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Original Article

Long-term safety evaluation of doum fruit (*Hyphaene thebaica* L.) aqueous extract on the female reproductive parameters, fetal formations and mutagenic potential

Avaliação de segurança a longo prazo do extrato aquoso da fruta doum (*Hyphaene thebaica* L.) nos parâmetros reprodutivos femininos, formações fetais e potencial mutagênico

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Abstract

Several studies have been reported about the distinct activity of the doum fruits as hypotensive, anti-diabetic, antimicrobial, antioxidants and hypolipidemic. This study evaluated the safety of the aqueous extract of doum fruits (*Hyphaene thebaica* L.) on the female reproductive parameters, fetal formations, and normal chromosomes. Acute toxicity studies (LD_{50} values) were evaluated in mice. Single doses of the tested material were given orally, and abnormal behavioral changes were firm through 14- day observation period. The reproductive parameters (no. of pregnancy survival, corpora lutea, implantations, and resorptions and weight at birth), fetal formations (external, skeletal, and visceral) and the normal chromosomes were also investigated. Toleration and absence of mortality or morbidity were observed among animals treated with the decoction of doum fruits until the dose of 5 g/kg of body weight (b. w). Also, repetitive oral treatment of doum fruits to pregnant mice, rabbits and rats at 3 g/kg b. w was not effective on the female reproductive parameters, fetal formations, and the chromosomes (chromosomal aberrations and micronuclei). The doum fruit under test may be of value in treatment of various health problems, with no side effects on the female reproductive parameters, fetal formations and chromosomes have been reported.

Keywords: doum, reproduction, fetal malformation, chromosomal aberrations, micronuclei.

Resumo

Vários estudos foram relatados sobre a atividade distinta dos frutos do doum como hipotensores, antidiabéticos, antimicrobianos, antioxidantes e hipolipidêmicos. Este estudo avaliou a segurança do extrato aquoso dos frutos do doum (*Hyphaene thebaica L.*) sobre os parâmetros reprodutivos femininos, formações fetais e cromossomos normais. Estudos de toxicidade aguda (valores de DL50) foram avaliados em camundongos. Doses únicas do material testado foram administradas oralmente, e mudanças comportamentais anormais foram firmes durante o período de observação de 14 dias. Os parâmetros reprodutivos (nº de sobrevivência à gestação, corpos lúteos, implantações e reabsorções e peso ao nascer), as formações fetais (externas, esqueléticas e viscerais) e os cromossomos normais também foram investigados. Tolerância e ausência de mortalidade ou morbidade foram observadas entre os animais tratados com a decocção dos frutos do doum até a dose de 5 g/kg de peso corporal (p.c.). Além disso, tratamento oral repetitivo de frutas doum para camundongos, coelhos e ratas grávidas na dose de 3 g/kg b. w não foi eficaz nos parâmetros reprodutivos femininos, nas formações fetais e nos cromossomos (aberrações cromossômicas e micronúcleos). A fruta doum em teste pode ser valiosa no tratamento de vários problemas de saúde, sem relatos de efeitos colaterais nos parâmetros reprodutivos femininos, formações fetais e cromossomos.

Palavras-chave: doum, reprodução, malformação fetal, aberrações cromossômicas, micronúcleos.

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1. Introduction

Numerous herbals that are studied and described to have beneficial properties against numerous illnesses were later shown to possess damaging effects on well-being as normal reproduction, fetal formation (Ainehchi and Zahedi, 2014; Yakubu and Afolayan, 2009; Mishra and Singh, 2008). And chromosomes (Abdou et al., 2011; Pugliesi et al., 2007; Sobita and Bhagirath, 2005). The toxic properties of most of the plants on the reproductive system were documented under therapeutic use. Even though a few plants have entered the clinical trials phase, most of them failed the trails because of their toxicity (Yakubu and Afolayan, 2009; Mishra and Singh, 2008).

Hyphaene thebaica has been globally considered as one of the most beneficial fruits for its antioxidant, antibacterial, antifungal, anti-inflammatory and anticancer potential for its flavonoid and phenolic content (El-Beltagi et al., 2018; Farag and Paré, 2013; Irobi and Adedayo, 1999) It comes from the family Palmae, which is known as a doum palm. The tree is found in tropical and subtropical countries. For improvement and breeding approaches of doum, a recent study was carried out on its genetic diversity (Khalil et al., 2020) Doum fruit is a well-known source of needed B-complex vitamins and minerals such as calcium, magnesium, potassium, sodium, and phosphorus (Aboshora et al., 2014). The fruits of doum showed different activities including hypolipidemic, antidiabetic, antihypertensive, and antimicrobial activities (Abd El-Moniem et al., 2015; Shehu et al., 2014; Abou-Elalla, 2009; Eldahshan et al., 2009; El-Gendy et al., 2008; Dosumu et al., 2006). The main hypotensive activity was through the mechanisms of inhibition of angiotensin converting enzyme and renin (Khalil et al., 2018). Traditionally, *Hyphaene thebaica* extracts are used for bilharzia, hematuria, bleeding and as a hematinic agent (Kamis et al., 2003). At high concentration, the ethanol extract is hypolipidemic, hepatotoxic and nephrotoxic (Kamis et al., 2000). However, changing the extraction method produced a nontoxic compound without amending its properties as observed by (Modu et al., 2001, 2000). Where the aqueous extract of *Hyphaene thebaica* was hypolipidemic with no toxic effect on both liver and kidney. Analysis of the phytochemical constituents of the water extract of doum fruit showed the presence of 14 polyphenols and a total of 31 volatile complexes (Aboshora et al., 2017; Habib et al., 2014) The doum fruit aqueous extract is rich with flavonoids and phenols. It possesses a potent antioxidant and anticancer potential, due to their water- soluble phenolic (Faten, 2009; Aamer, 2016; Hsu et al., 2006). The lower levels of doum enhanced spermatogenesis and higher levels made contrary properties Hetta et al. (2005) A few studies regarding the safety of doum (Hassan et al., 2018; Bayad, 2016),and there is no available data concerning the influence of doum fruits on female reproduction, fetal formation, and mutagenic potential. This experiment was designed to evaluate the safety of the decoction of doum fruits on the reproductive parameters, the fetal malformations and mutagenicity impacts.

2. Materials and Methods

2.1. Materials

The doum fruits were obtained from the local market, Alexandria, Egypt, and the other chemicals were of analytical grade and purchased from commercial companies.

2.1.1. Animals

Adult male and female Wistar rats weighted 180±10 g was used. The animals were obtained from the animal house at the College of Veterinary Medicine, Alexandria University, Alexandria, Egypt. Animals were maintained on food and water ad libitum. The animals were subjected to acclimatization period of 2 weeks prior to start. The investigation subjected to the Guide for the Care and Use of Laboratory Animals published by US National Institutes of Health (NIH publication no. 85-23, revised 1996), and the study was approved by the local ethics committee.

2.1.2. Preparation of the aqueous extract of doum fruits

Cleaning, removal of debris and separation of the fruit into pulp and seed was carried out and followed by drying and grounding the pulp into fine powder. The aqueous extract of doum fruits was prepared through boiling the powdered pulp in distilled water 1:5 (w/v) for 10 min. (rich in water-soluble phenolic contents) (Aamer, 2016; Hsu et al., 2006), then drained through two layers of cheesecloth with constant pressing until producing the complete free running extract, which was filtered using Whatman filter paper (no.1). The clear filtrate was then evaporated to dryness under vacuum using rotary evaporator (Buchi, Switzerland) at 60 °C and stored at -20 °C before administration.

2.2. Experimental design

2.2.1. Acute toxicity studies

The study was carried out in rats. The animals were fasted overnight prior to the actual experimental procedure. Single graded doses of the aqueous extract of doum fruits (from 0.1 g to 5 g/kg bw) and the vehicle by gavage. Animals were observed for clinical signs, symptoms, and mortality continuously for the first 2 hours and then for every four hours up to 14 days. Eventually, with the aid of Finney (1985), the LD_{50} values were determined.

2.2.2. Effect on the reproductive parameters:

This design was carried out in female pregnant animals (16 New Zealand rabbits, and 40 albino Swiss mice divided equally into groups). Twice daily doses of 0 and 3 g/kg bw of the tested drug were administrated to mice and rabbits from day 6-15 and day 7-19 of pregnancy respectively and were sacrificed on day 18 and 29 of pregnancy respectively. Fetuses were delivered through the caesarean section. Observation and recording of corpora lutae number, number and position of implantations, resorptions, live and dead fetuses was carried out. The uteri were subjected to

ammonium sulfide 10% staining (Kopf and Salewski, 1964), when no visible implantations observed, for examinations and signs of early resorptions.

2.2.3. Fetal observations

Twenty pregnant female rats have been placed in two groups. They were subjected to the mentioned treatment regime within mice but were not sacrificed on the nineteenth pregnancy day. The caesarean section was used to attain the fetus delivery. Assessment was made of the visceral organs and fetal skeleton where each fetus was assessed, weighed, sexed, and analyzed thoroughly to extract external variations or malformations along with palatal defects assessments. Within each litter, visceral malformations/variations assessments were made for nearly half of the fetuses (Staples, 1974).

The visceral assessment was made upon the fetus by injecting Bouin's solution of 2 mL using the abdominal injection. They were then left in the Bouin's fixative overnight before it was turned into 10% formalin solution and then sectioning and assessment was done (Wilson, 1965) When eviscerating and processing the rest of the fetuses, straining was done for the ossified skeletal structures and the cartilaginous parts using alizarin red S, and Alcian blue stain respectively (Dawson, 1926).

2.2.4. Mutagenicity studies

The drug was tested for its mutagenic potential and analysis was made using the mitotic index, chromosomal aberrations, and micronucleus technique. Two equal groups were formed for twenty mice, male and female. The drug was administered twice in 0 and 3 g / kg bw doses for 3 months.

2.2.5. Chromosomal aberrations

The femur bone marrow was analyzed for chromosomal aberrations. After 1 to 2 hours of the colchicine (4 mg/kg bw) injection, the animals had been sacrificed. The bone marrow cell was extracted for the bone marrow preparation using the hypotonic solution that is 5-7 mL of K Cl (0.65%). The process was carried out for twenty minutes at 37°. For nearly 5 minutes, centrifuging was done for the cells at 1000 rpm. They were then fixed within methyl acetic acid (Carnoy) 3:1, and again for 5 min were centrifuged at 1000 rpm. Discarding was done of the supernatant and 5 mL fixative was used for the suspension of the pellets. The centrifugation was then repeated, and the process was carried out twice. The cells were distributed into clean slides and Giemsa was used for staining (Giri et al., 1986).

2.2.6. Micronucleus technique

Flushing was carried out of the bone marrow using 1-1.5 ml fetal calf serum and for 5 min it was centrifuged at 1000 rpm. Smearing was done of the sediment cells upon the clean slides (3 for each animal). When it dried, for 10 min, fixing was done of the slides within absolute methyl alcohol and 10% Giemsa stain was used for staining (Schmid, 1976).

2.3. Statistical analysis

ANOVA has been used to analyze data, and it is stated by (Snedecor and Cochran, 1980). The control and mean values of several treatments have been compared. The presentation of results was done as mean ± S.E. and statistical significance maintained at $p < 0.05$.

3. Results and Discussion

3.1. Acute toxicity studies

Various researchers have analyzed the advantages of the doum fruits influences. The research indicates that there is richness of polyphenolic compounds within the aqueous extract (Sharaf et al., 1972) Additionally, it maintains potent hypotensive, hypolipidemic, hypocholesterolemia, antidiabetic, and antioxidant potential (Abd El-Moniem et al., 2015; Shehu et al., 2014; Abou-Elalla, 2009; Eldahshan et al., 2009; El-Gendy et al., 2008; Dosumu et al., 2006). A safety margin was presented by the acute toxicity research conducted upon the doum fruits aqueous extract. This safety margin is quite wide and excludes the presence of toxic as well as lethal influences at the time of clinical usage even if there is overdosing. In rats, the acute oral LD_{50} was higher than 5 g/ kg bw and there was no observation of the abnormal behavior influences. In a similar manner, for the kidney and liver functions, a wide safety margin was brought forward by (Bayad, 2016; Kamis et al., 2003) They also presented a high rise within red blood corpuscles count, packed cell volume and hemoglobin concentration after occurring of the doum fruit extract administration.

3.2. Reproductive parameters

For the treatment group, pregnancy persistence control number, corpora lutea, implantation sites, number of fetuses, implantation loss, and resorptions or dead fetuses per mice and rabbits' dams, there was not much alteration. Additionally, the fetal body weight from mice and rabbit dams that were treated using doum fruit did not have a significant influence when compared to the control group (Table 1). Similarly, when the alloxan-diabetic rats were treated using doum extract, there was a significant rise within the serum testosterone level along with marked modulation as part of the level of total acid phosphatase and prostatic acid phosphatase activities. On the other hand, research also indicated that spermatogenesis is progressed using small doses and a large dose would decrease it (Hetta et al., 2005). Furthermore, the reproductive procedure within several animal species would be delayed by various plant products (Ainehchi and Zahedi, 2014; Mortazavian et al., 2012; Nordeng and Havnen, 2004).

3.3. Fetal observations

At the time of pregnancy, when herbal products are used, more attention must be paid. Synthetic drugs have several side effects, they are not advised to be used during pregnancy, however, for herbal agents; the data considering perinatal and maternal undesirable issues is quite limited.

Since there is limited, toxicological data related to herbs, it is quite a concern, since it has made a place within livestock and its use should be monitored (Mandlik and Namdeo, 2020; Qin et al., 2020; de Moura et al., 2020; Efferth and Kaina, 2011) Until now, the doum fruit safety report has not been generated for the normal fetal formations and female reproduction.

During the research, fetal data were collected for pregnant rabbits, mice, and rats, which have been summarized within (Table 2-3). The gross anomalies were none and the attained fetuses from the dams that were treated using doum fruit extract did not have stunted growth. Furthermore, there was a normal attachment to dams. The group, which was treated with the doum fruit extract, when compared to the control, had a high amount of subcutaneous hemorrhage. Additionally, the presence of the visceral and skeletal differences within the doum fruit extract treatment group was also insignificant when compared to the control group. Dilated cerebral ventricles, mal-positioned thymus, and heart and lung hypoplasia were present in the visceral malformations. The skeletal malformations that were most prominent had delayed skull ossifications, vertebrae and stern brae and the fused ribs were not present (Table 3). The laboratory has reported similar results after a thorough analysis was carried out upon the commercial use of specific herbs (El-Ashmawy and Bayad, 2016; Mandour et al., 2013).

Drugs through direct action upon the embryo could produce teratogenicity or then drug metabolite since there is interference with the biochemical procedures using competitive inhibition of the essential cellular components, which leads to birth defects (Sisodia, 1972). Additionally, teratogenic drugs would state their influence through affecting of metabolism of proteins or then nucleic acids. Fetal malformations may have a correlation with chromosomal anomalies and the cell division could be interfered Additionally, the maternal/fetal placental barrier includes the P-glycoprotein gene expression alteration since the drug created teratogenesis (El-Ashmawy et al., 2011).

3.4. Mutagenicity studies

Toxic constituents, component pharmacologic activity, herbal remedy interactions or overdose may lead to herbal remedy adverse influences. There is much controversy present regarding literature related to adverse effects of such herbal medicines as Jan-On et al. (2020), Murbach et al. (2019), Patra et al. (2020), Yoshino et al. (2019) and Bayad (2016).

Much change was not observed in the chromosomal aberrations (fragment, deletion, ring, and gap) and

Table 1. Reproductive parameters in mice and rabbits give doum fruit aqueous extract.

Reproductive Parameter	Mice		Rabbits	
	Dose (g/kg)		Dose (g/kg)	
	0	**3**	**0**	**3**
No. bred	20	20	8	8
No. pregnant	16	18	6	6
No. aborted	0	0	0	0
No. litters totally resorbed	0	0	0	0
No. corpora lutea	7.0±0.25*	7.2±0.30	7.83±0.47	8.61±0.49
No. implantation	6.37±0.23	6.17±0.21	6.63±0.42	7.67±0.40
Dead fetuses	0	0	0	0
Litter size	6.0±0.20	6.17±0.22	6.73±0.40	7.5±0.62
Mean fetal body weight (g)	1.1±0.02	1.06±0.02	36.0±1.29	37.67±1.48

*Values are means ± SE.

Table 2. External abnormalities of rat's fetuses obtained from doum fruit extract treated dams at 6th to 15th days of gestation.

Treatment	Control	Parameters
No of fetuses (no of litter) examined		
80 (10)	80 (10)	External alterations
No of fetuses (no of litters) affected		
External observations		
2 (1)	2 (1)	Stunted (≤ 3 g)
0 (0)	1 (1)	Subcutaneous hemorrhage
1 (1)	1 (1)	Multiple facial anomalies

Table 3. Visceral and skeletal abnormalities of rat's fetuses obtained from doum fruit aqueous extract treated dams at 6th to 15th days of gestation.

No. of fetuses (No. of litters)		Parameters
Treatment	Control	
40 (10)	40 (10)	Visceral observations
0 (0)	0 (0)	ë Dilated cerebral ventricles
1 (1)	1 (1)	ë Mal-positioned thymus
2(1)	2 (2)	ë Heart hypoplasia
1 (1)	0 (0)	ë Lung hypoplasia
40 (10)	40 (10)	Skeletal observations
	ë Skull	
1 (1)	1 (1)	ë Delayed ossifications
	ë Vertebrae	
2 (1)	2 (2)	ë Delayed ossifications
	ë Ribs	
0 (0)	1 (1)	ë Fused
	ë Sternebrae	
1 (1)	2 (1)	ë Delayed ossifications

Table 4. Different kinds of chromosomal aberrations in mice treated with doum fruit aqueous extract by gavage twice daily for 3 months.

Dosage Group (g/Kg)	Parameter*			
	Fragment	Deletion	Ring	End to end association
0	1.4±0.2	0.6±0.02	0	2.2±0.3
3	1.2±0.5	0.8±0.03	0	1.8±0.3

*250 metaphases examined per treatment. Values are means ± SE, *n*=10.

Table 5. Micronucleus (MN) and mitotic index in mice are treated with doum fruit aqueous extract by gavage twice daily for 3 months.

Dosage Group (g/kg)	Parameter		
	Mitotic index*	Micronucleus** (Nuclear budding)	Micronucleus** (Nuclear fragmentation)
0	9.2±0.5	1.6±0.24	0.8±0.3
3	8.4±0.9	1.2±0.49	0.8±0.4

*No. of dividing cells in 500 cells; **Micronucleus incidence in 500 polychromatic erythrocytes. Values are means ± SE, n= 10.

micronuclei due to the Doum fruit extract even though it was given daily over a period of 3 months to the mice (Tables 4 and 5). Doum has been researched upon, and focus was maintained upon fruit based on the nutritional values present. It is considered a healthy tonic due to the dried fruit pulp containing hot water infusion. The *H. thebaica* fruit pulp was analyzed and nutritionally essential linoleic acid, nutritional trace minerals, proteins and fatty acids were found (Kamis et al., 2003), Saponins, coumarins, hydroxycinnamates, essential oils and flavonoids were also abundantly present in the fruit. Within animal models, blood pressure was also lowered by the fruit (Sharaf et al., 1972). An antioxidant activity was indicated by the doum fruits aqueous extract mainly because there is a significant amount of water-soluble phenolic contents, (Hsu et al., 2006; Islam et al., 2022).

5. Conclusions

It could be concluded that, the doum fruit under test may be of value in treatment of various health problems, with no side effects on the investigated female reproductive parameters (no. of pregnancy survival, corpora lutea, implantation, resorption, and weight at birth), fetal parameters (external, skeletal and visceral)

and the mutagenicity impacts (chromosomal aberrations and micronuclei).

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