

In-vitro bioassays of aqueous and ethanol extracts of *Aseel* dates

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Abstract: *Aseel* is amongst the most vital date variety of Pakistan. Beside its nutritional value, it also got remedial uses therefore for the first time different *in-vitro* bioassays were performed to assess its medicinal value. Aqueous (DFAE) and ethanol (DFEE) extracts of fresh *Aseel* dates were used for the purpose. Microplate alamar blue assay was done for antibacterial activity, Brine shrimp lethality test for cytotoxicity and MTT assays with different cancer cell lines were used for anti-cancer activity. Antioxidant and anti-inflammatory activity were also evaluated by free radical scavenging bioassay and chemiluminescence technique. Alamar blue assay of both extracts exhibited weak antibacterial activity against *E.coli*, *S. flexenari* and *S. aureus*. Brine shrimp lethality revealed absence of cytotoxicity at 1000µg/mL concentration. DFEE 50 µg/mL was effective against MCF-7,MDA-MB-231, PC3, 3T3 and Hela cancer cell lines showing 17.59%, 20.90%, 37.60%, 22.35% and 36.70% inhibition whereas DFAE exhibits 20.46%, 30.86%, 15.21%, 29.70% and 16.40 % inhibition respectively. Similarly both extracts also showed varying degree of anti-oxidant and anti-inflammatory activity against standard drug. The results are suggestive of weak bioactivity of *Aseel* date extracts might because of reduced potency however further studies are required for better understanding of observed results and separation of active ingredients from *Aseel* dates.

Keywords: *Aseel*, *in-vitro* bioassays, antibacterial activity, cytotoxicity, antioxidant, anti-inflammatory activity.

INTRODUCTION

Since ancient times, natural compounds and plants provide maintenance of human health and wellbeing. *Phoenix dactylifera* Date palm, is amongst the early cultivated trees widely spread in Southern and Central America, southern Europe, Africa, Middle East, Pakistan and India (Zaid, 1999; Chandra *et al.*, 1992; Al-Shahib and Marshall, 2003, Naeem *et al.*, 2018). The date fruit is highly nutritious due to the abundance of macronutrients like proteins, carbohydrates, vitamins, fibers and minerals (Zaid, 1999). It might have several health benefits because of its bioactive constituents however its true medicinal potential is still unexplored.

Dietary components of date fruit includes carbohydrates (approx. 70%), dietary fibers, proteins and fats. It contains essentials nutrients like thiamine, riboflavin, biotin, folic acid, vitamin C (Al-Farsi and Lee, 2008), while also contains potassium, calcium, iron, phosphorus, zinc and copper in noticeable quantities (Al-Farsi *et al.*, 2005a; Ali-Mohamed and Khamis, 2004). Additionally, bioactive compounds like phenols, sterols, carotenoids, anthocyanins, flavonoids and procyanidins are also reported in different date varieties in varying proportions (Allaith, 2008; Al-Farsi *et al.*, 2005b).

In present days, the medicinal plants have drawn much attention for prevention and cure of several ailments due to high degree of adverse events and increased risk of resistance to pathogens. Thus natural and plant derived

medicine offers great advantage in the therapeutic treatment of several infectious diseases (Nagesh and Shanthamma, 2009). They are generally cheaper and got greater acceptance in general population in comparison to modern medicines (Patra, 2012; Shakiba *et al.*, 2011).

Cancers or neoplasms are caused by genetic mutation of normal cells that deregulates the fine balance between apoptosis and cell division (Valastyan and Weinberg, 2011). Tumors are formed due to genetic alterations changing normal cells to cancer cells that leads to uncontrolled proliferation (Bray *et al.*, 2014).The modern anticancer drugs not only targets the cancerous cell but also destroy rapidly growing normal cells like bone marrow stem cells, hair follicles and growing fetus. However natural bioactive compounds give us chance to explore novel anticancer agents that may destroy only cancerous cells without harming normal cells.

Inflammation is natural reaction in response of tissue injury that starts with leukocytes activation. It replenish the damages caused exogenous and endogenous agents (Markiewski and Lambris, 2007; Hawiger and Zienkiewicz, 2019). Oxidative stress is one of the major causes that ultimately results in inflammation. The inflammatory response tries to neutralize the stimulus and favors healing of injured tissue by reconstruction to maintain homeostasis (Tepole and Kuhl, 2013). On the other hand, certain diseases are caused due to chronic inflammation like rheumatoid arthritis, hypersensitivity, diabetes mellitus, allergies, obesity, atherosclerosis, and certain cancers (Scriver *et al.*, 2011).

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Purpose of the study

Recently several date fruit varieties all around the world were evaluated for their antioxidant, anti-hyperlipidemic and anti-inflammatory potentials however no preclinical or clinical data is available on the bioactivity of Pakistani date varieties. Thus, present study was conducted to evaluate antibacterial, anti-cancer, antioxidant and anti-inflammatory potential of aqueous and ethanol extracts of *Aseel* dates.

MATERIALS AND METHODS

Identification of *Aseel* dates

Aseel dates (fresh) were obtained from local market of Khairpur (Sindh) identified and voucher specimen saved in Plant Conservation Center, Department of Botany, University of Karachi under Herbarium no G.H# 92189.

Preparation of extracts

Date fruit aqueous extract (DFAE) was prepared by mixing 100 g date without seeds in 500 mL distilled water and left for two days with intermittent shaking. After that mixture was subjected to filtration. Rotary evaporation was carried out in order to obtain concentrated crude aqueous extract. The obtained extract was reconstituted with 3%DMSO and left in the freezer at -4°C for further use. DFEE (Date fruit ethanol extract) was also prepared in similar fashion by replacing ethanol in place of distilled water.

Antibacterial assay

Antibacterial activity for DFEE and DFAE was assessed through 96 well plate technique used by Petit and Sarkar (Sarkar *et al* 2007; Pettit *et al* 2005; Tyc, *et al* 2016). To cultivate bacterial strains of *E. coli*, *S. aureus*, *P. aeruginosa* and *S. flexenari*, Mueller Hinton medium was utilized keeping McFarland turbidity index = 0.5. DFEE and DFAE stock solutions were prepared in DMSO in the ratio of 1:1. The media was added to all wells in triplicate fashion followed by extracts addition only excluding control wells that don't contain test sample. The final volume was makeup to 200 μ L in all wells. Bacterial cells in the strength of 5×10^6 were delivered subsequently in each test and control wells. Parafilm sealed plates was then kept for 18-20 hours in incubator. After adding Alamar Blue Dye the plates were shaken at 80 RPM in a shaking incubator for 120-180 min. Blue color transformation to red was the indication of bacterial growth. In the end, the antibacterial activity was estimated by taking the final absorbance at 570nm and 600nm on ELISA reader. The authentication numbers of bacterial strains used are as follows:

S. No.	Bacterial strains	Authentication numbers
1.	<i>P. aeruginosa</i>	NCTC 10662
2.	<i>S. flexenari</i>	ATCC 12022
3.	<i>E. coli</i>	ATCC 2599
4.	<i>S. aureus</i>	NCTC 6571

Brine shrimp lethality assay

DFEE and DFAE were assayed for their *In-vitro* LD₅₀ by *Artemia salina* (Brine shrimp) Lethality test. It is quick and cost-effective method for identification of bioactive and toxic natural products (Carballo *et al.*, 2002; Mayer *et al.*, 1982; Madjos and Luceño, 2019). Previously filtered Brine solution having brine shrimp eggs (50mg) was spread over hatching tray already half filled with water. The tray was then incubated at 37°C. These conditions provide excellent environment to produce enough quantity of larvae in just 48 hours. 20mg of DFEE and DFAE were incorporated in 2 mL solvents, from which 5, 50 and 500 μ L volume was added to 3 vials per concentration and finally the volume was made up to 10, 100 and 1000 μ g/mL respectively. Two days were given for hatching of larvae and transformation to mature nauplii stage. The solvent was then evaporated overnight and with Pasteur pipette 10 larvae/ vials were inoculated. The final volume of vials were made up to 5mL with seawater and placed in illuminated incubator for 24 h at room temperature. Etoposide was used as a reference cytotoxic drug and added to standard control vials along with solvent. Finney computer software was analyze and calculate LD₅₀ values with 95% confidence interval.

Anticancer assay MDA-MB-231 and MCF-7

Anticancer potential of DFEE and DFAE were analyzed by MTT bioassay to estimate living or dividing cancer cells quantitatively. The principle of the technique was the reduction of MTT (3-(4, 5-dimethylthiazol-yl)-2,5-diphenyl tetrazolium bromide) which is a tetrazolium salt. The reaction was carried out using different dehydrogenase enzymes of living origin. MTT tetrazolium ring was supposed to be broken down in active mitochondria which resulted in the formation of formazan crystals that appeared as purple color. DMSO was used to dissolve these crystals and ELISA reader was used to read optical density of these colored crystals (Scudiere *et al.*, 1988).

Fetal bovine serum 10% (FBS) along with Dulbecco's modified Eagle medium was utilized to culture cancer cell lines (MCF-7 and MDA-MB-231) in 75mL container and left in 5% CO₂ incubator at 37°C. These cancer cells were then harvested in 96-bored tissue culture plate in the seeding density of 8,000 and 10,000 cells/well for MCF-7 (ATCC#HTB-22) and MDA-MB-231 (ATCC#HTB-26) respectively in the medium (100 μ L). Next day, DFAE and DFEE were poured in triplicate in concentration of 50 μ M, and kept in incubator for 2 days followed by addition of 200 μ L MTT at 0.5mg/mL in each well and re-incubated at 37°C for 3 hours. Formazan crystals thus formed were dissolved in 100 μ L DMSO and absorbance was analyzed using micro-plate reader (Spectra Max plus, Molecular Devices, CA, USA) at 570nm. The standard drug used was doxorubicin and the percent inhibition (decrease in viable cells) was calculated by equation 1.

$$\text{Percent inhibition} = 100 - \frac{(\text{Mean O.D of test drug} - \text{mean O.D of negative control})}{(\text{Mean O.D of standard} - \text{mean O.D of negative control})} \times 100 \quad \text{eq 1}$$

Where O.D = optical density

Anticancer assay 3T3, PC3 and Hela cell line

Cytotoxicity and anticancer potential of DFEE and DFAE was assessed on breast, prostate and cervical cancers in 96-well micro-plates by standardized MTT colorimetric assay (Mosmann, 1983). 3T3, mouse fibroblast (ATCC#CRL-1658) and PC3, Prostrate Cancer cells (ATCC#CRL-1435) were harvested in the mixture of Dulbecco's Modified Eagle Medium, FBS5%, streptomycin (100µg/mL) and penicillin (100 IU/mL) in the flasks of 75mL capacity. It was then placed at 37°C in CO₂ incubator. However Hela cells that represent cervical cancer were cultivated in Minimum Essential Medium Eagle, added with the same components and kept in the same conditions as above. Cells exhibiting rapid exponential proliferative pattern were thus harvested and counted with the help of hemocytometer. Dilution with respective medium were made and cells in the concentration of 5x10⁴ cells/mL was obtained and poured into 96-well plates in the concentration of 100 µL/well.

This overnight incubation was followed by removal of used medium and addition of 200µL of fresh medium with different strengths of test compounds (1-30µM). Rest of the procedure is same as followed in above MTT assay. The anticancer potential was recorded in terms IC₅₀ for 3T3, PC3 and Hela cells and calculated by same equation 1.

In-vitro anti-inflammatory assay

Anti-inflammatory activity of DFEE and DFAE was assessed through *in-vitro* luminol-enhanced chemiluminescence assay (Helfand *et al.*, 1982). 25 µL of whole blood diluted in HBSS⁺⁺ (Hanks Balanced Salt Solution; Sigma, St. Louis, USA) was put to incubator after addition of 25 µL of three different concentrations of DFAE and DFEE (1, 10 and 100µg/mL), in triplicate fashion. Control contains HBSS⁺⁺ and cells without test extracts. The test was carried out in white half area of plates (Costar, NY, USA) followed by incubation for 15 minutes at 37 °C in the thermostat chamber of luminometer (LabSystems, Helsinki, Finland). 25µL of SOZ (serum opsonized zymosan) (Fluka, Buchs, Switzerland) and intracellular ROS detecting probe (25 µL) and luminol (Research Organics, Cleveland, OH, USA) were thus poured into each well, except controls (containing only HBSS⁺⁺). The levels of ROS were than recorded in lumino meter in term of RLU (relative light units). Ibuprofen was used as standard drug in this bioassay with IC₅₀ value= 11.2±1.9.

Antioxidant assay

The antioxidant potential of DFEE and DFAE was assessed through DPPH (2, 2-diphenyl-1-picryl hydrazyl) radical scavenging assay. This is a rapid and cheap assay employed for the assessment of antioxidant potential of crude compounds. The compounds having antioxidant potential have capability to scavenge free radicals. DPPH utilization by tested compound is a good indicator of their antioxidant property (Uddin *et al.*, 2011). DFAE and DFEE were solubilized in 100% DMSO whereas 300 µM solution of DPPH was prepared by dissolving in 100% ethanol. 5µL of test sample was then poured to 96 plate and read at 515nm followed by addition of DPPH solution to make up the final volume to 100µL in each well. The plate was then covered with parafilm and was kept in incubator for 30 minutes at 37°C. Microplate reader was used to read the absorbance at 515 nm. 100% DMSO and Gallic acid /N-acetyl cysteine were used as the control and standard respectively. The % RSA was estimated by following formula:

$$\% \text{ RSA} = 100 - \frac{\text{Optical. Density of sample}}{\text{Optical Density of control}} \times 100$$

Where

% RSA= percent radical scavenging activity

STATISTICAL ANALYSIS

Values are presented as mean ± SD and data was analyzed statistically by using IBM SPSS Statistics (version 20) with P≤0.05 was considered to be significant.

RESULTS

Antibacterial assay

Table 1 reveals the antibacterial potential of DFAE and DFEE against standard antibiotic ofloxacin and negative control in the concentration of 150µg/mL. Both extracts revealed slight antibacterial potential against *S. aureus*, *E. coli* and *S. flexenari*, however no activity was exhibited against *P. aeruginosa* by the both extracts.

In DFEE maximum growth inhibition (34.27%) was seen against *S. flexenari* followed by *E. coli* (27.20%) and then *S. aureus* (22.26%). In DFAE, the growth inhibition was (24.21%) against *S. aureus* followed by *S. flexenari* (17.27%) and *E. coli* (16.72%).

Brine shrimp lethality assay

Table 2 depicts in-vitro LD₅₀ for DFAE and DFEE through brine shrimp lethality assay, both of the extracts did not revealed cytotoxic potential at 10, 100 and 1000 µg/mL concentrations, however standard cytotoxic drug etoposide revealed LD₅₀ value of 7.46µg/mL in the period of 24 hours.

Table 1: Antibacterial activity of DFAE and DFEE using Alamar blue Assay

% inhibition	<i>E. coli</i>	<i>S. flexenari</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>
DFAE	16.72	17.27	24.21	0.0
DFEE	27.2	34.27	22.26	0.0
-ve control	0.0	0.0	0.0	0.0
Ofloxacin	84.75	85.25	88.7	82.46

n=3, DFAE: date fruit aqueous extract, DFEE: date fruit ethanol extract, Concentration of extracts= 150µg/mL, Negative control = water

Table 2: Brine shrimp lethality assay of aqueous and ethanol extracts of *P. dactylifera*

Concentration µg/mL	No. of larvae/ extract	survivors in DFAE	Survivors in DFEE	LD ₅₀ µg/mL	Standard cytotoxic drug	LD ₅₀ Etoposide µg/mL
10	30	29	29	-	Etoposide	7.46
100	30	29	27	-		
1000	30	27	27	-		

n=3, DFAE: date fruit aqueous extract, DFEE: date fruit ethanol extract

Table 3: MB-231 and MCF-7 anticancer-assay of DFAE and DFEE

Concentration (µg/mL)	DFAE	DFEE	Doxorubicin
	50	50	50
% inhibition MDA-MB-231	30.86	20.90	89.19
% inhibition MCF-7	20.46	17.59	89.59
IC ₅₀ ±SD	-	-	0.92±0.1

n=3, DFAE: date fruit aqueous extract, DFEE: date fruit ethanol extract

Table 4: Anticancer 3T3, PC3 and Hela assay of extracts of *P. dactylifera*

Concentration (µg/mL)	Tested drugs			
	DFAE	DFEE	Doxorubicin	Cyclohexinide
	50	50	50	50
% inhibition 3T3	29.70	22.35	N.A	65.95
% inhibition Hela	16.40	36.70	96.20	N.A
% inhibition PC3	15.21	37.60	99.17	N.A
IC ₅₀ ±SD	-	-	0.19±0.03Hela 0.31±0.03 PC3	0.61± 0.17 3T3

n=3, DFAE: date fruit aqueous extract, DFEE: date fruit ethanol extract

Table 5: *In-vitro* anti-inflammatory assay of aqueous and ethanol extracts of *P. dactylifera*

Drugs	Concentration µg/mL	% inhibition	IC ₅₀ ± SD
DFAE	25	21.40	-
DFEE	25	31.40	-
Ibuprofen	25	73.42	11.20 ± 1.90

n=3, DFAE: date fruit aqueous extract, DFEE: date fruit ethanol extract

Table 6: DPPH RSA antioxidant assay of DFAE and DFEE

Drugs	DFAE	DFEE	Gallic acid	N-acetyl cysteine
Concentration µM	500	500	500	500
IC ₅₀ ±S.E.M	-	-	23.44 ±0.43	111.44±0.70
% RSA	23.96	35.88	93.93	95.95

n=3, DFAE: date fruit aqueous extract, DFEE: date fruit ethanol extract, RSA: Radical scavenging activity

Anticancer assay MDA- MB-231 and MCF-7 bioassay

The anticancer activity of DFAE and DFEE is represented in table 3 against the cell lines *MCF-7* and *MDA-MB-231*. Both the extracts revealed weak anticancer potential as compared to control at the concentration of 50µg/mL, the percent inhibition value was 30.86% and 20.90% for DFAE and DFEE respectively when assessed against MDA-MB-231 cell line. The percent inhibition value were 20.46% and 17.59% when assessed against MCF-7 cancer cell lines for DFAE and DFEE respectively. However standard drug doxorubicin was revealed 89.19 % inhibition for MDA-MB-231 and 89.59% inhibition for MCF-7.

Anticancer assay 3T3, PC3 and Hela cell line

The anticancer activity of DFAE and DFEE was assessed against 3T3, Hela and PC3 cancer cells and is depicted in table 4. Both the extracts showed 29.70 and 22.35% inhibition against 3T3 cancer cells at 50µg/mL as compared to 65.95% inhibition by cyclohexinide. Whereas% inhibition against Hela and PC3 cancer cells by DFEE was 36.7% and 37.6% as compared to 96.2% and 99.17 % by doxorubicin at 50µg/mL.

Anti-inflammatory assay

Table 5 shows *in-vitro* anti-inflammatory activity results of DFAE and DFEE through Luminol-enhanced chemiluminescence assay. Both extracts demonstrate considerable anti-inflammatory activity but the inhibitory response was less than 50%, however inhibitory response of ibuprofen was 73.42%. The inhibitory response of DFAE was 21.40% and DFEE was 31.40%.

Antioxidant activity

Table 6 revealed antioxidant potential of both the date extracts through DPHH free radical scavenging bioassay. The anti-oxidation potential of both extracts was 23.96% and 35.88%. Both DFAE and DFEE exhibited 23.96% and 35.88% anti-oxidation potential as compared to 93.93% and 95.95% by gallic acid and N- acetyl cysteine respectively.

DISCUSSION

Various bioactive substances obtained from plants are reported to bear pharmacological potential (Gurib-Fakim, 2006). Thousands of higher plants exist on the earth but a very little fraction has been tested for their medicinal use. Plants are known to have various secondary metabolites such as terpenes, flavonoids, alkaloids and glycosides that have important role in protecting them from animals and other parasitic plant growth. These compounds present in plants for their intrinsic safety could be highly advantageous if utilized for therapeutic options (Da Rocha et al., 2001) in the treatment of different ailments hence leading to the development of new drugs from plant source.

Date palm scientifically known as *Phoenix dactylifera* is one of the earliest trees planted by humans. It is well established and has excellent dietary potential but there is lack of scientific studies to evaluate its medicinal potential. Thus extensive studies are required to establish its advantages among health care professionals and general population, since exploration of biological activity will facilitate the therapeutic use of dates (Ahmed et al., 2017). Hence present study is an indication to validate its use as antimicrobial, cytotoxic, anticancer, antioxidant and anti-inflammatory agent.

The aqueous extract of *P. dactylifera* showed maximum susceptibility against *S. aureus* followed by the strains of *S. flexenari* and *E. coli*, while its ethanol extracts again showed maximum susceptibility against *S. flexenari* and to a lesser extent on *E. coli* and *S. aureus*, however both the extracts fails to produce inhibition against *P. aeruginosa*.

The results of the present study clearly depicts that DFEE and DFAE have tendency to inhibit both gram positive and negative bacteria but the inhibition was not pronounced. This is in contrast to some previous studies that reported highly significant antibacterial potential of alcoholic extracts of date varieties against a wide variety of pathogens (Selim et al., 2012; Al-Daihan and Bhat, 2012;). This may be due to decrease concentration of antibacterial component in *Aseel* date or may be some other solvent with different polarity is required to concentrate that antibacterial moiety.

Safety evaluation of herbal products is vital before the prophylactic, therapeutic or nutritional use medicinal compounds (Parra et al., 2001; Cáceres, 1996). Brine shrimp lethality (*In-vitro* LD₅₀) is relatively newer technique to judge the cytotoxic and bioactive ability of natural and synthetic compounds. Hence, both aqueous and ethanol extracts of *Aseel* dates were put to test. Results revealed absence of cytotoxicity in both extracts at a concentration of 10, 100 and 1000µg/mL against Etoposide (standard cytotoxic drug). These observations are in accordance with previously conducted *in-vivo* oral toxicity of *Aseel* dates that revealed no animal death within 24 hours even at the dose of 5000mg/kg. Hence his study provides strength to the safety profile of tested date specie (Agbon et al., 2014).

Carcinogenesis is one of the leading global health concerns and cancer cases are projected to rise from 14 million new cases in 2012 to 24 million in the year 2035 (Stewart et al, 2016). Vincristine, vinblastine, paclitaxel, docetaxel, irinotecan, topotecan, and etoposide are all herbal derived frequently used drugs available for treatment of cancers (Shoeb, 2006). Anticancer activity of the date extracts were screened by using *MCF-7*, *MDA-MB-231*, *PC3* and *3T3* and *Hela* cancer cell lines.

MCF-7 cell and MDA-MB-231 cancer cell lines were meant for assessing activity against breast carcinomas (Pozo-Guisado *et al.*, 2002). Both extracts showed anti-carcinogenic activity at 50 µg/mL. DFAE revealed 20.46% and 30.86% growth inhibition against MCF-7 and MDA-MB-231 respectively, however DFEE revealed 17.59% and 20.90% against MCF-7 and MDA-MB-231 respectively.

DFAE and DFEE were also checked against PC3, 3T3 and Hela cell lines. PC3 demonstrate prostate cancers, 3T3 represent mouse fibroblast and Hela cells represents cervical cancers respectively (Mosmann; 1983). Though previous studies were suggestive of anticancer potential of dates but *Aseel* date extracts did not reveal significant activity against above cancer cell lines attested 50 µg/mL concentration. Perhaps more concentration was required to produce significant anticancer activity. Ishurd and Kennedy in a study in 2005 claimed anti-cancer effects of glucans extracted from date fruit in preclinical studies. The present data gives some possibility of active anticancer activity in date fruit but there is a need to use different solvents to isolate secondary metabolites having significant cancer cell inhibition.

In-vitro anti-inflammatory studies exhibited insignificant results in comparison with ibuprofen; although recent *in-vivo* study revealed significant anti-inflammatory potential of *Aseel* dates in carrageenan induced paw edema. DPPH antioxidant assay also exhibited insignificant activity in both the extracts. Previous preclinical studies demonstrated that bioactive compounds like *p*-coumaric acid present in date fruit enhance antioxidant enzymes producing gene expression in rats (Ferguson *et al.*, 2004; Yeh *et al.*, 2009). In short it may be understood that anti-inflammatory and antioxidant potential of date fruit might depend on the presence of bioactive compounds like procyanidins, flavonoid, anthocyanins, glycosides and selenium that possess free radical scavenging activity hence resists oxidation process. Further investigation for analysis and separation of active ingredients in *Aseel* dates should be done for better understanding of activity and further development of new natural drugs.

CONCLUSION

The outcomes of current study reveals that fresh *Aseel* dates might have several medical effects since *in-vitro* analysis of ethanol and aqueous extracts have showed substantial anticancer, antioxidant, anti-inflammatory and antibacterial responses however additional preclinical and clinical studies using other solvents for extraction may be carried out to evaluate dose dependent responses. It is also imperative that brine shrimp lethality assay did not reveal any cytotoxicity however revealed selective anti-cancer activity targeting cancerous cells without damaging normal cells.

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