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Effects of Al-Zahdi date palm seed extract on the AKT and PTEN genes expression in testicular tissues of mature and immature albino rats

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Abstract

This work was dedicated to the influence of oily and macerated Al-Zahdi date palm seed extracts (DPSE) on AKT and PTEN genes expression in testicular tissues and their relation to apoptosis. Sixty (30 mature and 30 immature) male albino rats were used for this study after being divided into six subgroups for each. Two subgroups were daily dosed with oily DPSE using two doses (200 and 400 mg/kg body weight) orally via micropipette over two successive months. Similarly, the other two subgroups were dosed with macerated DPSE, while the control received tap water. Expression of AKT and PTEN genes extracted from testis tissue was detected using the Preb Total RNA Extraction Kit. The Bcl-2 marker was tested using immunohistochemical staining to evaluate the incidence and spreading of the anti-apoptotic protein within the testis. Results revealed a significantly high expression of mRNA associated with both studied genes of both mature and immature rats treated with oily and macerated DPSE in comparison to control, with significant elevation of AKT level in oily treated rats, particularly with 4 hundred doses. Immunohistochemical results corroborated the genetic results as demonstrating a distinct presence of anti-apoptotic Bcl-2 in testicular cells, with greater clarity observed in mature testis, especially those treated with 400 mg/kg B. W. The study concluded that date palm seed extract acts as a natural stimulator for AKT in testicular tissue, which in turn contributes to prolonging the lifespan of testicular cells. Bcl-2 expression is present in testicular cells, with greater evidence in mature ones that received 400 mg/Kg.B.W.

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Introduction

Testes are the basic organs of the male reproductive system that are responsible for performing vital tasks, especially secreting sex hormones (1). The male infertility disorder is highly associated with oxidative stress and free radicals that affect spermatozoa, causing injury of nucleic acids, loss of motility, and even reduction in its fusion capacity. These oxidative factors, reactive oxygen species, and free radicles disturb plasma membrane fluidity and DNA integrity through oxidation and peroxidation (2). Consequently, the use of antioxidant materials to regulate, balance, and prevent male infertility has drawn much attention (3). AKT is an activator of survival and proliferation pathways, while PTEN acts as a negative regulator of AKT by dephosphorylating phosphoinositides, which accordingly inhibits the signaling pathway of PI3K/AKT. So, an increase in AKT typically causes a decrease in PTEN expression, While DPSE PTEN negatively adjusts immature Sertoli cell production, spermatogenic cell proliferation, and testis size by impeding the PI3K/AKT signaling. In contrast, the PI3K/AKT signaling pathway can enable propagation and anti-apoptosis within immature Sertoli (4,5). The

(AKT/PTEN) pathway itself is involved in numerous phases of male reproduction, including the regulation of the hypothalamus-pituitary-gonad axis during spermatogenesis, the multiplication and differentiation of the spermatogonia, as well as the direction of the sperm autophagy and testicular-endocrine functions (1). Additionally, the AKT/PTEN signaling pathway further directly or indirectly affects spermatogenesis in the male testis in completely different ways, depending on whether it is mature or immature (5). Anti-apoptotic family protein expression is a very common indicator that is widely used to measure the condition of various cells, whether dead or alive (6,7). Furthermore, other researchers proved the anticancer activity of the Bcl-2 family in their biological and phytochemical evaluation of two different types of date fruit (8,9). The natural antioxidants present in some plants are very important in improving the content of unsaturated fatty acids in ruminants' meat; therefore, the food industries should use these constituents in different products to enrich food quality and diet value, add synthetic antioxidants and delay lipid oxidation (10,11). Phytomedicine is a strategy that emphasizes the chemical substances naturally present in plants to improve health conditions by managing, preventing, and treating several diseases. Herbal phenolics are normal antioxidant agents that hold electrons that form relatively constant phenoxy radicals and subsequently interfere with the redox reactions inside the cells (12). Polyphenolic compounds in plants often interact with various signaling molecules and can alter gene expression (13). These compounds could activate or inhibit certain kinases or transcription factors that lead to the expression of AKT and PTEN (14). Date palm seeds consume about 10% of the total fruit's weight, and they are usually discarded. Nevertheless, current research shows that the seeds and their oil could be considered as a new good basis for diverse elements, especially phenolic and dietary fibers, many minerals other than natural antioxidants, and unsaturated and saturated fatty acids (15-17). The balance between AKT and PTEN is critical for normal cellular function, and an imbalance can lead to diseases such as cancer, diabetes, or neurodegenerative disorders (18). The increased AKT activity would lessen the percentage of cell apoptosis, raise the sperms' count, and improve sperms' motility (19). The apoptosis process is essential for maintaining the balance between germ cells and sustentacular Sertoli cells by eliminating the abnormal and damaged germ cells during their life (20). Uncontrolled or excessive apoptosis in male germ cells can lead to infertility (21-23). AKT signaling could be activated to stimulate the proliferation of immature porcine Sertoli cells and consequently inhibit their apoptosis by suppressing the PTEN gene (24,25). Sertoli cells' proliferation and apoptosis amount directly to their final number, which determines the seminiferous epithelial structure and spermatogenesis progress (5,26,27).

The benefits of date seeds, especially their fatty acids and antioxidant activity, are highly recommended for more advanced procedures, including molecular and biomarker analysis, to obtain more precise data. Thus, the present work was planned to study the influence of oily and macerated extracts of Zahdi DPS and their effects on the testicular tissue, levels of AKT, PTEN genes, and the apoptotic phenomena accordingly.

Materials and methods

Ethical approve

This study was permitted by (ACUC) of the Faculty of Vet. Med. of Baghdad University, under P.G./1354, dated 24/7/2024.

Animal model and housing

Healthy male rats of *Rattus norvegicus* species were purchased from Mosul University/animal breading house and acclimatized under controlled conditions for two weeks before the experiment, then kept in the same conditions throughout the experiment, including a well-ventilated and cleaned room at 20-25ºC temperature 30-70% humidity with 12 hrs. dark/light cycle. Each 5 rats were kept collectively in one plastic cage (5*15.5*10.5 inch). Commercial pellets fed and fresh tap water were available ad libitum (28-30).

Experimental design

Sixty male albino rats (*Rattus norvegicus*) half of them were immature (30 days old), and the other half were mature (75 days old), and they were used for this experiment. All studied groups were submitted to the same living conditions. The animals were equally divided into two primary groups, 30 rats for each; these primary groups represent the main two groups of this experiment in which an oily DPSE was orally given via micropipette as the first primary group (group 1), While the second primary group (group 2), macerated DPSE was orally given via micropipette. Then, these two main groups (N=30) were further divided into two secondary groups of immature (a) and mature (b) of 15 rats for each. Then, these secondary groups ((N=15) were extra divided into 3 subgroups of 5 rats for each (N=5). Later, the first subgroups from both secondary groups, which represent the control groups, were given oral tap water for two months on a daily basis. The second subgroups were orally administered 200 mg/kg of DPSE; meanwhile, the third subgroups were dosed orally with 400 mg/kg of DPSE. The rats were euthanized after 60 days of DPSE administration and then dissected; testes were taken using sterile tools under sterile conditions. The size of testes samples for the molecular study was 60-70 mg weight, taken from the right testis, using sterile sharp scissors; the remaining part was fixed with the left testis in 10% neutral buffered formalin for 72 hours and then

dehydrated with gradual ethanol concentrations and cleared by xylene, finally infiltrated then embedded within paraffin. Sections of 5 µm were prepared and stained with immunohistochemical staining using Bcl-2 for anti-apoptotic protein demonstration (31-33).

Preparation of date palm seed extract

Al-Zahdi date fruits were purchased from local markets and taxonomies. The date seeds peeled off from their fleshy coat manually were washed, cleaned, and dried for 10-15 days, grinding with an electrical grinder; then, the extraction process was achieved using two procedures, maceration and Soxhlet extraction using petroleum ether 40-60 solvent for each (34-36).

RNA extraction

RNA was extracted from testicular tissue using Preb Total RNA Extraction Kit/Addbio Inc company/Korea. Its procedure is based on total RNA extraction via the application of a specific RNA binding spin column procedure using distinct buffer solutions and an on-column. DNase handling to exclude DNA traces throughout the RNA extraction procedure was applied. The cDNA was synthesized with AddScript RT Master (Addbio, Korea). Temporarily, the reaction mixture was prepared in a total volume of 20 µl (10 µl AddScript RT Master, 7 µl Nuclease-Free D.W, 3 µl Extracted RNA). After mixture preparation, the PCR tubes were placed in the Thermocycler (Biorad, USA), and the program was set (Table 1). qPCR was performed to measure the expression of the AKT and PTEN genes using specific primers listed in Table 2. Briefly, the total reaction volume was 20 µl, as 10 µl Syber green, 1 µl both forward and reversed primers, 2 µl cDNA templet, and finally, 6 µl PCR water). The qPCR cycling conditions were set using QuantStudio™ 5 Real-Time PCR System (Thermofisher, USA), as presented in Table 3. After amplification was completed, the expression data was saved for the later Ct value method (37,38).

Table 1: Temperature cycling for cDNA synthesis

Step	Temp °C	Time
Priming	25	10 min
Transcription Reverse	60	60 min
RT inactivation	80	5 min
Hold	12	∞

Immunohistochemical protocol

The Bcl-2 marker was identified through immunohistochemical staining using FLEX Monoclonal Mouse Anti-Human Oncoprotein, Clone 124, Ready-to-Use (Link), Code IR614, in combination with the Dako EnVision FLEX detection system and Auto-stainer Link equipment**.** The numerous cell types were recognized according to the external appearance of these cells throughout the series of divisions, the cells' sizes, and the nuclei shapes (39).

Table 2: Primer sequences used for qRT-PCR

Primer name	Primer Sequence 5' – 3'
PTEN-F	CAATGTTCAGTGGCGGAACTT
PTEN-R	GGCAATGGCTGAGGGAACT
Akt1-F	CTCATTCCAGACCCACGAC
Akt1-R	ACAGCCCGAAGTCCGTTA
β-actin-F	GGAGATTACTGCCCTGGCTCCTA
β-actin-R	GACTCATCGTACTCCTGCTTGCTG

Table 3: Cycling conditions of qRT-PCR

Step	Temp °C	Time	Cycle
Polymerase activation	95	10 min	1X
Denature	95	45 sec	
Annealing	*60	45 sec	35X
Extension	72	1 min	
Melting analysis	60	1 min	1X
Dissociation	90	0.01	1X
Hold	10	2.0	∞

*An annealing temperature of 60ºC was used for all genes.

Statistical analysis

The statistically significant differences among the subgroups were determined using one-way ANOVA. The data is presented as Mean±SEM, highlighting the significant differences among the subgroups at $P<0.0001$ (40).

Results

The results indicated that the expression of AKT and PTEN genes were significantly evaluated in immature male rats treated with both oily and macerated DPSE, using both doses (200 mg/kg B.W. and 400 mg/kg B.W.) corresponding to control animals. The real-time qPCR assay revealed that the testes tissue appeared to have a high expression of the AKT gene in the oily DPSE groups, with significant ($P<0.0001$) increased to 2.41±0.10 in the subgroup that treated with 200 mg/kg B.W., whereas increased to 3.64±0.07 in subgroup treated with 400 mg/kg B.W., in comparison with the control subgroup, where AKT expression level was 0.59±0.04. In addition, the real-time qPCR assay showed that the PTEN gene expression level in the same treated groups was significantly ($P<0.0001$) raised to 1.30±0.11 in the subgroup treated with 200 mg/kg, whereas it raised to 2.12±0.08 in subgroup treated with 400 mg/kg, in comparison to the control subgroup, where the PTEN expression level was 0.55±0.02 (Figure 1).

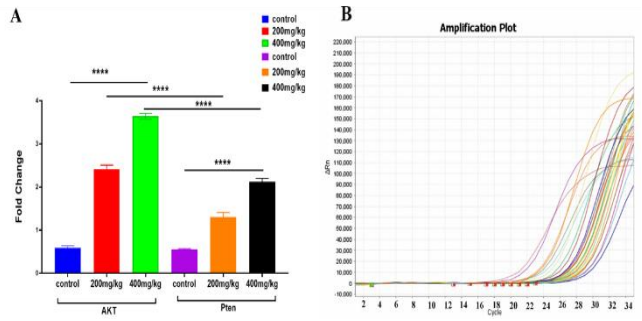

Figure 1: The qRT-PCR validation for the mRNA expression of AKT and PTEN genes in the testis of immature rat subgroups treated with oily DPSE. The significant differences among the subgroups are exposed with the asterisks (****P<0.0001) (A). The qRT-PCR amplification curve for mRNA expression of AKT and PTEN genes in testes ofoilyDPSE–treated immature rats of all subgroups (B).

While in subgroups that got macerated DPSE, the real-time qPCR assay results showed that the AKT gene seemed to express itself at varying levels. In comparison to the other subgroups, AKT gene level was significantly (P<0.0001) higher to 1.94±0.10 in the subgroup treated with 400 mg/kg B.W., and 3.22±0.22 in the subgroup treated with 200 mg/kg B.W. when compared with the control subgroup, where AKT expression level was 0.61±0.05. However, the PTEN gene expression level was considerably elevated to 1.40±0.04 in the subgroup treated with 200 mg/kg B.W., and to 1.92±0.05 in the group treated with 400 mg/kg B.W.in comparable to the control group 0.47±0.06 (Figure 2).

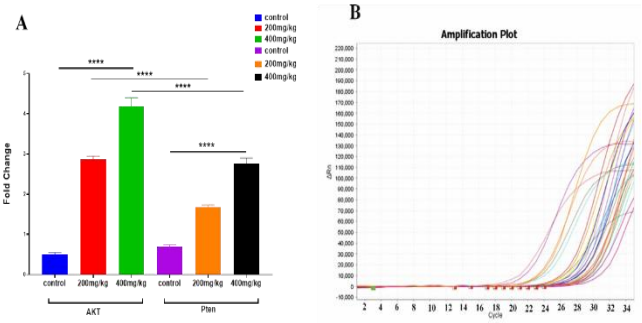

Figure 2: The qRT-PCR validation for the mRNA expression of AKT and PTEN genes in the testis of immature rat subgroups treated with macerated DPSE. The significant differences among the subgroups are exposed with the asterisks (*P<0.05, ****P<0.0001) (A). The qRT-PCR amplification curve for the mRNA expression of AKT and PTEN genes in testes of macerated DPSE-treated immature rats of all subgroups (B).

Meanwhile, the mature male rats given the same dosage of oily and macerated DPSE had varying expression levels of the AKT and PTEN genes in comparison to the control subgroup. The AKT gene expression levels in both subgroups that received oily DPSE were 2.88±0.07 and 4.19±0.22 in subgroups treated with 200 and 400 mg/kg B.W., respectively. They were significantly rise compared to the control subgroup of 0.52±0.03. Furthermore, both treated subgroups with oily DPSE had significantly higher PTEN gene expression levels, 1.69±0.04 and 2.77±0.13 in subgroups treated with 200 and 400 mg/kg B.W., respectively, in comparison to the control subgroup level 0.70±0.04 (Figure 3).

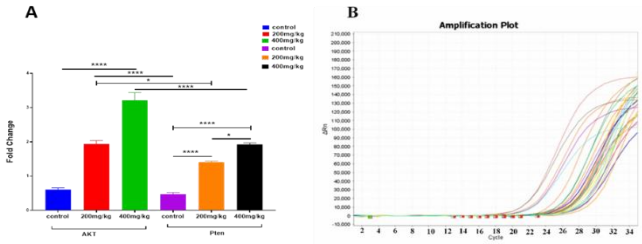

Figure 3: The qRT-PCR validation for the mRNA expression of AKT and PTEN genes in the testis of all mature rat subgroups treated with oily DSPE. The significant differences among the subgroups are exposed with the asterisks (****P<0.0001) (A). The qRT-PCR amplification curve for mRNA expression of AKT and PTEN in testes of oily DPSE-treated mature rats of all subgroups (B).

The AKT gene was also found to express itself at variable degrees in subgroups that obtained macerated therapy; it showed 2.22±0.13 and 3.45±0.07 in subgroups treated with 200 and 400 mg/kg B. W., respectively, when compared to level 0.43±0.07 of control subgroup. Additionally, the real-time PCR experiment revealed that the PTEN gene levels were significantly increased in treated subgroups. The gene expression level was 1.71±0.08 and 2.72±0.11 in the subgroups treated with 200 and 400 mg/kg B.W., respectively. Whereas it was 0.62±0.05 in the control subgroup (Figure 4).

Immunohistochemical results

The immunohistochemical staining of the Bcl-2 marker indicated the presence of high expression and localization of it within spermatogenic cells, Leydig and Sertoli cells of the treated group with 200 mg/kg B.W. and 400 mg/kg B.W. of oily DPSE of both immature and mature rats in comparable with control groups. Besides, that expression was much denser in the groups treated with 400 mg/kg B.W. than 200 mg/kg B.W. however, no clear differences were noticed between mature and immature groups (Figure 5).

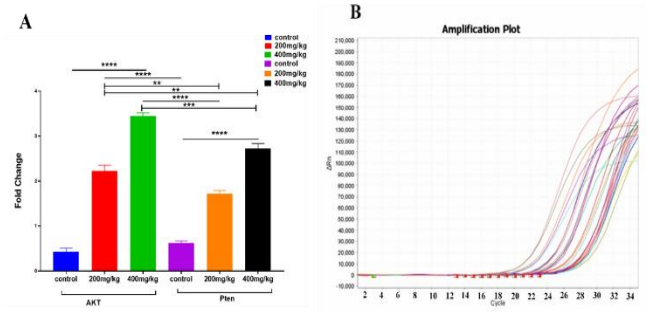

Figure 4: The qRT-PCR validation for the mRNA expression of AKT and PTEN genes in testes of mature rat subgroups treated with macerated DSE. The statistically significant differences among the subgroups are shown with asterisks. (*P>0.05, **P<0.01, ***P<0.001, ****P<0.0001) (A). The qRT-PCR amplification curve for mRNA expression of AKT and PTEN in testes of macerated DPSE-treated mature rats of all subgroups (B).

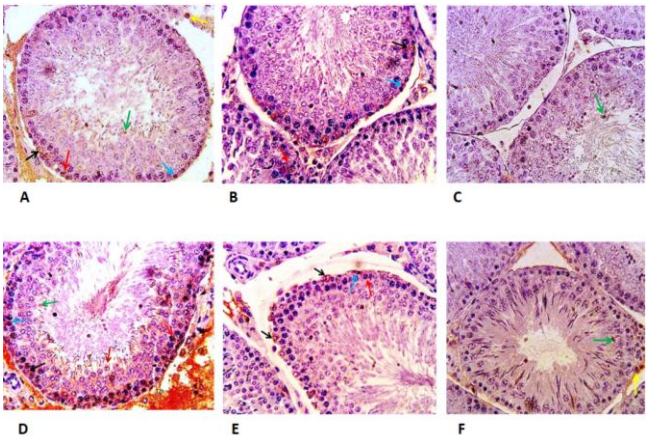

Figure 5: Immunohistochemical section for Bcl-2 marker expression of rat testis treated with oily DPSE of immature rats (top), using 400 mg/kg B.W. (A), 200 mg/kg B.W. (B) and control (C). Mature rats (down) using 400 mg/kg B.W. (D), 200 mg/kg B.W. (E), and control (F), where spermatogonia (a blue arrow), primary spermatocytes (a red arrow), secondary spermatocytes (a brown arrow), spermatid (a green arrow), Sertoli cells (a black arrow) and Leydig cells (a yellow arrow), show the presence of good expression and localization of Bcl-2 marker within both treated subgroups comparable to the control subgroups.

The immunohistochemical staining of the Bcl-2 marker for both groups of macerated DPSE showed the existence of dense localization and expression of marker within spermatogenic cells, Leydig and Sertoli cells of testicular seminiferous tubules treated with 200 and 400 mg / Kg B.W. of both immature and mature rats in comparable with control subgroups. Besides, that expression was denser at 400 mg/kg B.W. than 200 mg/kg B.W.; however, no clear differences were noticed between the mature and immature groups (Figure 6).

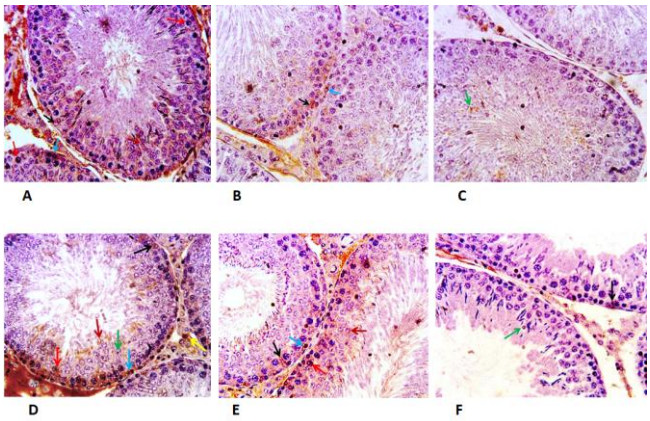

Figure 6: Immunohistochemical section for Bcl-2 marker expression of rat testes treated with macerated DPSE of immature rats (top), using 400 mg/kg B.W.(A), 20 mg/kg B.W (B) and control (C). Mature rats (down), using 400 mg/kg B.W. (D), 200 mg/kg B.W. (E), and control (F). Where spermatogonia (a blue arrow), primary spermatocytes (a red arrow), secondary spermatocytes (a brown arrow), spermatid (a green arrow), Sertoli cells (a black arrow), and Leydig cells (a yellow arrow). Notice the presence of good expression and localization of the Bcl-2 marker within both treated subgroups comparable to the control subgroups.

Discussion

Fortunately, all animal cells, including reproductive cells, have been lucky to have an astonishing monitoring mechanism that strictly regulator their proliferation, differentiation, survival, and even their death and life story through the presence of PI3K/AKT signaling pathway (18,41), and since the balance between AKT and PTEN is critical for normal cellular function, and an imbalance such as too much AKT activation and insufficient PTEN can lead to diseases or disorders (42,43). The investigation of DPSE's role in the AKT/PTEN signaling pathway within testicular tissue since it represents the most critical reproductive organ in males that accomplishes the basic generative functions, particularly sperm production via spermatogenesis, which comprises many molecules plus signaling pathways. The male reproductive cells, as part of that whole animal body, are also affected and regulated by that amazing PI3K/AKT signing pathway either directly or indirectly (5).

The current study results declared that the AKT gene value in male rats treated with 400 mg/kg B.W. of oily and macerated DPSE was significantly higher than the same

gene level in male rats treated with 200 mg/kg B. W. of the same DPSE. These results indicate a dose-dependent increase in gene expression for both PTEN and AKT, with higher levels observed at the 4 hundreds mg/kg treatment compared to 200 mg/kg across both oily and macerated extract forms. This suggests that the treatment significantly ($P<0.0001$) influences both gene expression, potentially in form and dose-dependent manner. These results concurred with the results of the study on female albino rats, administered with daily DPSE for 60 days, with 200 and 400mg/ kg (43). The increased expression of AKT might be a result of enhanced cell survival or growth signaling in response to the DPS extract; this could indicate that the extract has a protective effect on cells or tissues, promoting survival and/or proliferation (44). The germ cells suffering from apoptosis could form the treatment basis of several gonadal syndromes (45). In fact, the date fruit and pits are rich sources of nutritive and protective substances like proteins, fats and oil, vitamins, minerals, dietary fibers, and energy. The phenols, sterols, carotenoids, and anthocyanins are the chief phytonutrients and phytochemicals stated in the date palms (6).

The results of the Immunohistochemistry section for Bcl-2 marker expression within cells show the presence of good expression and localization of Bcl-2 marker within both treated groups comparable to the control groups. That may be due to the fact that date fruit and pits are rich sources of nutritive and protective substances like proteins, fats and oil, vitamins, minerals, dietary fibers, and energy. DPS extract possesses antioxidants like phenols and flavonoids that could enhance cell resilience to stress, leading to an upsurge in the production of the Bcl-2 protein known for safeguarding cells against factors triggering cell death (46). Furthermore, the extract from date palm seeds might boost signals that support cell survival by affecting protein pathways involved in controlling lifespan, like the Bcl-2, BAX pathways enhancement of Bcl-2 levels could indicate the extracts' ability to decrease rates of cell apoptosis. Furthermore, extract from date palm seeds potentially boosts the system or lessens tissue inflammation, resulting in levels of Bcl-2 expression for cell stabilization and decreased cell mortality (47,48). AKT initiatives cell survival over multiple mechanisms: it suppresses pro-apoptotic factors directly, activates transcription factors that favor survival, and boosts the production or function of anti-apoptotic proteins such as Bcl-2; this various regulatory influence highlights AKT's serious role in maintaining the balance between cellular life and death (49).

The date pollen has estradiol, estriol, and estrone, which were documented to improve male subfertility complications via their gonadotrophic action (50). The PI3K/AKT signing way plays crucial monitoring mechanisms throughout spermatogenesis, which can mostly regulate the cell multiplying, survival, and anti-apoptosis aptitude in immature Sertoli cells and cell lines, especially through encouraging spermatogonia multiplication and differentiation, spermatocytes meiosis, sperm maturing, and spermiation (5,18,51)

The study revealed that oily DPSE has a stronger effect than the macerated one, which in turn has a good improving influence; this may be attributed to the high antioxidant value in the oily extract in comparison to macerated extract; this result corresponded with the study that proof the resilient effect of the date palm oil as strong radical scavengers that eliminate free radicals from cells, thus avoiding or decreasing their harmful impact caused by oxidation and can be considered as a good antioxidants natural source for medicinal, functional, and commercial uses (52-55). Spermatozoa are highly susceptible to oxidative stress due to their limited antioxidant defense capacity and low repair ability (37). Consequently, using antioxidant materials to regulate, balance, and prevent male infertility is of major importance; this supports the major benefit of adding date pits to animal diets.

Conclusion

The increase in AKT and PTEN gene expression in rats treated with date palm seed extracts reflects a complex interplay of cellular signaling pathways. It could indicate a compensatory mechanism where an increase in AKT activity is counterbalanced by an upregulation of PTEN to adjust cell survival and growth. AKT's ability to coordinate blocking cell death signals, activating survival-promoting transcription factors, and elevating anti-apoptotic proteins like Bcl-2 opens the way for broad distance plans for benefit from manufacturing byproducts of the date palm to support the diets with important antioxidants, minerals, and fibers necessary in animal's nutrition.

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Conflict of interest

None.

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تأثير مستخلص نوى تمر الزهدي على مستوى جيني الفُوسفاتيز والتنسين المتماثل في نسيج خصية الجرذان البيضاء الناضجة وغير الناضجة

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الخلاصة

ركز البحث على تأثير المستخلص الزيتي والمعطن لنوى تمر الزهدي على تعبير جيني الفُوسفاتيز والتِنْسين المتماثل في أنسجة الخصية وعلاقتها بالموت المبرمج للخلايا. استخدم لهذه الدراسة ستون من ذكور الجرذان البيضاء (ثلاثون منها ناضجة وثلاثون غير ناضجة)، قسمت كل منها الى ست مجاميع فرعية، جرعت مجموعتان منها يوميا بالمستخلص الزيتي لنوى التمر بجرع ٢٠٠ و٤٠٠ ملغم/كغم من وزن الجسم عن طريق الفم باستخدام ماصة دقيقة على مدى شهرين متواليين، وبنفس الطريقة جرعت المجموعتان الثانيتان يوميا بالمستخلص المعطن لنوى التمر، في حين أخذت مجموعات السيطرة الماء الجاري. تم تحديد التعبير الجيني لكل من جيني الفُوسفاتيز والتِنْسين المتماثل في نسيج الخصية باستعمال عدة استخلاص الحمض النووي الرايبوزي. تم استخدام صبغة البروتين المناعي المضاد للموت الخلوي المبرمج للكشف عن تعبير وموقع البروتين المضاد للموت الخلوي المبرمج في نسيج الخصية. أظهرت نتائج الدراسة وجود مستويات معنوية عالية من الحمض الرايبوزي المراسل لجيني الفُوسفاتيز والتِنْسين المتماثل في خصى الجرذان الناضجة وغير الناضجة المعاملة بالمستخلص الزيتي والمعطن لنوى التمر مقارنة بمجموعة السيطرة مع وجود زيادة معنوية في مستوى جين الفُوسفاتيز لصالح مجموعة المستخلص الزيتي وخاصة الجرعة ٤٠٠. عززت نتائج الكيمياء النسيجية المناعية نتائج الجينات حيث ظهر البروتين المناعي المضاد للاستماتة في خلايا الخصية وبنسبة أعلى في الخصية الناضجة، خاصة المعاملة بجرعة ٤٠٠ ملغم/كغم. خلصت الدراسة الى أن مستخلص بذور النخيل يعتبر محفز طبيعي لرفع مستوى جين الفُوسفاتيز في الخصية مما يزيد في عمر خلاياها، فضلا عن رفع مستوى البروتين المضاد للموت المبرمج. كما وظهر تعبير البروتين المضاد للموت الخلوي المبرمج في خلايا الخصية وكان أكثر وضوحا في الجرذان الناضجة المعاملة بجرعة ٤٠٠ ملغم/كغم.