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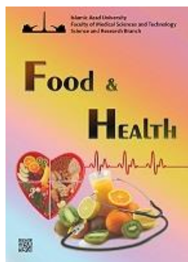

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Evaluation of the acute oral toxicity of a *Crocus sativus* (Iranian saffron) nanoemulsion in rat

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ABSTRACT

Crocus sativus (Iranian saffron) is a widely consumed spice that has been historically attributed to various therapeutic properties. It is commonly used as a food colorant and flavoring agent in the food industry. Encapsulating spices using nanoemulsions can enhance properties such as stability and antibacterial activity. In this study, we developed a *C. sativus* nanoemulsion using ultrasonication. Toxicological assessment is imperative for validating the incorporation of spice nanoemulsions into foods. Therefore, the aim of this study was to evaluate the potential acute oral toxicity of this nanoemulsion in albino rats. All assessments were conducted using a single prototype batch representative of projected human exposure levels. Results indicated no treatment-related mortalities or statistically significant changes in food or water intake. Additionally, no gross abnormalities were observed in vital organs upon macroscopic examination at doses exceeding the projected human intake. Overall, this nanoemulsion demonstrated a satisfactory acute oral toxicity profile under the conditions tested.

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1. Introduction

Crocus sativus L., commonly known as saffron, is a member of the family Iridaceae and is cultivated in countries such as Iran, India, and Greece (1). Iran accounts for more than 90% of global saffron production (2). Saffron consists of dried red stigmas and is widely used as a culinary spice. It contains several volatile compounds, including safranal and non-volatile ones, such as crocins and the glycoside picrocrocin (3,4). The bitter taste of saffron stems from picrocrocin, while safranal imparts its distinctive hay-like aroma and crocins lend its golden color (5). Saffron is considered the world's most expensive spice due to its labor-intensive harvesting, and it is used for food coloring, flavoring, and various other purposes (6). It is also employed as a fabric dye in the garment industry (7). Traditionally, saffron has been used medicinally to treat conditions such as depression, seizures, bronchitis, cardiovascular disorders, and cancers (8). Considering saffron's myriad benefits, maximizing its utilization is paramount. However, limitations such as low solubility hamper efficiency in food/pharmaceutical applications. Nanotechnology can be leveraged to enhance saffron's efficacy (9). There has been growing interest from industry and academia in nanoemulsions' applications (10). Exhibiting intrinsic properties like augmented transport across membranes and increased surface area:volume ratios, and nanoemulsions confer manifold advantages for industries (11). Comprising droplets 20-200 nm, they are classified as mini-emulsions, nanomolecules, and microemulsions (12). Nanoemulsions are thermodynamically unstable colloidal dispersions of two immiscible liquids, with one phase sequestered in nano-sized droplets (13). Surfactant coatings stabilize oil droplets, yielding robust systems (14). Due to their minute size, nanoemulsions augment bioactives' stability and antimicrobial impacts (15). Studies evidenced cumin and black pepper nanoemulsions' antibacterial/biofilm-inhibiting effects

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(16). Multiple techniques yield nanoemulsions, including high-pressure homogenization, spontaneous emulsification, and ultrasonication (17). Growing nanomaterial interest necessitates toxicity assessments. This study aimed to preliminarily evaluate the acute toxicity of a saffron nanoemulsion suitable for functional foods and nutraceutical applications.

2. Materials and methods

2.1. Preparing saffron

Saffron flowers (*Crocus sativus*) were collected from fields near Tehran, Iran. Approximately 300 g of pure saffron stigmas were obtained from 2100 g of plant material. Species identification was performed according to descriptions in the European flora (18).

2.2. Saffron extract preparation

A saffron extract was prepared by mixing 4.60 g saffron with 250 mL of an 80:20 ethanol:water solution in a sealed container. The mixture was agitated at 100 rpm for 24 h at 25°C using an orbital shaker (Heidolph Unimax 1010 Inkubator 1000, Germany). Magnetic stirring (Heidolph Instruments, Germany) was then performed at 650 rpm for 6 h to maximize extraction. The extract was purified using Whatman filter paper, and ethanol was removed by heating. The extract was stored refrigerated until use.

2.3. Saffron nanoemulsion preparation using ultrasonication

An oil phase was prepared by mixing the emulsifier span 80 (sorbitan monooleate) with N-octane and stirring for 15 min at 200 rpm using a magnetic stirrer (Heidolph Instruments, Germany). The saffron extract was added dropwise, and the mixture stirred for 30 min at 600 rpm. The beaker was immersed in an ice bath during ultrasonication (SONICS 500W, 20 kHz) applied for 5 min using 7 sec pulses alternating with 3 sec rests. Samples were transferred, sealed, and refrigerated to the toxicology laboratory for analysis.

2.4. Acute oral toxicity testing

2.4.1.Test items

The test substance evaluated was identical to the formulations expected for human intake. The vehicle used to formulate the test article was also the intended vehicle for human consumption. When possible, the vehicle alone serves as the control, dosed at equivalent volumes and concentrations as the high-dose group. All assessments used a single prototype batch (batch no. 3) representative of expected consumer exposure, provided in January 2021 with excess doses to account for potential loss while retaining study samples.

2.4.2. Vehicle and control

Appropriate control groups were included per toxicity study standards. Here, sterile saline served as the negative control vehicle for oral gavage.

2.5. Test animals

Per ISO 10993-11:2016 guidance, a single species/strain facilitates comparative analyses across study durations (e.g., acute, subacute, subchronic, chronic). Rodents are typically selected for acute studies. Accordingly, healthy young adult female albino rats of a defined microbiological health status were obtained from the Pasteur Institute of Iran. Thirty rats were used in the study. At initiation, the rats weighed 118-136g with ≤20% variance from the mean weight. Rats were acclimated prior to the study. All animal procedures received institutional ethics approval and complied with guidelines for animal welfare and protection.

2.6. Housing and care

Good laboratory practices were followed for high-quality, valid data. Rats were acclimated to laboratory conditions for 14 days prior, with environmental conditions monitored and controlled per standards. Temperature was maintained at 23 ± 3°C with 40% ± 10% relative humidity under a 12:12 hour light/dark cycle. Technicians were trained in humane animal care and treatment. Rats (n=5/cage) were housed in polyurethane cages with rice husk bedding changed daily. Non-toxic standardized commercial diet (Pars Co., Iran) and water were provided ad libitum (Table 1). Animal use complied with institutional ethics approval and guidelines for welfare and humane treatment.

Table 1. Standardized commercial laboratory diets (Feed AS2).

Name	Percentage
Crude protein	NLT 19-21%
Ether Extractive	NLT 04-05%
Crude Fiber	NMT 01%
Ash	NMT 08%
Calcium	1.3%
Phosphorus	NLT 0.6%
NFE	55%
ME	Kcal/kg 3800
Pallet size	13.2mm
Vitamins and Minerals	A, D3, B12, Thiamine, Riboflavin, Folic acid, all minerals, and microelements

2.7. Dose administration and group allocation

Animals were randomly allocated into 7 groups of 5 subjects each (1 vehicle control group and 6 treatment groups) using a computer-generated randomization scheme. Prior to group assignment, all animals underwent a 14-day acclimation period upon arrival to ensure general health and the absence of discernible abnormalities. Randomization was validated to confirm no statistically significant differences between groups regarding baseline physical characteristics such as body weight. The vehicle control and 6 test article treatment groups were established based on the results of pre-study examinations and evaluations. This experimental design was

employed to distribute any potential confounding factors evenly across test and control populations, thereby facilitating unbiased comparisons between dosing conditions.

2.8. Test preparation and administration

The required dosage volumes were obtained from the provided sample. Given the prototype's intended use, the oral route best simulated potential human exposure and was selected for this study.

2.9. Observation parameters

Per ISO 10993-11:2016 guidance, body weight changes and clinical observations were recorded:

2.9.1. Body weight and food/water intake

Weights were measured within 24 hours prior to dosing and at regular intervals/termination. Individual food and water consumption (estimated per cage based on division by number of rats) was also monitored. Generally, >10% weight reduction indicates toxicity.

2.9.2. Clinical observations

Trained personnel conducted daily examinations for consistency. Observation frequencies/durations were tailored based on reaction severity. Rats were evaluated for symptoms listed in Table 2, with occurrences documented. This standardized, well-described methodology provides reproducible, translational data on potential adverse effects following oral exposure. Procedures complied with ethical care and use of laboratory animals.

Table 2. List of evaluated clinical observations and signs in the tested animals.

Clinical observation	Observed sign
Respiratory	Abnormal breathing, gasping, apnea, cyanosis, tachypnea, nostril discharges
Motor activities	Decreased/Increased drowsiness, loss of righting, anesthesia, catalepsy, ataxia, unusual locomotion, prostration, tremors, fasciculation
Convulsion	Clonic, tonic, tonic-clonic, asphyxia, opisthotonos
Reflexes	Corneal, writing myotatic, light, startle reflex
Ocular signs	Lacrimation, miosis, mydriasis, exophthalmos, ptosis, opacity, iritis, conjunctivitis, chromodacryorrhea, relaxation of nictitating membrane
Cardiovascular signs	Bradycardia, tachycardia, arrhythmia, vasodilation, vasoconstriction
Salivation	Excessive
Piloerection	Rough hair
Analgesia	Decrease reaction
Muscle tone	Hypotonia, hypertonia
Gastrointestinal	Soft stool, diarrhea, emesis, diuresis, rhinorrhea
Skin	Edema, erythema

2.10. Histopathological examinations

Upon completion of the dosing/recovery periods, animals were humanely euthanized and underwent complete gross necropsy examinations. Histopathological analysis was conducted on tissues from major organ systems to investigate potential toxic effects, with a focus on common target organs. Unless indicated by gross findings, full histopathology is not typically performed in acute toxicity studies due to resource constraints. For this study, histopathological examination targeted the liver, kidneys, lungs, and spleen. The liver and kidneys are frequently impacted due to their roles in xenobiotic metabolism and excretion, respectively. Evaluation of the spleen and lungs allowed for assessment of potential immunotoxic and respiratory effects. Tissues were fixed in 10% neutral buffered formalin, with lungs infused post-weighing. Samples were processed routinely through graded alcohols, xylene, and paraffin before sectioning at 3-5 microns. Hematoxylin/Eosin staining was applied to identify cellular structures and histopathological changes. Proper fixation and slide preparation are critical for accurate pathological assessments. Histopathological examination facilitates the characterization of temporal and dose-dependent lesion profiles, allowing the identification of target organ systems and no observed adverse effect levels. Findings from this study further the understanding of compound safety and mechanisms of toxicological response.

2.11. Statistical analysis

Group findings were prioritized over individual variability due to the larger sample sizes used in rodent studies. Statistical analyses could thus be appropriately applied. If none of the rats treated with the test substance exhibited significantly greater reactivity than vehicle controls during observation, the sample met test requirements. Additionally, the sample did not meet the criteria if two or more rats died, two or more showed signs like convulsions or prostration or three or more lost >10% body weight. If slight signs of reactivity occurred in only a few rats, the test was repeated using groups of ten. If none of the repeat test rats displayed scientifically meaningful increased reactivity compared to controls, the sample met test requirements. Evaluation of dose-response relationships and effect incidences/severities included behavioral, clinical, gross lesions, and body weight changes. A one-way ANOVA model with $\alpha=0.05$ tested for statistically significant differences between dose levels and controls. The ANOVA F-test ratio compared between- versus within-group variations. If deaths occurred, the LD50 was determined using moving average methods. The statistical analysis allowed an objective interpretation of the toxicological findings.

3. Results

3.1. Mortality

No mortalities occurred at any tested dose levels over the 14-day observation period (Table 3). This demonstrates doses up to a full human equivalent amount caused no mortality in rats, well above expected human intakes.

3.2. Clinical signs

Daily clinical observations of rats detected no treatment-related abnormalities associated with the saffron nanoemulsion (batch no. 3) compared to controls (Table 4).

3.3. Gross necropsy findings

Necropsy examination and histopathological analysis of selected vital organs showed no significant irreversible tissue damage potentially attributable to test substance administration (Fig. 1). The results indicate the saffron nanoemulsion (batch no. 3) was well-tolerated with no observable adverse effects on mortality, clinical status, or gross/histological organ morphology at dose levels far exceeding expected human exposure levels. The test article can thus be considered non-toxic under these acute oral testing conditions.

Table 3. Mortality rates of animals subject to different doses of saffron nanoemulsion.

Dose mg/kg	**1000**	**2000**	**3000**	**4000**	**5000**	**6000**
Group	**1**	**2**	**3**	**4**	**5**	**6**
Hr 1	Nil	Nil	Nil	Nil	Nil	Nil
Hr 2	Nil	Nil	Nil	Nil	Nil	Nil
Hr 3	Nil	Nil	Nil	Nil	Nil	Nil
Hr 4	Nil	Nil	Nil	Nil	Nil	Nil
Day 1	Nil	Nil	Nil	Nil	Nil	Nil
Day 2	Nil	Nil	Nil	Nil	Nil	Nil
Day 3	Nil	Nil	Nil	Nil	Nil	Nil
Day 4	Nil	Nil	Nil	Nil	Nil	Nil
Day 5	Nil	Nil	Nil	Nil	Nil	Nil
Day 6	Nil	Nil	Nil	Nil	Nil	Nil
Day 7	Nil	Nil	Nil	Nil	Nil	Nil
Day 8	Nil	Nil	Nil	Nil	Nil	Nil
Day 9	Nil	Nil	Nil	Nil	Nil	Nil
Day 10	Nil	Nil	Nil	Nil	Nil	Nil
Day 11	Nil	Nil	Nil	Nil	Nil	Nil
Day 12	Nil	Nil	Nil	Nil	Nil	Nil
Day 13	Nil	Nil	Nil	Nil	Nil	Nil
Day 14	Nil	Nil	Nil	Nil	Nil	Nil
Mortality	0.5	0.5	0.5	0.5	0.5	0.5

Table 4. List of abnormalities in animals subject to saffron nanoemulsion.

No.	**Parameters**	**Cage side observation**
1	Condition of the fur	Normal
2	Skin	Normal
3	Subcutaneous swellings	Nil
4	Abdominal distension	Nil
5	Eyes-Dullness	Nil
6	Eyes-opacities	Nil
7	Pupil diameter	Normal
8	Ptosis	Nil
9	Color & consistency of the feces	Normal
10	Wetness or soiling of the perineum	Nil
11	Condition of teeth	Normal
12	Breathing abnormalities	Nil
13	Gait	Normal

4. Discussion

Saffron and its derivatives have wide applications in food and pharmaceutical industries. Saffron is used as a spice and food coloring. Its main active compounds, crocin, crocetin, and safranal, also exhibit pharmacological activities including antioxidant, anticancer, and neuroprotective effects (19). Nanotechnology-based formulations have shown promise in enhancing the solubility, bioavailability and targeted delivery of poorly water-soluble plant extracts like saffron. Nanomulsification methods can enhance the delivery and efficacy of the main bioactive compounds in saffron, such as crocin, crocetin, and safranal (12). While several studies have evaluated the toxicity profile of saffron (20), to our knowledge no prior studies have specifically assessed the toxicity of saffron nanoemulsions. Therefore, the present study aims to characterize for the first time the toxicity and safety profile of saffron nanoemulsions. Nanoemulsions

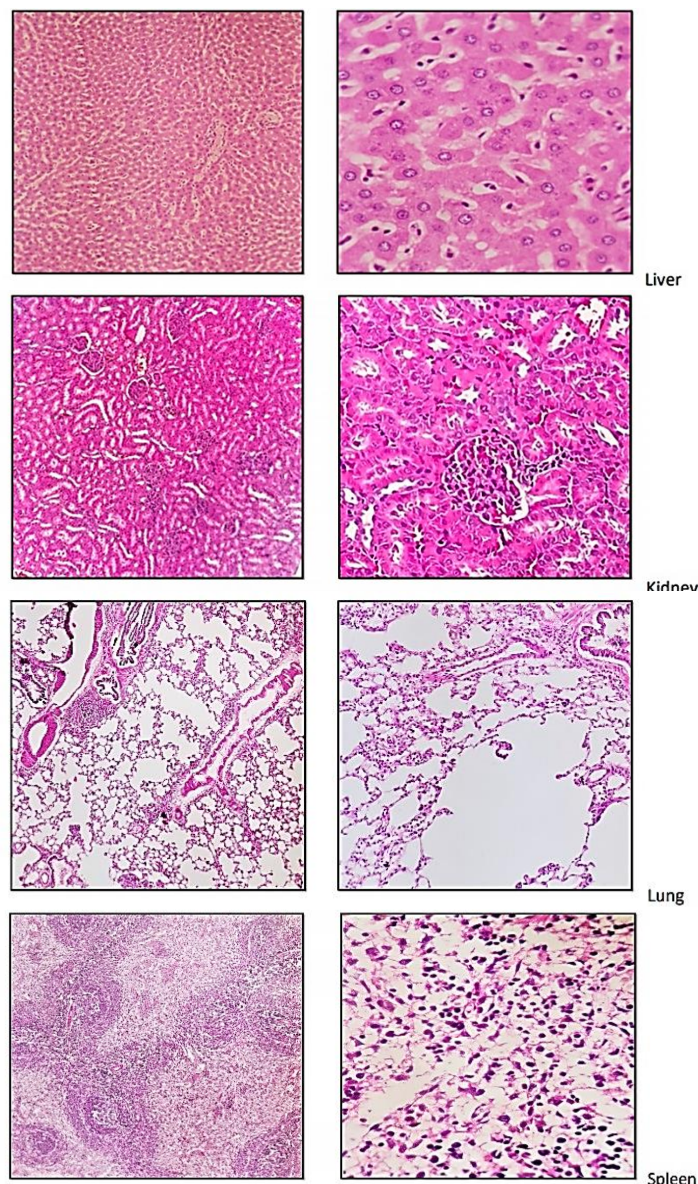

Fig. 1. H&E staining of liver, kidney, lung, and spleen tissues from toxicity study slides.

possess several advantageous properties owing to their small particle size. However, their nanoscale dimensions may also

facilitate potential toxicity through alternative absorption and distribution mechanisms compared to microparticles (21). Factors like particle size, shape, surface charge, and degradability influence the toxicity potential of nanoemulsions. Most food-grade nanoemulsions are readily digested by gastrointestinal enzymes like lipase and protease upon reaching the upper GI tract, thus precluding systemic absorption and toxicity. However, exceptions may exist. Non-degradable nanoemulsions containing mineral oils could evade enzymatic breakdown. Additionally, incompletely digested lipid droplets may traverse the stomach and enter the intestine, enabling epithelial uptake and potential toxicity (22). Prior studies show that non-degradable nanomaterials such as titanium dioxide and silicon oxide, upon epithelial penetration, can distribute systemically and accumulate in organs (23). Therefore, characterizing the toxicity profile of each nanoemulsion formulation is prudent. One previous in vivo study orally administered nanoemulsions to Wistar rats at doses up to 800 mg/kg for 21 days, finding no changes in biochemical parameters or organ weights, suggesting oral safety (24). Conversely, Martins et al. observed inflammatory cell infiltrates in mouse livers after nanoemulsion treatment, though other health indicators were unchanged (25). Key organs implicated in detoxification and clearance like the liver and kidneys, were histologically normal in this study, indicating saffron nanoemulsion does not impair their function or induce toxicity (25). General health markers like water consumption, food intake, and body weight also remained unaltered versus controls, supporting the non-toxic characteristics of this nanoemulsion (26). In summary, this work provides the first characterization of saffron nanoemulsion toxicity, finding it to be safe and well-tolerated based on these preclinical safety parameters.

5. Conclusion

In conclusion, this acute oral toxicity study found Iranian saffron nanoemulsion (Crocus sativus, batch no. 3) to be non-toxic. No mortalities occurred at doses up to 2000 mg/kg, well above a full human equivalent amount administered to rats. No abnormalities were observed clinically or in pathology assessments up to 14 days post-exposure. Histopathological examination of major organs revealed no evidence of irreversible damage. General health indicators like weight and food/water intake were unchanged. Therefore, based on the absence of adverse outcomes even at doses far higher than human exposure levels, this saffron nanoemulsion formulation can be considered safe for its intended uses.

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