coli LPS and then methanolic date palm extracts were added into it therapeutically and incubated for two different time periods, 1hour and 24 hour. Taking control as 100% viability data was calculated ( $p < 0.01$ ). Dexamethasone (9nM) was used as positive control (a= 250,b=100,c=50,d=10  $\mu\text{g/ml}$ ). (B) RAW 264.7 Cells were treated with 1 $\mu\text{g/ml}$  of E.coli LPS and then acidic ethanolic date palm extracts were added into it therapeutically and incubated for two different time periods, 1hour and 24 hour. Taking control as 100% viability data was calculated ( $p < 0.01$ ). Dexamethasone (9nM) was used as positive control. (a= 250,b=100,c=50,d=10  $\mu\text{g/ml}$ ). (C) RAW 264.7 Cells were treated with 1 $\mu\text{g/ml}$  of E.coli LPS and then basic ethanolic date palm extracts were added into it therapeutically and incubated for two different time periods, 1hour and 24 hour. Taking control as 100% viability data was calculated ( $p < 0.01$ ). Dexamethasone (9nM) was used as positive control. (a= 250,b=100,c=50,d=10  $\mu\text{g/ml}$ ) (\* for 1 hour group, # for 24 hour group in comparison to the untreated control group).



**Figure 13. Super oxide scavenging assay, following NBT inhibition method, using three different date palm extracts show that for twenty four hour LPS induced model, both acidic ethanolic extract and methanolic extract is more capable to inhibit LPS induced inflammatory oxidative burst than the basic ethanolic tested extracts in a dose dependent manner. In lower doses basic ethanolic extract shows proinflammatory activities by inducin oxidative stress on murine RAW macrophages.** (A) Super oxide Ion scavenging activities of methanolic date palm extracts on LPS (1 $\mu\text{g/ml}$ ) induced RAW macrophages, after 24 hour incubation, were determined by using NBT inhibition methods, taking control group as 100% inhibition ( $p < 0.05$  in comparison to the untreated control group). (B) Super oxide Ion scavenging activities of acidic ethanolic date palm extracts on LPS (1 $\mu\text{g/ml}$ ) induced RAW macrophages, after 24 hour incubation, were determined by using NBT inhibition methods, taking control group as 100% inhibition ( $p < 0.05$  in comparison to the untreated control group). (C) Super oxide Ion scavenging activities of basic ethanolic date palm extracts on LPS (1 $\mu\text{g/ml}$ ) induced RAW macrophages, after 24 hour incubation, were determined by using NBT inhibition methods, taking control group as 100% inhibition ( $p < 0.05$  in comparison to the untreated control group). Dexamethasone (9nM) was used as positive control. (a= 250,b=100,c=50,d=10  $\mu\text{g/ml}$ ).

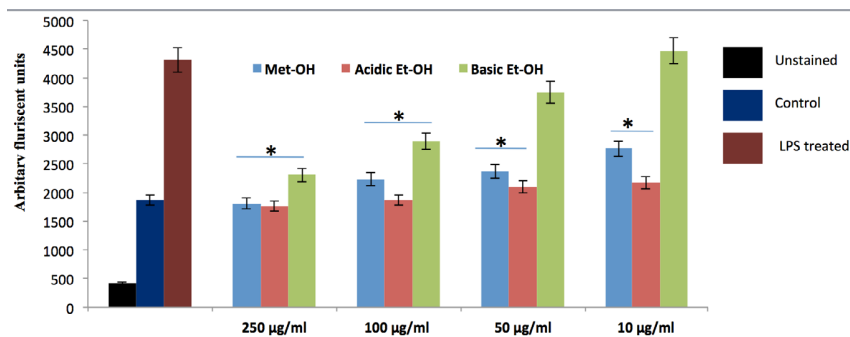
**Determination of reducing power**

The reducing power of different date extract samples using the potassium ferricyanide reduction method was evaluated. It has been investigated from the Fe<sup>3+</sup>-Fe<sup>2+</sup> transformation in the presence of three different extract samples. Highest activity was found in basic ethanolic extracts, followed by acidic ethanolic extracts of date and the lowest activity was found in methanolic extract of date (Fig. 8).

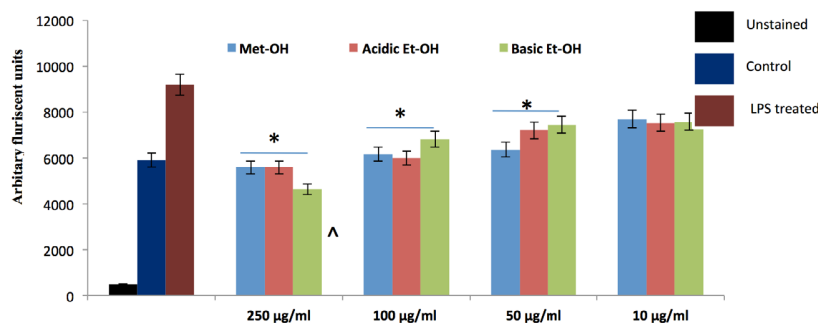
**Total antioxidant capacity (equivalent to ASA/mg of plant material)**

Total antioxidant capacity of date extract is expressed

as the number of equivalents of ascorbic acid. The assay is based on the reduction of Mo(VI) to Mo(V) by the extract and subsequent formation of a green phosphate/Mo(V) complex at acid pH. The phosphomolybdenum method is quantitative since the antioxidant activity is expressed as the number of equivalents of ascorbic acid (Prieto et al., 1999). Acidic ethanolic extract had a higher capacity than the other two varieties. The results from various free radical-scavenging system revealed that the three date extract samples collected by three different methods had significant antioxidant activity. The extracts were found to have different levels of antioxidant activity in the systems tested. The antioxidant activities of the three varieties were in the order acidic ethanolic ex-



**Figure 16. Intracellular ROS measurement in RAW macrophages (all three date palm extracts are capable to inhibit LPS induced oxidative stress in RAW macrophages in a dose dependent manner).** Intracellular formation of ROS was assessed by using oxidation sensitive dye DCFH-DA as a substrate where non-fluorescent DCFH-DA dye, that is freely penetrate into cells get hydrolyzed by intracellular esterase to 207-dichlorofluorescin (DCFH), and traps inside the cells. The formation of 207-dichlorofluorescin (DCF) due to oxidation of DCFH in the presence of ROS was read after 30 min at an excitation wavelength of 485 nm and emission wavelength of 525 nm using a spectrofluorometer. Intracellular ROS scavenging activities of three date palm extract followed a dose dependent manner where at 250 µg/ml, 100 µg/ml and 50 µg/ml doses all three extracts are capable to inhibit LPS induced oxidative stress in RAW macrophages significantly, but at lower dose, i.e. 10 µg/ml only methanolic and acidic ethanolic extracts are capable to reduce oxidative burst significantly. (\*p < 0.05 in comparison to the untreated control group) From this data, it can be concluded that all three date palm extracts are capable to inhibit LPS induced oxidative stress in RAW macrophages in a dose dependent manner.



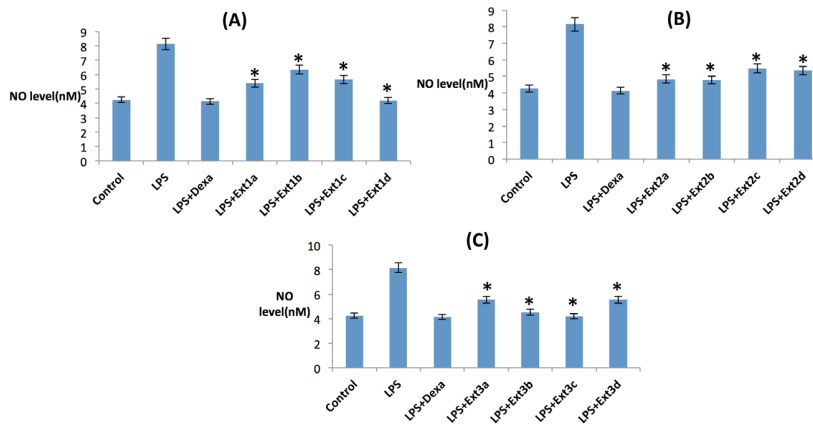
**Figure 17. Mitochondrial ROS measurement in RAW macrophages (all three date palm extracts are capable to inhibit LPS induced mitochondrial oxidative stress in RAW macrophages in a dose dependent manner).** Mitochondrial ROS production was monitored by the fluorescent dye, Rhodamine123 where due to oxidative stress depolarization of mitochondrial membrane potential results in the loss of Rhodamine123 from the mitochondria and a produce the fluorescence intensity. Mitochondrial ROS scavenging activities of three date palm extract followed a dose dependent manner where at 250 µg/ml, 100 µg/ml and 50 µg/ml doses all three extracts are capable to inhibit LPS induced Mitochondrial oxidative stress in RAW macrophages significantly, but at lower dose, i.e. 10 µg/ml though they reduce mitochondria oxidative burst in comparison t the LPS treated group, but as the values are so much higher than the control group, so the values are not counted (\*p < 0.05 in comparison to the untreated control group). From this data, it can be concluded that all three date palm extracts are capable to inhibit LPS induced mitochondrial oxidative stress in RAW macrophages in a dose dependent manner.

tract>basic ethanolic extract>methanolic extract (Table 7) (Fig. 9).

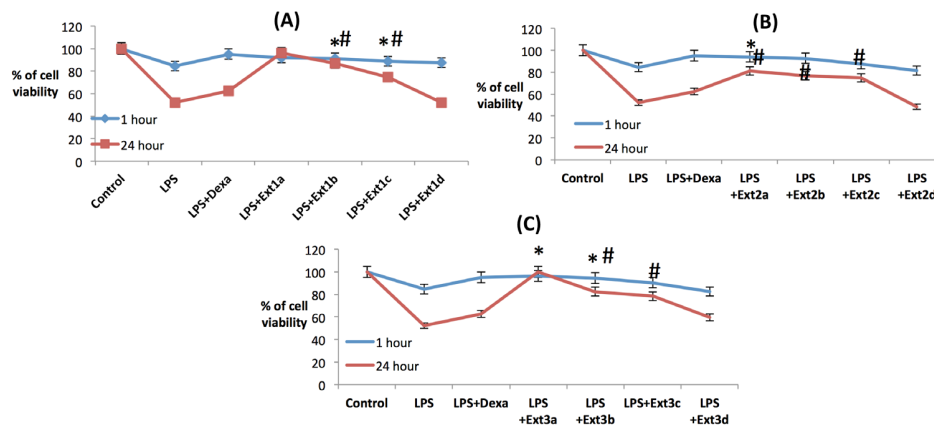
**Total Flavonoid and phenol content.**

Total flavonoid content expressed as Fisetin equivalents was 15.44±0.872 mg/g plant material in methanolic extract, 60.96±0.996 mg/g plant material in acid-

ic ethanolic extract and 26.07±1.26 mg/g plant material in basic ethanolic extract respectively. Total phenolic concentration showed close correlation with the antioxidant activity, being highest in basic ethanolic extract and lowest in methanolic extract of date. Plant phenolics present in fruit and vegetables have received considerable attention because of their potential antioxidant activity (López-Vélez et al., 2003).



**Figure 14.** Nitric oxide (NO) scavenging assay, following Griess reagent method, using three different date palm extracts show that for twenty four hour LPS induced model, all three date palm extracts, viz. Methanolic, acidic ethanolic extract and basic ethanolic extract is capable to inhibit LPS induced NO production in a dose dependent manner on murine RAW macrophages. (A) NO levels (nM) were determined using Griess reagent from LPS (1µg/ml) stimulated RAW macrophages supernatant, where methanolic date palm extracts at various concentrations were added therapeutically and incubation time was 24 hours. (p<0.05). (B) NO levels (nM) were determined using Griess reagent from LPS (1µg/ml) stimulated RAW macrophages supernatant, where acidic ethanolic date palm extracts at various concentrations were added therapeutically and incubation time was 24 hours. (C) NO levels (nM) were determined using Griess reagent from LPS (1µg/ml) stimulated RAW macrophages supernatant, where basic ethanolic date palm extracts at various concentrations were added therapeutically and incubation time was 24 hours. (p<0.05, in comparison to the untreated control group). Dexamethasone (9nM) was used as positive control. (a= 250,b=100,c=50,d=10 µg/ml).



**Figure 15.** Cell viability assay using three different date palm extracts show that for one hour LPS induced model, there is no significant variation to maintain cell viability of all three extracts where as in 24 hour model, both acidic and basic ethanolic extract is more capable to inhibit LPS induced inflammation than the methanolic extract in a dose dependent manner on human embryonic kidney cell lines. (A) Cell viability determination using MTT assay. HEK 293 Cells were treated with 1µg/ml of E.coli LPS and then methanolic date palm extracts were added into it therapeutically and incubated for two different time periods, 1hour and 24 hour. Taking control as 100% viability data was calculated (p<0.01). (B) Cell viability determination using MTT assay. HEK 293 Cells were treated with 1µg/ml of E.coli LPS and then acidic ethanolic date palm extracts were added into it therapeutically and incubated for two different time periods, 1hour and 24 hour. Taking control as 100% viability data was calculated (p<0.01). (C) Cell viability determination using MTT assay. HEK 293 Cells were treated with 1µg/ml of E.coli LPS and then basic ethanolic date palm extracts were added into it therapeutically and incubated for two different time periods, 1hour and 24 hour. Taking control as 100% viability data was calculated (p<0.01). Dexamethasone (9nM) was used as positive control. (a= 250,b=100,c=50,d=10 µg/ml) (\* for 1 hour group, # for 24 hour group).

Natural polyphenols have chain-breaking antioxidant activities and are believed to prevent many degenerative diseases, including cancer and atherosclerosis (Roginsky, 2003) (Table 8) (Fig. 10a,b).

#### UV based spectrophotometric Analysis:

When different extracts of date was analysed in a UV-VIS spectrophotometer to assess their solubility in different solvents to determine actual active compounds remain in that solution, we got a striking result where date extracts in basic ethanolic solvents yielded best solubility in comparison to their acidic ethanolic solvent, whereas the methanolic extracts failed to show any such significant peak, indicating very low or no solubility of the date extract into it which states that ethanolic solvent is the best to exhibit the potent anti-inflammatory biological activities of the date which correlates with the cellular analysis further (Fig 11).

#### Cell viability of RAW macrophages:

In order to ascertain the anti-inflammatory activities of the date extracts, collected by three distinct methodologies, LPS induced preclinical in vitro models, using both RAW macrophage like murine cell lines and HEK, human embryonic kidney cell lines were used. Cells were seeded into the 96 well plate and incubated for 12 hours, then 1 µg/ml LPS was added into it following the various doses of the extracts and incubated for one hour and twenty four hour time periods respectively. In both the models using RAW macrophages, three extracts possess their anti-inflammatory activities by regulating the cell viability in a dose dependent manner where methanolic extracts are not potent to maintain cell viability in 24 hour inflammatory models in comparison to the other two extracts where acidic ethanolic extracts retain the cell viability significantly in its lower doses, i.e. 100 µg/ml, 50 µg/ml, 10 µg/ml and 1 µg/ml respectively in both one hour and 24 hour models (Figure 12a, 12b), but the basic ethanolic extracts maintain the cell viability only in higher doses, i.e. 250 µg/ml, 100 µg/ml and 50 µg/ml, where in lower doses (1 µg/ml) it acts as pro-inflammatory causing the cell death (Fig 12c). So all these extract may act as either anti-inflammatory or pro-inflammatory depending on their doses and nature of extraction.

#### Assay of hydroxyl radical (OH)-scavenging activity

As the *in vitro* study suggested that all three date crude extracts have potent hydroxyl radical and superoxide ion scavenging activities, the *ex vivo* result,

using RAW macrophages, also advocated the same where acidic ethanolic extract has been shown to have strongest hydroxyl radical scavenging power, >50% inhibition (Fig. 13b), followed by basic ethanolic (Fig. 13c) and methanolic extracts (Fig. 13a). This may have been accomplished by modulating the mitochondria associated NADPH oxidase activity.

#### NO estimation

None of the tested compounds induced changes in NO basal levels, when incubated without LPS (data not shown). To evaluate the nitrosative stress, Griess reagent was used. After 24 h LPS treatment, cellular NO production increases nearly two folds (Figure 14a) in comparison to the untreated control samples, whereas the three date extracts ameliorate the LPS induced NO production to the basal level like untreated group in a dose dependent manner which strongly showing their NO scavenging capabilities which is already been established by the in vitro assays. Here dexamethasone has been taken as positive control (Fig. 14a,b,c).

#### Cell viability of HEK 293 cells

To investigate the cytotoxicity of the three date extracts MTT assay was performed as MTT is reduced by mitochondrial dehydrogenase to form formazan, an insoluble purple compound and one can measure the cytotoxicity in terms of the intensity of the purple compound. Whereas on the other hand, dead cells do not form any purple formazan because the enzyme is degraded and lack regular function. Among the three extracts of date, acidic ethanolic extracts ameliorated the LPS induced cell death to retain the cell viability in both one hour and twenty four hour inflammatory models (Fig. 15b) followed by basic ethanolic (Fig. 15c) and methanolic extracts (Fig. 15a) in a dose dependent manner. In lower doses all these three extracts fail to inhibit LPS induced cell death where as they are active only in the higher concentrations, i.e. in 250 µg/ml and 100 µg/ml doses which is similar to the LPS induced inflammatory models applied on murine macrophage cell line. This data suggests that acidic ethanolic extracts contain potent anti-inflammatory compound(s) which is/are able to inhibit the LPS induced cytotoxicity in a dose dependent manner. Dexamethasone was used as positive control in the entire study.

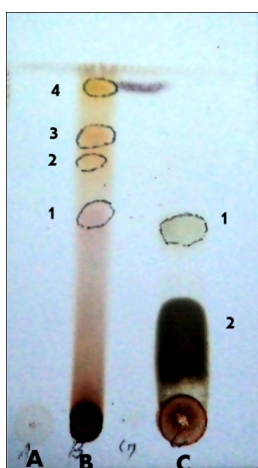
#### ROS measurement

The evidence presented above suggests that date extracts mediate its effects through the anti-oxidant pathway. We used a DCF-DA probe to examine whether this mechanism can scavenge ROS inside the RAW macrophage cells. Cells were treated with E.coli LPS and then various concentrations of date extracts were added into it therapeutically and incubated for 6 h, and analyzed by florescent spectrophotometer. Date extracts ameliorate LPS induced ROS levels significantly over the control in a dose dependent manner. This effect was observed best in acidic ethanolic extracts where it inhibited the ROS generation in higher doses, i.e. in 250  $\mu\text{g/ml}$  and 100  $\mu\text{g/ml}$  and increased ROS levels slightly thereafter. ROS levels decreased significantly in methanolic and basic ethanolic extracts also but increased significantly in its lower doses compared to control cells (Fig. 16).

#### Measurement of mitochondrial membrane potential (MMP)

After the assessment of total cellular anti-oxidant property, in the next phase we targeted mitochondria, the most important site inside the cell for ROS generation. Here we have found that all three extracts of date is capable of reducing LPS induced oxidative stress in murine macrophage like cells significantly, in comparison to the control group in a dose dependent manner, which advocates that the date extracts ameliorate the LPS induced oxidative stress by targeting the mitochondria in a dose dependent manner and thus maintain the cell viability (Fig. 17).

#### TLC analyses



**Figure 18. TLC profile of A,B & C extract.** There is no band was observed in extract A. In extract B & C there is 4 and 2 band were observed respectively. *R<sub>f</sub>* values of sample B & C are as below:

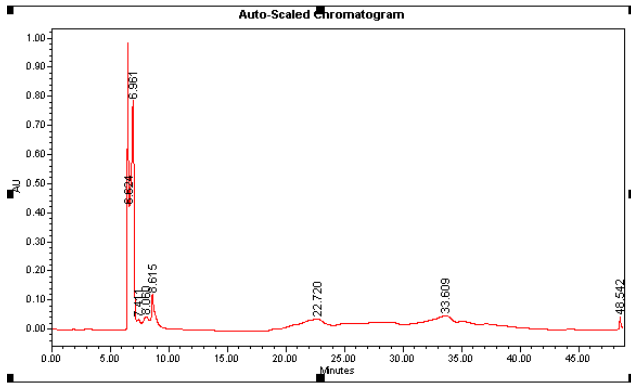
Sample Name	Number of Bands	Rf value
B	4	1. 0.58
		2. 0.73
		3. 0.81
		4. 0.95
C	2	1. 0.32
		2. 0.56

**Sample A:** The extract A showed several peaks as shown in the Figure 18. However only two peaks show appreciably high absorbance when observed with 254 nm wavelength. The peak at 23.5 min is the major peak while that at 34.0 min is a minor peak. The peak at 23.5 min appears about 20 fold attenuated when observed with 370 nm wavelength while the peak at 34.0 min is not observed at this wavelength.

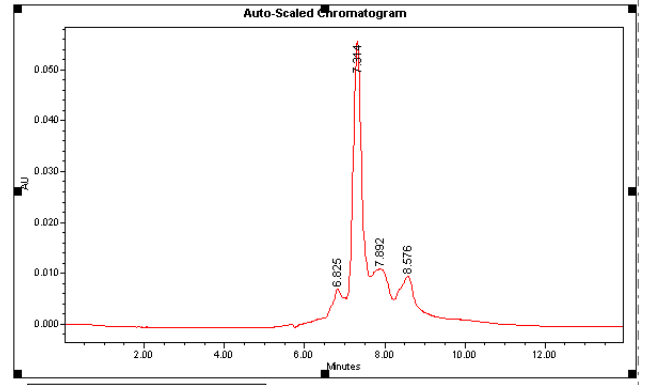
**Sample B:** Extract B shows four closely spaced peaks between 6.0 – 9.0 min appearing at 6.7, 7.3, 7.8 and 8.4 min with major peak being at 7.3 min when observed with 254 nm wavelength. These peaks were also observed with 370 nm wavelength but with about 4 fold attenuation in peak absorbance values.

**Sample C:** The extract C showed roughly three distinct peaks. They were at 5.1-5.2 min, 6.3 min and 7.9 min using 254 nm wavelength. The first two peaks are major peaks but are not well separated. The third peak at 7.9 min is a minor peak in this sample. There is another minor peak at about 6.8 min observed with 254 nm wavelength which may be a spurious peak as it lies on the shoulder of the 6.3min peak and didn't appear in repeat scan. The absorbance maxima of these peak positions remained similar when seen in 370nm wavelength although their intensities were attenuated by about 10 fold. Comparing the absolute absorbance values of peak maxima of sample C with others samples (A & B), it indicates that sample C contains much higher concentration of compound(s) as similar values of samples (100-200  $\mu\text{l}$ ) were injected for HPLC analysis in each case.

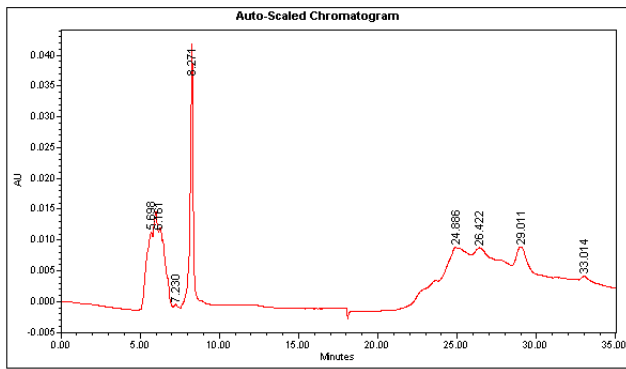
#### HPLC analyses



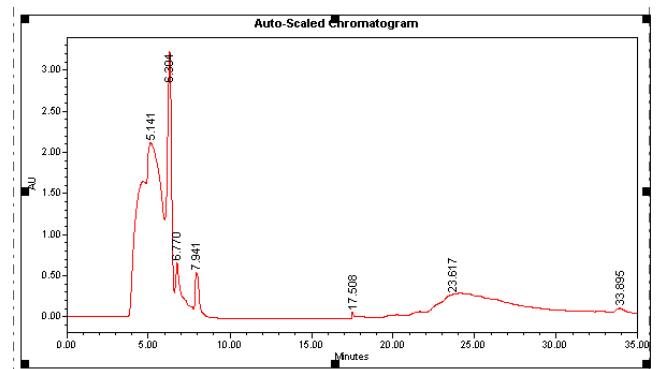
**Figure 19A.** HPLC profile of methanolic extract (A) of *Phoenix sylvestris*L). Crude methanolic extract was analysed by HPLC, detecting several peaks at 254 nm. Their HPLC retention times are 6.62,6.96,7.41,8.06 & 8.61 min, respectively.



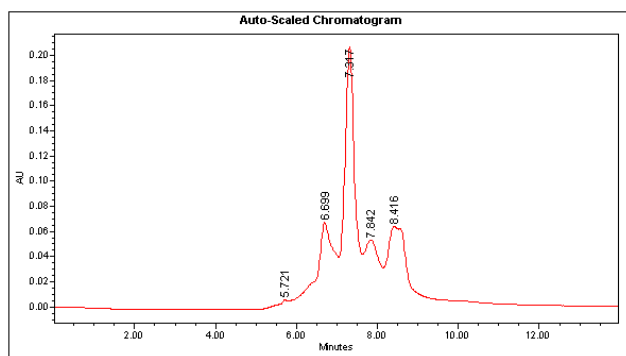
**Figure 19D.** HPLC profile of basic ethanolic extract (B) of *Phoenix sylvestris*L). Crude basic ethanolic extract was analysed by HPLC, detecting several peaks analysed by HPLC, detecting several peaks at 370 nm. Their HPLC retention times are 6.82,7.31,7.89,8.57 min, respectively.



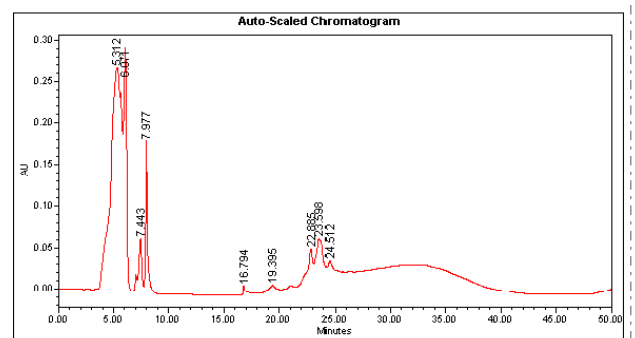
**Figure 19B.** HPLC profile of methanolic extract (A) of *Phoenix sylvestris*L). Crude methanolic extract was analysed by HPLC detecting several peaks at 370 nm. Their HPLC retention times are 5.69, 6.16, 7.23, 8.27, 24.88, 26.42, 29.01, & 33.01 min, respectively.



**Figure 19E.** HPLC profile of acidic ethanolic extract (C) of *Phoenix sylvestris* L. Crude acidic ethanolic extract was analysed by HPLC, detecting several peaks analysed by HPLC, detecting several peaks at 254 nm. Their HPLC retention times were 5.14,6.30,6.77,7.94,23.61,33.89 min, respectively.



**Figure 19C.** HPLC profile of basic ethanolic extract (B) of *Phoenix sylvestris*L. Crude basic ethanolic extract was analysed by HPLC, detecting several peaks analysed by HPLC, detecting several peaks at 254 nm. Their HPLC retention times are 5.72, 6.67, 7.31, 7.84 & 8.41 min, respectively.



**Figure 19F.** HPLC profile of acidic ethanol extract (C) of *Phoenix sylvestris*L. Crude acidic ethanolic extract was analysed by HPLC, detecting several peaks at 370 nm. Their HPLC retention times were 5.31,7.44,7.97,16.79,19.39,22.88,23.59 and 24.51 min, respectively.



## DISCUSSION

In the present study to evaluate anti-inflammatory potential of the extracts of date collected by three different extraction procedures (**Fig. 1**) where one or more fractions of the mesocarp tissue is likely to be included and the rest excluded, various inflammatory markers were used against a powerful and well characterized pro-inflammatory stimulus-LPS, a major component of the outer membrane of Gram-negative bacteria. This molecule shows potent pro inflammatory action on various cell types, including macrophages, endothelial cells, and fibroblasts (Allaith, 2008) induced inflammation in RAW 264.7 and HEK 291 cell lines by interacting with TLR4, a trans-membrane receptor on macrophages, which recognizes molecules derived from gram negative bacteria activating the downstream NF- $\kappa$ B (nuclear factor kappa) signaling pathway leading to increased production of cytokines, and/or ROS/RNS generation following the activation of mitogen-activated protein kinases (MAPK) and NF- $\kappa$ B mediated signaling pathway which ultimately drive the tissue degeneration.

Fruits constitute an important part of a balanced diet as they are natural sources of food nutrient needed by human and animals, being rich in proteins, carbohydrates, minerals and dietary fibers. *Phoenix dactylifera*, belongs to tree palm family Arecaceae, consumed throughout the world and is an important part of the diet in the Middle East (Barh and Mazumdar, 2008). Phytochemical screening has revealed dried date pollen to contain sterols and other supplements such as vitamins and co-factors (Ishurd et al., 2003) and lack volatile substances (Hussein et al., 1998). Phytochemical analyses of fruit flesh and seed of date revealed the presence of flavonoids (luteolin, methyl luteolin, quercetin, and methyl quercetin), flavanols (catechin, epicatechin) (Al-shahib and Marshall, 2003; Bennett et al., 1966; Heftmann et al., 1965), free phenolic acids (protocatechuic acid, vanillic acid, syringic acid, and ferulic acid) (Mahran et al., 1976; Tabeta et al., 2002), tannins, glycosides, cardiac glycosides and steroids (cholesterol, stigmaterol, campesterol and  $\alpha$ -sitosterol), essential amino acids (Biglari et al., 2008; Hong et al., 2006; Ziouti et al., 1996); and nine bound phenolic acids (gallic acid, protocatechuic acid, p-hydroxybenzoic acid, vanillic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid, and o-coumaric acid) (Hong et al., 2006; Hussein et al., 1998) respectively. In the past few years, many pharmacological studies have been conducted on *Phoenix dactylifera* (Abdulla;

Al-Qarawi et al., 2003; Ishurd and Kennedy, 2005). Various in vitro and in vivo antioxidant assays have been carried out on various extracts of different parts of *Phoenix dactylifera* (Vayalil, 2002). Oral administration of the methanolic and aqueous extracts of edible portion of *Phoenix dactylifera* fruits suppressed swelling in the foot, while the methanolic extract of date seeds showed significant reduction in adjuvant arthritis in rats (Bastway Ahmed et al., 2010) and on gentamicin induced nephrotoxicity in rats (Javanmardi, 2003).

In the present study, datefruit was extracted using various organic solvents, and their anti-oxidative and anti-inflammatory activities were evaluated. Phenolic compounds as well as Flavonoids, which are a group of poly phenolic compounds possessing free radical scavenging properties, potential inhibitory actions of hydrolytic and oxidative enzymes as well as anti-inflammatory action. Obtained data have clearly demonstrated that date extracts, depending on their mode of collection possess potent anti-oxidative properties indicated by significant inhibition of lipid peroxidation (**Fig. 7**), superoxide radical scavenging, hydroxyl radical scavenging, DPPH radical scavenging and nitric oxide (NO) scavenging activities. The calculated IC<sub>50</sub> values of date indicate strong anti-oxidative properties of these crude extracts.

Data obtained from studies using these date extracts, collected by three different methods indicate potent anti-oxidative properties by showing significant inhibition of lipid peroxidation, superoxide radical scavenging, hydroxyl radical scavenging, DPPH radical scavenging and nitric oxide (NO) scavenging activities. The calculated IC<sub>50</sub> values of these three extracts of date having revealed strong anti-oxidative properties (**Fig. 9, Table 7**), their anti-inflammatory potencies warranted confirmation through additional studies on cells. Both human and murine cell lines were assessed. Oxidative stress being the main driving force in any inflammatory cascade, studies were designed to evaluate their ameliorative effect during and post-oxidative stress induced inflammation.

As we have identified the anti-oxidative capacity (**Fig. 9**) of dietary date extracts, collected by three different extraction procedures, i.e. methanolic, basic ethanolic and acidic ethanolic methods, (**Fig. 1**) as an interesting lead that can stabilize the intracellular reactive oxygen and nitrogen intermediates in both murine and human cell lines *in vitro* at non cytotoxic concentrations, we were therefore interested to evaluate the *in vivo*

anti-inflammatory activities of these compounds in preclinical models, but before this cytotoxic and anti-inflammatory properties of these three extracts have been evaluated thoroughly, targeting the mitochondrial reactive oxygen and nitrogen intermediates (Fig. 17).

The acidic ethanolic extracts of date extract ameliorates the LPS induced oxidative stress by inhibiting the NO production (Fig. 6) and subsequently protecting the mitochondrial respiratory chain complex function, thus maintaining the cellular homeostasis or cell viability per se. As we have mentioned earlier that oxidative stress and mitochondrial dysfunction are key features for any inflammatory cascade and here the date extracts are capable of inhibiting this, thus, these crude extracts must have potent anti-inflammatory power. Another important point to be noted here is that the acidic ethanolic extract of the date has shown a more potent anti-inflammatory properties in murine RAW macrophages (Fig. 12b) in comparison to other two extracts (Fig. 12a, 12c), whereas in case of LPS induced human HEK cells, the methanolic extract (Fig. 15a) is better at inhibiting the LPS induced oxidative stress and in maintaining the cellular homeostasis, than either acidic or basic ethanolic extracts. From studies done by various researchers as stated and referred to in the preceding paragraphs, we now know that plants have various phenolic compounds and flavonoids, which are basically secondary metabolites of the pentose phosphate, shikimate, and phenylpropanoid pathways, exhibit strong anti-oxidative redox properties allowing them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. Also, many of them have been shown to exhibit the antagonism for the classical NF- $\kappa$ B signaling pathways and for this reason in past few years there has been an upsurge of interest in the therapeutic potentials of medicinal plants as antioxidants in reducing such free radical induced tissue injury. Although several phytochemicals have been shown to possess pharmacological properties of potential interest anti-inflammatory properties and/or therapy, their activity in the *in vitro* and *in vivo* preclinical model organisms is still not well understood due to variations of their extraction and collection procedures, heterogeneous solubility in different solvents and most importantly, route of administration of these compounds in a dose dependent manner in different disease models. The mechanism by which various

date extracts mediates its antioxidant effects remains unclear.

Mitochondria are the major source for ROS generation in the cell. Evidence from our studies suggests the role of mitochondria in date induced ROS scavenging pathways (Fig. 17). It is possible that date extracts inhibit the mitochondrial enzymes that lead to production of ROS and thus maintain the oxidative stress induced cell damage (Fig. 16). The inhibition of ROS by date extracts could occur through its interaction with thioredoxin reductase thus changing its activity to NADPH oxidase, which could then lead to the scavenging of ROS. It is not clear yet which structural group or compound of date extracts is responsible for inhibiting the ROS production. Present extracts of date also has shown a high level of phenol (Fig. 10b) and flavonoid (Fig. 10a) content and this may be the reason behind their anti-inflammatory activities.

We can conclude that the inhibitory effect of date extracts on LPS induced inflammation in RAW macrophages is via the downstreaming of the classical TLR4 mediated signaling cascades scavenging the mitochondrial ROS production (Fig. 17). Until phytochemical screening of these extracts is carried out, this will be very early to conclude. However, it is true that the compounds present in these extracts can inhibit the endotoxin induced oxidative stress (Fig. 16) in both murine and human cell lines in a dose dependent manner. The phytochemical screening and the structure activity relationship of these extracts are under investigation now along with the detailed anti-inflammatory signaling pathways. But the question is, what compound (s) are exactly present in these extracts that are truly exhibiting the anti-inflammatory properties and the signaling pathways that they follow, is still not clear yet. For this HPLC and mass spectrometry of the samples are being carried out and from database analysis individual eluents shall be used in anti-inflammatory and anti-oxidative assays to assess their oxi-inflammatory potential. This is ongoing work in the lab.

Acting as second messengers, transient free radicals, synthesized during regular metabolism often trigger further downstream sequence of activation networks. But produced in unregulated catabolic cycles, they demonstrate deleterious compounds such as toxins and wastes which further contribute to the disease etiology. Studies on intermediate free radicals, especially reactive oxygen species (ROS) and their action on cellular physiology, have shown that they play a

pivotal role in causing secondary tissue degeneration in various inflammatory diseases, such as rheumatoid arthritis (Filippin et al., 2008), multiple sclerosis (Offen et al., 2004), thyroiditis (Burek and Rose, 2008), and type 1 diabetes (Chen et al., 2008).

ROS are known to perform essential roles in immune response to pathogens, including bacterial killing via induction of superoxide anion during respiratory burst in activated macrophages and neutrophils (Kanayama and Miyamoto, 2007; Lambeth, 2004). Further studies on patients with chronic granulomatous disease (CGD) or genetically engineered mice lacking components of the NADPH oxidase enzyme (NOX) (Morgenstern, 1997; Shiloh et al., 1999) provide corroborative data.

Reactive oxygen species (ROS) oxidatively modify DNA, proteins, lipids, and small intracellular molecules. Lipids, for example, pulmonary surfactants, react with ROS to produce lipid peroxides that perpetrate increased membrane permeability and inactivation of surfactants (Yang et al., 2006). Further, ROS react with cellular proteins and inhibit protein synthesis by disabling proteins involved in translation and translocation. This effectively impairs cellular metabolism (Tuder et al., 2003). ROS also damage nucleic acids by modifying purine and pyrimidine bases and by causing DNA strand breakage (Dizdaroglu et al., 2002). Overproduction of ROS and oxidative stress has been found to be critical in pathophysiology of other complex syndromes like cancer, asthma, cystic fibrosis, ischemia-reperfusion injury, drug-induced toxicity, and aging (Jeziarska-Drutel et al., 2013).

Natural products have yielded as many as 70% of the drugs used today for inflammation and degeneration (Newman et al., 2002). Consumption of large portions of fruits and vegetables have been recommended for reduction of risk of cardiovascular diseases, autoimmune diseases, cancer, and various other chronic illnesses (Neuhouser, 2004) although little is known about the actual active compounds or moieties which are beneficial. It is an important project to identify and validate direct action and participation of such active ingredients in modulating disease.

Figures 18-20 show data on proteomic analyses by TLC, HPLC and MS. On preliminary investigation TLC data reveals that extract A may not contain important compounds although anti-inflammatory activity was seen in the other two extracts. Clear peaks were not obtained in scan data of the other two which

necessitates further purification steps and MS analyses of the individual peaks and NMR. This work is ongoing and is beyond the scope of this work.

## CONCLUSION

In this study, three different types of date extracts, methanolic, acidic ethanolic and basic ethanolic produce effects on reactive oxygen species where scavenging of hydroxyl radicals, superoxide radicals, nitric oxide scavenging activity, and inhibition of lipid peroxidation were found to occur in a dose dependent manner. Moreover, they have a significant inhibition of anti-inflammatory activities. *In vitro* experiments prove that the effects of the extracts is likely mediated through inhibition of mitochondria derived (ROS) scavenging pathways. This is an important finding as it shows not only the extracts' pathway of action but also proves the direct involvement of a sub-cellular signaling network that can be interfered with, using natural products without the possibility of drastic side effects. Ongoing proteomic analysis has already revealed scan data using various sensitive chromatographic techniques of certain specific peaks. Further analyses are needed to conclusively identify the peaks to reveal identity of one or more compounds for detailed characterization and development into novel drug entities.

## ABBREVIATIONS

Human embryonic kidney cell line (HEK); murine RAW macrophages; lipopolysaccharide (LPS); reactive oxygen species (ROS); nitric oxide (NO); 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH); di-hydro rhodamine (DHR); super oxide dismutase (SOD).

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## COMPETING INTERESTS

The authors declare that they have no competing interests.

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