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Toxicological Evaluation of Pomegranate Fruit Extract in Male and Female Rats

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POmegranate (*Punica granatum* L.) fruit is often consumed as fresh fruit and juice. Its varied medicinal benefits in traditional medicine. The current study aimed to assess the acute and subacute toxicity profiles of pomegranate extract in male and female Sprague Dawley rats. In acute toxicity investigation, rats of both sexes were administered 1g/kg body weight of extract intraperitoneally. In the subacute toxicity trial, that was administered the extract (1g/kg body weight) intraperitoneally every 24 hours for 15 days, whereas the control group received distilled water. Daily food consumption and drink intake and rats' body weight changes were carefully recorded and documented for two weeks. Hematological measures of complete blood count (CBC), serum biochemical parameters of liver, heart, and kidney function, and lipid profile were evaluated to identify the principal harmful effects on tissues. There were no behavioral changes or mortality recorded in the treated groups. The LD50 value was greater than 1g/Kg body weight. There were no significant differences ($p<0.05$) in body weight gain, food, and water intake in groups. The hematological and biochemical parameters and the organ weights showed no significant alterations ($p<0.05$) between the treatment and control groups. Group 1, which received doses of 1 g/kg body weight of extract 2, exhibited superior outcomes in most of the parameters under investigation. This included improvements in behavior, food and water intake, average body weight, relative organ weights, and serum biochemical markers. The overall finding of this study reveals that pomegranate extract is safe up to 1g/Kg body weight intraperitoneal administration in male and female Sprague Dawley rats, and we conclude from prior results that pomegranate extract is safe.

Keywords: Punica granatum (L), pomegranate extract, acute toxicity, subacute toxicity, safety.

Introduction

Many plant extracts appeared to have therapeutic values with respect to diseases [1]. Clinical pharmacologists have distinguished natural products and herbal medicines with antimicrobial and antiviral effects with increasing interest. Traditional medicine represents the sole healthcare option available to millions of individuals. The safety and reduced occurrence of adverse effects associated with numerous herbal extracts have been cited as potential reservoirs for the development of novel pharmaceuticals. [2, 3].

Pomegranate belongs to a fruit family in the Mediterranean climate. Fruit contains a lot of saccharides, polyphenols, and essential minerals. The physical and chemical properties of pomegranate have been evaluated in Turkey and Italy [4, 5]. The

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peel (pericarp), seeds, and aril (around the seeds) comprise the fruit. The peel includes minerals and several bioactive polyphenolic chemicals, including structurally different ellagitannins and derivatives such as alpha-/beta-punicalagin, punicalin, and punigluconin. The arils are mainly composed of water and contain phenolics and flavonoids. Anthocyanins, flavonoids found in arils, are responsible for the fruit's and juice's red color [6]. Pomegranate is a rich source of anthocyanins. One of the main characteristics of its quality is the red color of its seeds and juice. The red color depends on the concentration and type of anthocyanins contained in the fruit. Delphinidin derivatives give blue and purple shades, whereas pelargonidin is responsible for orangish red shades [7].

Pomegranate juice contains more than 100 phytochemicals. For thousands of years, pomegranate fruit has been employed as a medicine, and pomegranate juice is currently under investigation for its multiple health benefits. It may aid in preventing cancer, fertility, and immunological support. Pomegranate juice has more antioxidants than the majority of fruit juices. Additionally, it contains three times the antioxidants found in green tea and red wine. Pomegranate juice contains antioxidants that help eradicate free radicals, defend cells from damage, and reduce inflammation [6]. Pomegranate juice has broader antiviral activity against enveloped viruses like influenza, including the potential pandemic H5N1, pox). The Efficacy of Pomegranate on herpes viruses can also be ultrafiltered to separate low-molecular-weight nutraceuticals called enveloped virus-neutralizing compounds (EVNCs). Pomegranate juice includes HIV-1 entrance inhibitors, which adsorb on maize starch. The resulting complex blocks virus binding to CD4 and CXCR4/CCR5 and inhibits infection by primary virus clades A to G and group O [8]. While no long-term human studies have been performed to indicate that pomegranate juice protects or reduces the risk of cancer, having it in your diet cannot harm you. Trials have given excellent outcomes, and researchers are conducting more thorough studies. The antioxidants in the juice, plus their high concentration, are thought to inhibit the progression of Alzheimer's disease and preserve memory [9]. Pomegranate juice is a powerful anti-inflammatory beverage due to its rich antioxidant content. It has the potential to alleviate inflammation across the body, safeguard against oxidative stress, and deter damage caused by it. Researchers are presently investigating its potential impact on conditions like osteoporosis, rheumatoid arthritis, as well as various forms of arthritis and joint inflammation. Furthermore, it is being considered as one of the most heart-friendly juices, with potential benefits for heart and artery protection. Consistent consumption of pomegranate juice on a daily basis might also contribute to reducing systolic blood pressure.

Nevertheless, more studies need to be done to determine if pomegranate juice can decrease overall blood pressure in the long term [10]. Pomegranate juice, rich in vitamin C and other immune-boosting elements like vitamin E, can help avoid disease and infection. They were also shown in laboratory tests as antibacterial and antiviral. They are being examined to determine how they impact common diseases and viruses. Pomegranate juice is high in folate, potassium, vitamin K, and vitamins C and E. It is fueled with many antioxidants and can affect oxidative stress, which makes it a potential fertility aid. Oxidative stress has been shown to cause sperm dysfunction and decrease fertility in women [11]. The juice was also shown to help reduce oxidative stress in the placenta. Pomegranate was traditionally used as a remedy for diabetes in the Middle East and India [12].

Pomegranate extracts represent one of various herbs that have been found to have significant therapeutic benefits. In ayurvedic medicine, pomegranate is reported as “a pharmacy unto itself and is used as a “blood tonic” to heal many health problems [8]. Pomegranate extracts are being investigated as a potential therapy for viral infections. The antimicrobial activity of pomegranate clearly demonstrated a broad spectrum against both bacteria and fungi, viruses, and parasites [13, 14]. In culture, pomegranate extracts preferentially prevent the growth of breast, lung, colon, and prostate cancer cells. In preclinical animal studies, oral consumption of pomegranate extracts inhibited the growth of lung, skin, colon, and prostate tumors [15, 16]. Studies in the realm of research indicate that pomegranates offer advantages for various health conditions, encompassing cardiovascular disease [17]. Pomegranates have positive effects on oral or dental health [18]. These activities could be attributed to the presence of phytochemical compounds in the extracts, such as phenols, tannins, and flavonoids, which are significant active ingredients. Certainly, it has been documented that in Brazil, the bark, leaves, blossoms, and fruits of the pomegranate tree are extensively employed as natural remedies [13].

Pomegranate and pomegranate extracts contained polyphenols in a hydro-ethanolic extract that prevented H5N1 and SARS-CoV-2 infections, according to Ganapathy [19], Maiti and Banerjee [20], Eggers, Jungke, Wolkinger, Bauer, Kessler and Frank [21]. Several studies have proved that polyphenolic compounds have many good characteristics, such as antibacterial, antioxidant, anti-inflammatory, antiproliferative, and broad-spectrum antiviral activity, by inhibiting viral DNA and RNA, and directly binding the viral particles, providing antiviral activity during intracellular replication, and DNA repair activities and thus can be used as a good alternative for systemic drugs problems [8, 22, 23]. Flavanols in pomegranate juice may help block the inflammation contributing to

osteoarthritis and cartilage damage [10]. Anthocyanins have been demonstrated to decrease the release of chemokine monocyte chemotactic protein 1, which attracts monocytes to inflammatory areas. The anthocyanins delphinidin and cyanidin have been shown to prevent the expression of vascular endothelial growth factor, a compound that stimulates atherosclerosis [24].

The primary objective of this research is to comprehensively evaluate the acute and subacute toxicity profiles of pomegranate extract in male and female Sprague Dawley rats. This assessment includes investigating the potential toxicity at a dose of 1g/kg body weight of the extract administered intraperitoneally, explicitly focusing on behavioral changes, mortality, and LD50 value determination. Additionally, the study aims to analyze the impact of pomegranate extract on daily food and water consumption, body weight changes, hematological parameters (complete blood count), serum biochemical parameters related to liver, heart, and kidney function, as well as lipid profiles. By assessing these various aspects, the study aims to establish the safety profile of pomegranate extract and provide evidence to support its safe administration at the mentioned dose in both male and female Sprague Dawley rats.

Material and Methods

Pomegranate fruit extract

Pomegranate fruits were peeled by hand; then, the seeds were stored in a refrigerator at -4°C. The seeds were mixed inside a high-speed blender at (2000 rpm) for 15 min. The mixture was filtered to gain the juice. A list of these components is presented in Table 1.

TABLE 1. Percentage of pomegranate fruit components

Type	Weight*	Percentage % to fruit	
Fruit	(5180g)	100%	
Seeds (2660g)	Juice (2010g)	Seeds 51.4%	38.87%
	Ash (400.2g)		7.73%
	Loose (249.8g)		4.80%
Peel	2340g	45.2%	
Loose	180g	3.4%	

*Each 1.015g of pomegranate juice = 1ml of pomegranate juice.
The ratio of pomegranate juice in fruit equals 38.87% but in seed = 75.60%.

The juice was divided into three parts: the first part (177ml) of natural pomegranate juice (P.J) was placed at -4°C as a stock. The second part (100ml) of natural juice was lyophilized at -50°C, 0.1mbar for three days, then was left for three days in glass container containing CaO to absorb humidity, and finally was placed under ambient conditions inside small jar (25ml) firmly closed pomegranate lyophilized juice (P.J.L). while the third part (902.875ml) of Pomegranate juice was centrifugated at 6573.84 Xg force for 15 minutes.

After centrifugation the mixture was categorised into aqueous pomegranate supernatant (Extract 2) and the dark pink colour pomegranate pellet (Extract 1) which was lyophilized at -50°C for three days at 0.1mbar before being placed under CaO for three days to absorb humidity. Thereafter Extract 1 was grinded into a powder, and was exposed to ambient conditions. The precipitate's colour changed from dark pink to brownish pink, as shown in Fig. 1 [25-29].

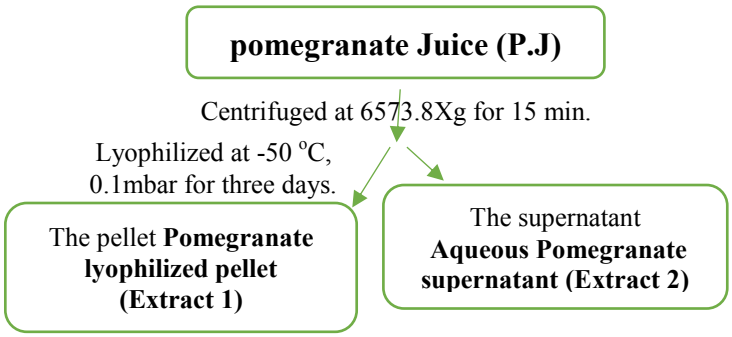


Fig. 1. Algorithm for preparation of pomegranate extracts.

Characterization and Identification Techniques

The functional groups and the structure of the compounds were identified by using FTIR. Thin films were formed by compression-molding the resultant products with KBr pellets, and FTIR spectra were obtained on (JASCO FTIR 6100 spectrometer, 64 scans with 4 cm^{-1} resolution) in the range of 4000–400 cm^{-1}. Nanomaterial size, concentration, and aggregation state evaluated by UV-Vis analysis. The UV–Vis's spectra were obtained using a double beam spectrophotometer Model JASCO V-670, Japan. The spectra were determined in the wavelength range 190-1000 nm. The compounds were dissolved in ethanol and measured in a quartz cuvette.

Based on the results obtained from both infrared (IR) and ultraviolet (UV) analyses, it was evident that extract 2 contained higher levels of flavones and polyphenolic compounds compared to the other extracts. Consequently, we opted to conduct a more in-depth investigation into its toxicological effects specifically related to polyphenols.

Experimental animals and housing conditions

Acute toxicity study

The toxicological studies on pomegranate extracts were carried out to determine the acute toxicity in male and female rats.

To achieve this objective, we divided a total of thirty rats into five groups, each consisting of six rats with a body weight ranging from 150 to 170 grams. These rats were administered the studied aqueous extract via intraperitoneal injection, with doses varying from 1 to 5 grams per kilogram of body weight.

Sub-acute toxicity study:

An animal experiment was performed to evaluate the toxicity of Extract 2; the investigation is a 2-week sub-acute toxicity study in vivo. All experimental protocols were in accordance with the national and international Guidelines for Ethical Care of Experimental Animals. The study was approved by the ethics committee of NRC (Approval number: NRC-18040), and all procedures and experiments were performed according to the approved protocol. Male and Female Sprague-Dawley (SD) rats (10 weeks old) weighing about 150-170g were purchased from the Holding Company of Production of Vaccines Sera and Drugs (VACSERA), Giza-Egypt. They were housed in the animal facility of the NRC, Egypt (3 per cage) in standard experimental conditions of temperature at 24±1°C, light of 12h dark-12h light cycle, free access to drinking water, and basic pelleted diet till the end of the experiment (2 weeks). Rats were deprived of food except water 12 h before blood sampling.

Sub-acute toxicity assessment of Extract 2 in male and female Sprague Dawley rats for 2 weeks

A total of thirty-six rats, comprising an equal number of males and females, were segregated into six male groups and six female groups, with each group consisting of three animals, as visually represented in Fig. 2, so that weight differences did not exceed ±20% of the average body weight among groups. The Extract 2 was administered intraperitoneally at concentrations of 1, 0.5, 0.25, 0.125, and 0.0625g/kg body weight daily for two weeks with a control group receiving an equivalent volume of distilled water. Food consumption and water intake were monitored daily. Rats' body weight changes were carefully observed and recorded day after day for two weeks. At the end of the experiment, animals were sacrificed under excess ketamine anesthesia, and the morphological look of the kidneys, liver, heart, and brain were carefully observed, and any specious or significant features or differences from the normal were recorded.

Hematological and biochemical analyses

Blood was taken in 2 ml plain tubes and 1 ml in heparinized tubes from the abdominal aortas of all sacrificed rats. The Plain tubes were centrifuged at 3000x, and sera were obtained and stored frozen at -20°C until use for biochemical parameters. While the heparinized tubes were used for hematological parameters of complete blood count (CBC) were analyzed using an automatic hematology analyzer (Sysmex-XT- 1800 Germany): red blood cells (RBCs); white blood cells (WBCs); hemoglobin concentration (HGB); mean corpuscular volume (MCV); mean corpuscular hemoglobin (MCH); mean corpuscular hemoglobin concentration (MCHC); platelet count (PLT); content of mixed cells MXD (monocyte, basophiles, eosinophils); Neutrophils (NEUT); hematocrit (HCT) and; lymphocytes (LYM). Later on, the serum biochemical parameters for liver function, alanine aminotransaminase (ALT), Aspartate aminotransferase (AST), and albumin (ALB) were estimated. Besides the determination of Creatine kinase MB (CK-MB), Troponin I for the heart as well as urea and creatinine for kidney function. Also, lipid profile were measured to investigate any toxic effects in tissues using a biochemistry auto analyzer (Olympus 640 Japan) and lipid profile.

Statistical analysis

Before proceeding with the statistical analysis, "data values were checked for normality using the Shapiro test and heteroscedasticity using the Brown-Forsythe test. The data are presented as means ± S.E.

Data were processed by one-way ANOVA followed by Bonferroni post-hoc testing test except for the scoring, statistical analysis was carried out by nonparametric Kruskal–Wallis H-test, followed by Dunn's test. GraphPad Prism software (version 9; GraphPad Software, Inc., San Diego, CA, USA) was employed to perform the statistical analysis and to establish the graphical representation".

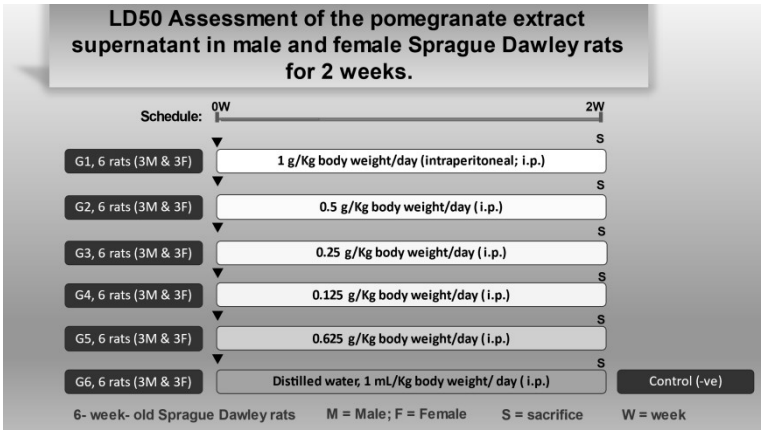


Fig. 2. Experimental Protocol 1 (2-week toxicity test).

Results

Characterization and identification of compounds

Fourier Transform-Infrared (FT-IR)Characterization

The strongest peaks of hydroxyl at 3419 cm^{-1}, α, β- unsaturated ketone band at 1710 cm^{-1}, olefin band at 1610 cm^{-1}, primary and secondary alcohols functionalities bands at 1043 cm^{-1} as well as the peaks around 3000 and 1400 cm^{-1} attribute to aliphatic C-H stretching and bending modes. Peaks at 3408 cm-1, 1717 cm-1, and 1629 cm-1 correspond to hydroxyl, oxidized ester carbonyl, and conjugated carbonyl groups, as illustrated in Fig. 3. In contrast to P.J.L. or Extract 1, Extract 2 has stronger absorption bands for the functional groups OH and C=O of polyphenolic acids and flavone, respectively.

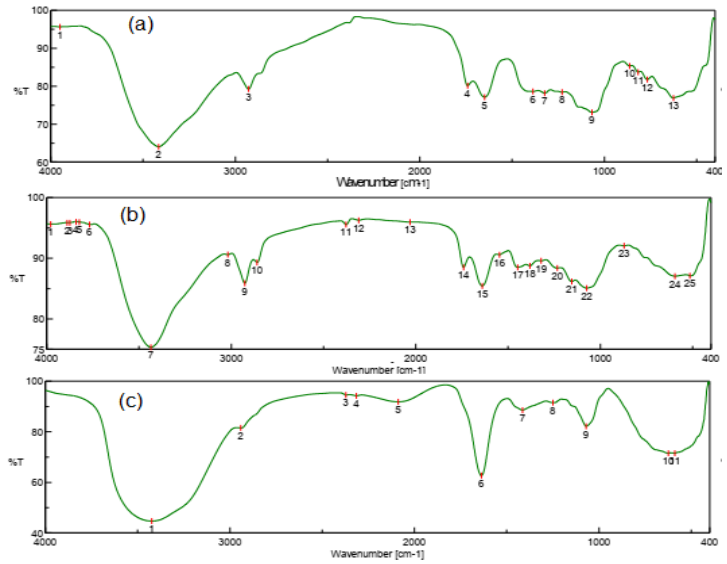


Fig. 3. FT-IR spectra of different compounds. FT-IR of (a) P.J.L. (b) Extract 1 (c) Extract 2.

UV–Visible spectrophotometer

The UV-Visible spectra illustrated that the potential source of the absorptions was explored in order to provide a chemical explanation of the loadings. Particularly, the spectral regions between 250 and 300, which are respectively characterized by pomegranate extract loading values, may be connected to ellagitannins specific to the Punica botanical gender (such as ellagic acid, punicalagin isomers, etc.) and to a pattern of antho-cyanins, which is characteristic of pomegranate (i.e., cyanidin, delphinidin, and pelargonidin 3-glucosides and 3,5-diglucosides). The polyphenols, which are abundant in the fruit and are phenolic rings with many

hydroxyl groups, are the primary class of pomegranate phytochemicals. Condensed tannins (proanthocyanidins), hydrolysable tannins (gallotannins and ellagitannins), and flavonoids (flavanols and anthocyanins) are all components of pomegranate polyphenols whereby strong absorption bands are greater in Extract 2 than P.J.L. or Extract 1, as shown in Fig. 4 with bathochromic shifted of ƛ max.

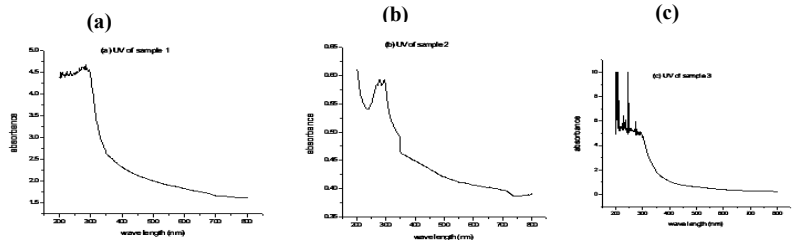


Fig. 4. UV-visible spectra of the different compounds. UV of (a) P.J.L. (b) Extract 1 (c) Extract 2.

Acute and Subacute toxicity study of Extract 2

In the present study, the 1g/kg body weight concentration used here was the Extract 2. Thus, the LD50 value for Extract 2 for intraperitoneal toxicity is greater than this value. The effect of 15 days of eleven intraperitoneal administrations of Extract 2 on the general behavior and hematological and biochemical parameters in rats were recorded. Administration of Extract 2 doses of 1, 0.5, 0.25, 0.125, and 0.0625g/kg body weight every 24h for 15 days did not record any mortality in the tested rats. There were no indications of apparent toxicity over the entire study period. There were no significant changes in the external physical structure of the tested rats. Behavioral changes like irritability, shivering, labored breathing, shock, and convulsion weren't noted in examined rats during the experiment Table 2 a & b till the end of the investigation. There were no significant differences in body weights, food consumption, or water intake between Extract 2 treated groups in relation to control Table 3. At the end of the experiment (2 weeks), Growth curves, average body weights of control and treated male rats with different doses of Extract 2 during the subacute toxicity test shown in Fig. 5 a & b and Table 4 a & b, the absolute and relative organs weights of liver, left and right kidneys, heart, and brain were recorded as shown in Table 5 a & b. Statistical analysis revealed no significant differences between treated and control rats.

Effect of (Extract 2) on complete blood count (CBC)

The majority of the assessed hematological factors listed in Table 6 for the treated groups, including WBC (white blood cell), RBC (red blood cell), HGB (hemoglobin), HCT (hematocrit), MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular hemoglobin concentration), PLT (platelet count), LYM (lymphocyte), MXD (mixed cell content, including monocytes, basophils, eosinophils), and NEUT (neutrophils), were examined for both male and female rats. Starting with the male rats, the hematological parameters were evaluated as shown in Table 6.a. in the treated groups such as WBC, LYM, RBC, HGB, MCV, MCH, MCHC, RDW-CV, PLT, MPV(F1), PCT (%) and P-LCR were not significantly different from control rats received distilled water. In contrast, LYM counts were significantly increased in group 1 by 11%. However, in groups 2, 3, and 5 by 9%, MID counts were significantly decreased by 3.5% in group 1 and by 1.5% in groups 2 and 3, GRAN% recorded a significant decrease with group 1 by 9%, GRAN recorded significant reduction with group 1 by 0.5 (10^3/µL), HCT% were decreased with group 1 by 3%, RDW-SD (fL) were reduced in group 1 and 4 by 2.5 (fL) and PDW (%) were decreased in group 1 by 3.23% with all examined doses as compared to control. On the other hand, in female rats, the evaluated hematological parameters Table 6.b. in the treated groups such as WBC, LYM, MID, GRAN, RBC, HGB, HCT, MCH, MCHC, MPV(F1), PDW, PCT (%) and P-LCR were not significantly different from control rats received distilled water. While LYM counts were significantly decreased in group 1 by 7%, groups 2 by 3%, and 4 by 6.5%, MID were significantly increased by 2.5% in group 1 and by 4.5% in groups 4, GRAN% recorded significant increase with group 1 by 4%, group 2 by 2% and group 4 by 1.6%, MCV recorded significant increase with group 1 by 2.2 (fL), group 4 by 3.5 (fL) and group 5 by 2.5 (fL), RDW-SD (fL) were increased in group 1 and 4 by 3.5 (fL) but group 5 increased by 3 (fL), RDW-CV were increased in group 2 by 1.8% and PLT(10^3/µL) were decreased in group 1 by 328.39 (10^3/µL) and group 2 by 345 (10^3/µL) with all examined doses as compared to control.

Serum Biochemistry

Effect on liver function parameters

The biochemical parameters of the liver function of all examined rats are shown in Table 7. Table 7

demonstrates the impact of Extract 2 on serum liver function parameters in untreated and treated male rats. Compared to the normal ones, statistical analysis revealed no significant differences in Alanine transaminase (ALT), Aspartate aminotransferase (AST), and Albumin (ALB). On the other hand, Table 7 displays the effect of Extract 2 on serum liver function parameters by control and treated female rats. Compared to the control ones, statistical analysis revealed no significant differences in Aspartate aminotransferase (AST) and Albumin (ALB), but Alanine transaminase (ALT) recorded a significant decrease in group 1 by 60.6µg/L and group 2 decreased by 46.5µg/L.

Effect on serum kidney function parameters:

The Urea and Creatinine levels of serum concentrations in the sera of all groups were measured as indicators for renal function in tested rats Table 8. Urea and creatinine displayed non substantial changes in tested doses among the tested groups recieving to male or female rats Table 8 with Extract 2 compared to the control group.

Effect on serum Heart function parameters:

The Troponin I, CKMB (creatine kinase myocardial band), and LDH (lactate dehydrogenase test) levels of serum concentrations in the sera of all groups were measured as indicators for heart function in tested rats Table 9. The effect of Extract 2 on serum heart function parameters by control and treated male rats is illustrated in Table 9. Troponin was significantly increased with a very weak positive in groups 1, 2, and 3, There were no notable variations in the administered doses of Extract 2 among the tested groups when compared to the control group or with respect to other parameters related to heart function. Secondly, the effect of Extract 2 on serum heart function parameters by control and treated female rats is illustrated in Table 9. Troponin was significantly elevated, with a very weak positive in group 2. Similarly, LDH was increased in groups 2 and 4 by 220 and 1200, respectively, while there were no significant differences in tested doses between the tested groups administered with Extract 2 in relation to the control group or other heart function parameters.

Effect on serum lipid profile:

The effect on the serum lipid profile of male and female rats is presented in Table 10. Firstly, the impact of Extract 2 on serum lipid profile parameters by control and treated male rats is illustrated in Table 10. a. Cholesterol (CHO) was significantly decreased in group 1 by 10µg/dL. Likewise, very low-density lipoprotein (VLDL) was significantly increased in groups 1 and 5 by 0.7 and 5µg/dL, respectively, and low-density lipoprotein (LDL) recorded a significant increase in group 4 by 4µg/dL. Counterwise, the triglyceride (TG) was significantly increased in group 5 by 8µg/dL, While the high-density lipoprotein (HDL) had no significant differences compared to the control. On the other hand, the effect of Extract 2 on serum lipid profile parameters by control and treated female rats is illustrated in Table 10. b. Cholesterol (CHO) were significantly increased in group 1 by 3.78µg/dL, and the high-density lipoprotein (HDL) recorded a significant increase in group 1, 2, 3, 4, and 5 by 6.99, 13.45, 15.23, 9.88, and 13.24µg/dL respectively. Triglyceride (TG), very low-density lipoprotein (VLDL), and low-density lipoprotein (LDL) had no significant differences compared to the control.

In the present study, the 1g/kg body weight concentration used here was the Extract 2. Thus, the LD50 value for Extract 2 for intraperitoneal toxicity is greater than this value. The effect of 15 days of eleven intraperitoneal administrations of Extract 2 on the general behavior and hematological and biochemical parameters in rats were recorded. Administration of Extract 2 doses of 1, 0.5, 0.25, 0.125, and 0.0625g/kg body weight every 24h for 15-days did not record any mortality in the tested rats. There were no indications of apparent toxicity over the entire study period. There were no significant changes in the external physical structure of the tested rats. Behavioral changes like irritability, shivering, labored breathing, shock, and convulsion weren't noted in examined rats during the experiment Table 2 a & b till the end of the investigation. There were no significant differences in body weights, food consumption, or water intake between Extract 2 treated groups in relation to the control Table 3. At the end of the experiment (2 weeks), Growth curves, average body weights of control and treated male rats with different doses of Extract 2 during the subacute toxicity test shown in Fig. 5 a & b and Table 4 a & b, the absolute and relative organs weights of liver, left and right kidneys, heart, and brain were recorded as shown in Table 5 a & b. Statistical analysis revealed no significant differences between treated and control rats.

Since no secondary symptoms were observed, and it was established that extract 2 is safe up to a dosage of 1g/kg in both male and female rats without any impact, it can be concluded that extract 2 does not affect the sexual characteristics of either gender.

TABLE 2. a. Gross observed behavioral observations accompanied with treatment with Extract 2 with male rats

Group	Dose (g/kg)	Symptoms	Score
G1	1g/kg	Irritability (0) Tremor (0) Labored breathing (0) Staggering (0) Convulsion (0) Death (0)	Good More active than control
G2	0.5g/kg	Irritability (0) Tremor (0) Labored breathing (0) Staggering (0) Convulsion (0) Death (0)	Good More active than control
G3	0.25g/kg	Irritability (0) Tremor (0) Labored breathing (0) Staggering (0) Convulsion (0) Death (0)	Good Normal activities
G4	0.125g/kg	Irritability (0) Tremor (0) Labored breathing (0) Staggering (0) Convulsion (0) Death (0)	Good Normal activities
G5	0.625g/kg	Irritability (0) Tremor (0) Labored breathing (0) Staggering (0) Convulsion (0) Death (0)	Good Normal activities
G6	1mL/kg (distilled water)	Irritability (0) Tremor (0) Labored breathing (0) Staggering (0) Convulsion (0) Death (0)	Normal

TABLE 2. b. Gross observed behavioral observations accompanied with treatment with Extract 2 with female rats

Group	Dose (g/kg)	Symptoms	Score
G1	1g/kg	Irritability (0) Tremor (0) Labored breathing (0) Staggering (0) Convulsion (0) Death (0)	Good More active than control
G2	0.5g/kg	Irritability (0) Tremor (0) Labored breathing (0) Staggering (0) Convulsion (0) Death (0)	Good More active than control
G3	0.25g/kg	Irritability (0) Tremor (0) Labored breathing (0) Staggering (0) Convulsion (0) Death (0)	Good Normal activities
G4	0.125g/kg	Irritability (0) Tremor (0) Labored breathing (0) Staggering (0) Convulsion (0) Death (0)	Good Normal activities
G5	0.625g/kg	Irritability (0) Tremor (0) Labored breathing (0) Staggering (0) Convulsion (0) Death (0)	Good Normal activities
G6	1mL/kg (distilled water)	Irritability (0) Tremor (0) Labored breathing (0) Staggering (0) Convulsion (0) Death (0)	Normal

TABLE 3. Food consumption and water intake by control and treated rats with different doses of Extract 2 during the subacute toxicity test (Mean± SD for 3 determinations).

Group	Dose (g/kg)	Food consumption (g/day)		Water intake (ml/2 days)	
		Male	Female	Male	Female
G1	1g/kg	22.65±0.3[a]	22.3±0.3[a]	41.8±0.4[a]	41.9±0.4[a]
G2	0.5g/kg	22±0.7	22.3±0.7	41.2±0.4	41.7±0.4
G3	0.25g/kg	21.5±1.0	21.8±1.0	40.9±0.5	41.7±0.5
G4	0.125g/kg	21.9±1.3	22.1±1.3	40.9±0.8	41.4±0.8
G5	0.625g/kg	21.9±0.7	22±0.7	41.0±0.4	41.5±0.4
G6	1mL/kg (distilled water)	22.4±1.3	22.5±1.3	42.5±0.4	42.4±0.4

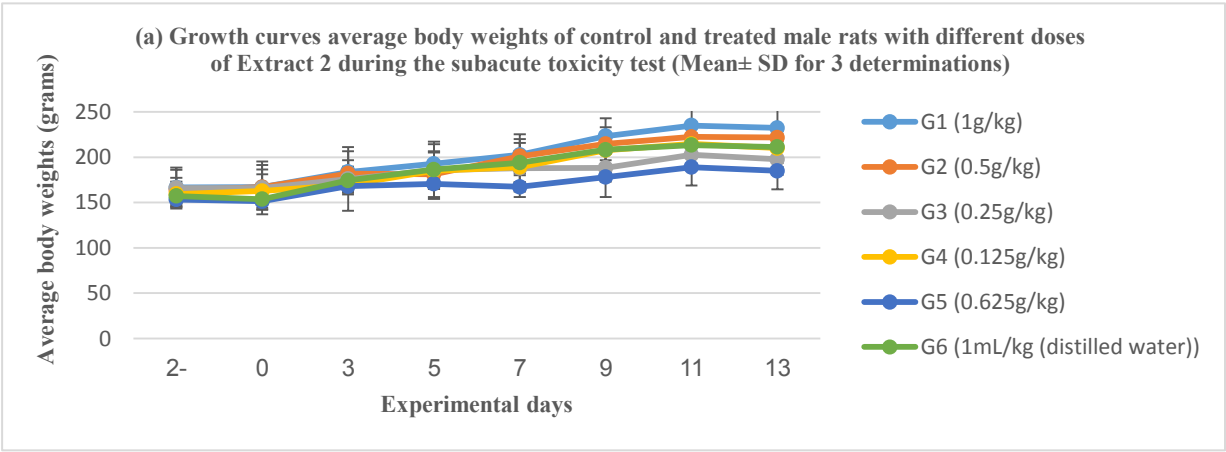
a: Means ± S.D.

TABLE 4. a. Growth curves, average body weights of control and treated male rats with different doses of Extract 2 during the subacute toxicity test (Mean± SD for 3 determinations)

Groups /does (g/kg)	Experimental days							
	-2	**0**	**3**	**5**	**7**	**9**	**11**	**13**
G1 (1g/kg)	166.66± 19.39	167± 24.43	183.66± 22.94	192.66± 21.77	202.66± 22.72	223.33± 19.60	235± 20.07	232.33± 20.84
G2 (0.5g/kg)	163.66± 13.793	167± 19.46	182± 14.73	181.33± 25.10	201± 6.082	214.66± 3.51	222.33± 4.04	221.66± 3.785
G3 (0.25g/kg)	166± 22.64	166± 29.13	176± 35.17	185.66± E31.65	188± 32.04	188.33± 32.33	202.66± 33.84	197.66± 33.17
G4 (0.125g/kg)	159.33± 13.86	162.66± 18.33	169.66± 8.082	185± 6.55	189± 6.92	207.66± 10.969	214.66± 9.60	210± 10
G5 (0.625g/kg)	153.33± 1.1541	151.33± 0.57	168± 4.00	170.66± 5.686	167.33± 5.13	178± 5	189± 2.64	185± 4.35
G6 (1mL/kg (distilled water))	157.33± 12.09	153.66± 11.71	174.33± 15.37	186.33± 18.82	194± 21.79	208.33± 24.84	213.33± 20.50	211.33± 17.925

TABLE 4. b. Growth curves, average body weights of control and treated female rats with different doses of Extract 2 during the subacute toxicity test (Mean± SD for 3 determinations)

Groups/does (g/kg)	Experimental days							
	-2	0	3	5	7	9	11	13
G1 (1g/kg)	177.67± 30.89	179.00± 37.32	190.00± 30.51	199.33± 31.34	209.00± 31.43	220.33± 32.62	224.00± 32.92	218.00± 30.32
G2 (0.5g/kg)	183.67± 17.01	189.67± 17.01	196.00± 30.32	188.33± 26.56	201.67± 26.65	219.33± 28.36	220.67± 28.04	215.67± 24.54
G3 (0.25g/kg)	176.00± 14.11	179.33± 20.55	191.33± 15.04	187.67± 14.19	183.67± 12.90	208.00± 16.52	208.33± 19.86	210.00± 22.72
G4 (0.125g/kg)	178.67± 42.77	178.00± 48.54	173.33± 43.04	190.67± 44.47	198.67± 41.88	213.00± 37.27	220.33± 34.44	214.67± 30.66
G5 (0.625g/kg)	176.00± 4.58	179.00± 8.54	195.33± 6.66	205.00± 10.15	213.00± 11.14	226.67± 14.01	231.67± 16.17	235.00± 14.53
G6 (1mL/kg (distilled water))	154.67± 12.90	171.33± 16.65	170.67± 16.17	169.33± 16.26	189.00± 21.28	200.33± 18.23	193.67± 12.90	189.67± 12.58



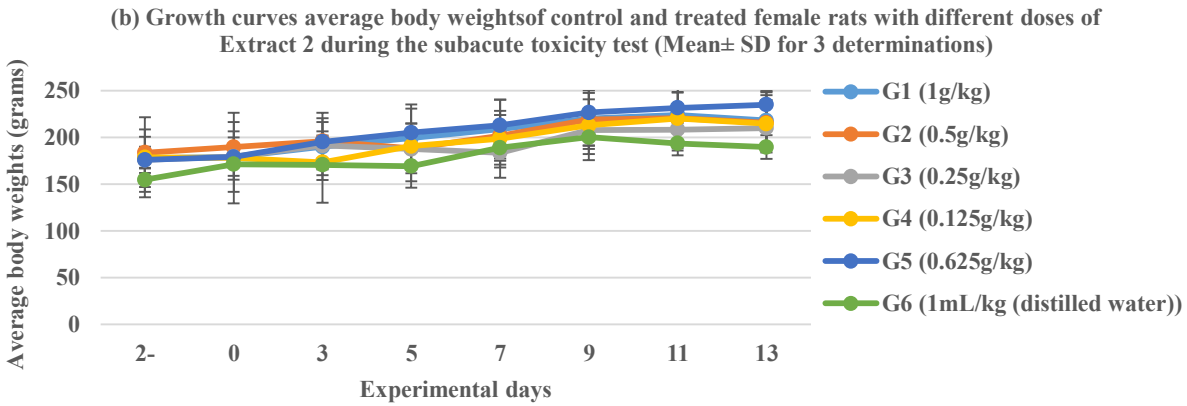


Fig. 5. (a).Growth curves, average body weights of control and treated male rats with different doses of Extract 2 during the subacute toxicity test. (b) Growth curves average body weights of control and treated female rats with varying doses of Extract 2 during the subacute toxicity test. Each value represents the mean of three samples ±SD.

Table 5. a. Absolute and Relative Organ Weights of Control and Treated Male Rats with Extract 2

Group	Dose (g/kg)	Avg. body weight(g)	Organs				
			Liver	L. Kidney	R. Kidney	Heart	Brain
G1	1g/kg	232.33±20.8	6.58±0.36[a] (2.8)[b]	0.66±0.06[a] (0.29)[b]	0.77±0.05[a] (0.33)[b]	0.78±0.08[a] (0.34)[b]	1.68±0.24[a] (0.72)[b]
G2	0.5g/kg	221.67±3.7	6.97±0.15[a] (3.14)[b]	0.72±0.01[a] (0.32)[b]	0.73±0.01[a] (0.33)[b]	0.72±0.01[a] (0.32)[b]	1.67±0.04[a] (0.75)[b]
G3	0.25g/kg	197.67±33.17	5.60±1.3[a] (2.83)[b]	0.58±0.12[a] (0.29)[b]	0.58±0.12[a] (0.30)[b]	0.58±0.12[a] (0.29)[b]	1.34±0.38[a] (0.68)[b]
G4	0.125g/kg	210.00±10	6.30±0.4[a] (3.0)[b]	0.65±0.03[a] (0.31)[b]	0.66±0.04[a] (0.31)[b]	0.65±0.04[a] (0.31)[b]	1.51±0.12[a] (0.72)[b]
G5	0.625g/kg	185.00±4.35	4.88±0.17[a] (2.63)[b]	0.50±0.02[a] (0.27)[b]	0.51±0.02[a] (0.28)[b]	0.50±0.02[a] (0.27)[b]	1.17±0.05[a] (0.63)[b]
G6	1mL/kg (distilled water)	211.33±17.92	6.38±0.71[a] (3.01)[b]	0.66±0.06[a] (0.31)[b]	0.67±0.06[a] (0.32)[b]	0.66±0.07[a] (0.31)[b]	1.53±0.21[a] (0.72)[b]

a: Values are Means ± S.D (for three determinations) of absolute weight.; b: Numbers between parenthesis is relative organs weights: Relative organ wt.= Mean organ wt. /Mean b. wt. XI00 (%); *: Significant vs. control (G6).

TABLE 5. b. Absolute and Relative Organ Weights of Control and Treated Female Rats with Extract 2

Group	Dose (g/kg)	Avg. body weight(g)	Organs				
			Liver	L. Kidney	R. Kidney	Heart	Brain
G1	1g/kg	218.00±30.31	6.71±1.20[a] (3.07)[b]	0.82±0.12[a] (0.38)[b]	0.84±0.13[a] (0.39)[b]	0.82±0.13[a] (0.38)[b]	1.70±0.44[a] (1.45)[b]
G2	0.5g/kg	215.67±24.5	6.58±0.97[a] (3.05)[b]	0.80±0.10[a] (0.37)[b]	0.82±0.10[a] (0.38)[b]	0.81±0.11[a] (0.37)[b]	1.68±0.36[a] (1.45)[b]
G3	0.25g/kg	210.0±22.7	6.25±0.90[a] (2.98)[b]	0.76±0.09[a] (0.36)[b]	0.78±0.10[a] (0.37)[b]	0.77±0.10[a] (0.37)[b]	1.60±0.33[a] (1.45)[b]
G4	0.125g/kg	214.67±30.66	6.52±1.22[a] (3.04)[b]	0.80±0.13[a] (0.37)[b]	0.82±0.13[a] (0.38)[b]	0.80±0.13[a] (0.37)[b]	1.67±0.45[a] (1.45)[b]
G5	0.625g/kg	235.00±14.52	7.68±0.578[a] (3.26)[b]	0.94±0.06[a] (0.40)[b]	0.96±0.06[a] (0.41)[b]	0.94±0.06[a] (0.40)[b]	1.70±0.21[a] (1.45)[b]
G6	1mL/kg (dist.water)	189.67±12.58	6.20±0.50[a] (3.28)[b]	0.76±0.05[a] (0.40)[b]	0.78±0.05a (0.41)b	0.70±0.05[a] (0.37)[b]	1.55±0.18[a] (1.45)[b]

a: Values are Means ± S.D (for three determinations) of absolute weight.; b: Numbers between parenthesis is relative organs weights: Relative organ wt.= Mean organ wt. /Mean b. wt. XI00 (%); *: Significant vs. control (G6).

TABLE 6. a. Effect of Extract 2 on the hematological parameters of Control and Treated Male Rats

Group No.	G1	G2	G3	G4	G5	G6
WBC (10^3/uL)	5.10± 0.20	10.10±6.08	9.80±1.84	6.45±1.20	8.30±0.20	10.90±5.94
LYM (%)	77.9± 0.20 *	74.10±4.38 *	73.60±1.27 *	58.80±12.16	72.70±0.20 *	62.15±4.17
MID (%)	11.30±0.20 *	10.65±2.90 *	11.85±1.34 *	13.75±1.63	14.30±0.20	16.90±1.84
GRAN (%)	10.80±0.20 *	15.25±1.48	14.55±0.07	27.45±10.54	13.00±0.20	20.95±2.33
LYM (10^3/uL)	4.00±0.20	7.35±4.03	7.25±1.48	3.85±1.48	6.00±0.20	6.90±4.10
MID (10^3/uL)	0.60±0.20	1.15±0.92	1.15±0.07	0.85±0.07	1.23±0.25	1.80±0.85
GRAN (10^3/uL)	0.57±0.12 *	1.60±1.13	1.40±0.28	1.75±0.35	1.13±0.15	2.20 ±0.99
RBC (10^6/uL)	5.68±0.20	5.94±0.78	5.79±0.06	5.58±0.06	6.21±0.02	5.55±0.27
HGB (G/Dl)	10.60±0.20	11.60±1.98	11.20±0.42	10.80±-	12.50±0.20	10.10±0.14
HCT (%)	33.10±0.20 *	34.45±4.60	31.10±0.42	31.55±0.21	35.50±0.20	29.35±0.92
MCV (fL)	58.30±0.20	58.00±0.14	53.85±0.21	56.70± 0.99	57.30±0.20	53.05±4.31
MCH (pg)	18.60±0.20	19.45±0.78	19.30±0.57	19.30±0.14	20.10±0.20	18.15±0.64
MCHC (g/dL)	32.00±0.20	33.50±1.27	35.95±0.92	34.15±0.21	35.20±0.20	34.40±1.56
RDW-SD (fL)	36.30±0.20 *	37.40±4.53	30.95±1.48	37.40±1.56 *	34.20±0.20	30.95±1.48
RDW-CV(%)	16.60±0.20	17.10±2.12	14.95±0.78	17.50±0.42	15.90± 0.20	15.25±0.21
PLT (10^3/uL)	1,503. 0±٢.٠0	858.0± 306.88	940.0±261.63	885.5±259.51	498.94±425.24	1,223.0±708.5
MPV(Fl)	9.10± 0.20	7.65± 0.64	6.95±0.64	7.20±0.14	7.30±0.20	7.55±0.64
PDW (%)	12.80±0.20 *	10.00±1.84	8.45±1.48	9.20±0.71	10.00±0.20	8.80±0.57
PCT (%)	1.36 ±0.20	0.67±0.29	0.66±0.24	0.63±0.17	0.54±0.02	0.94±0.61
P-LCR	16.70±0.20	5.55± 0	5.25 ±3.04	2.05±2.90	5.70±0.20	8.10±8.20

Values are means ± S.D. (for Three determinations); WBC, white blood cell; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelets count; LYM, lymphocyte; MXD, the content of mixed cells (monocyte, basophiles, eosinophils); NEUT, Neutrophils; g/dl (grams per deciliter); fL (femto liters); pg (picograms); *: Significant vs. control (G6).

TABLE 6. b. Effect of Extract 2 on the hematological parameters of Control and Treated Female Rats

Group No.	G1	G2	G3	G4	G5	G6
WBC (10^3/uL)	10.40±0.20	10.40±0.20	11.85±2.05	9.30±0.20	13.85±1.20	9.53±2.80
LYM (%)	69.30±0.20 *	72.80±0.20 *	74.75±1.20	69.40±0.20 *	٧٣,٩٠±5.09	78.07±1.58
MID (%)	13.80±0.20 *	12.50±0.20	13.80±1.13	16.20±0.20 *	11.95±0.64	11.13±0.46
GRAN (%)	16.90±0.20 *	14.70±0.20 *	11.45±0.07	14.40±0.20 *	14.15±4.45 *	10.80±1.73
LYM (10^3/uL)	7.20±0.20	7.60±0.20	8.90±1.70	6.50±0.20	10.20±0.14	7.43±2.16
MID (10^3/uL)	1.40±0.20	1.30±0.20	1.60±0.14	1.50±0.20	1.65±0.21	1.07±0.31
GRAN (10^3/uL)	1.80±0.20	1.50±0.20	1.35±0.21	1.30±0.20	2.00±0.85	1.03±0.35
RBC (10^6/uL)	5.88±0.20	6.13±0.20	6.34±1.47	5.96±0.20	5.66±0.37	6.27±0.27
HGB (G/Dl)	11.50±0.20	11.50±0.20	12.65±2.90	11.50±0.20	11.45±0.78	11.93±0.57
HCT (%)	33.10±0.20	32.60±0.20	35.80±5.80	34.30±0.20	33.95±0.35	33.20±1.82
MCV (fL)	56.40±0.20 *	53.20±0.20	57.05±4.03	57.70±0.20 *	60.25±3.46 *	53.03±1.01
MCH (pg)	19.50±0.20	18.70±0.20	19.95±0.07	19.20±0.20	20.20± -	19.00±0.56
MCHC (g/dL)	34.70±0.20	35.20±0.20	35.10±2.40	33.50±0.20	33.65±1.91	35.90±0.69
RDW-SD (fL)	29.90±0.20	34.20±0.20 *	34.20±6.08	34.20±0.20 *	36.35±3.04 *	29.20±1.21
RDW-CV (%)	14.10±0.20	16.70±0.20 *	15.70±1.98	15.70±0.20	16.05±0.49	14.30±0.46
PLT (10^3/uL)	1,116.0±0.20 *	1,099.0±0.20 *	1,485.0±714.18	1,814.0±0.20	1,466.0±186.68	2,151.6±707.21
MPV (Fl)	7.50±0.20	7.30±0.20	8.25±0.07	8.10±0.20	8.35±0.49	7.43±0.42
PDW (%)	10.00±0.20	8.70±0.20	11.10±1.27	9.70±0.20	12.40±1.27	9.20±0.30
PCT (%)	0.83±0.20	0.80±0.20	1.22±0.58	1.46±0.20	1.23±0.23	1.61± 0.60
P-LCR	7.90±0.20	-±.٠	10.65±2.05	11.90±0.20	12.75±1.20	7.43± 4.44

Values are means ± S.D. (for Three determinations); WBC, white blood cell; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin MCHC, mean corpuscular hemoglobin concentration; PLT, platelets count; LYM, lymphocyte; MXD, the content of mixed cells (monocyte, basophils, eosinophils); NEUT, Neutrophils; g/dl (grams per deciliter); fL (femto liters); pg (picograms); *: Significant vs. control (G6).

TABLE 7. Effect of Extract 2 on Serum Liver Function Parameters by control and treated rats.

Group	Dose (g/kg)	ALT (SGPT)		AST (SGOT)		ALB	
		Male	Female	Male	Female	Male	Female
G1	1g/kg	48.33±12.34	33.03±6.94 *	230.33±60.58	271.0±119.6	4.00±0.95	4.83±1.52
G2	0.5g/kg	46.13±7.38	50.1±4.24 *	242.66±72.50	250.33±16.50	5.16±1.20	4.11±0.20
G3	0.25g/kg	48.96±12.88	87.83±22.54	241.66±131.7	217.66±32.12	5.47±2.28	3.41±0.33
G4	0.125g/kg	39.77±13.88	99.7±25.68	252.66±102.7	207.86±124.15	4.16±1.48	3.94±0.60
G5	0.625g/kg	49.15±6.85	101.0±21.0	274.5±51.61	261.66±76.86	4.70±0.88	3.12±0.13
G6	1mL/kg (distilled water)	47.33±17.56	189.3±88.43	269.33±72.39	231.0±192.82	2.87±0.93	3.11±0.82

*Values are means ± S.D. (for 3 determinations); ALT: Alanine transaminase; AST: Aspartate aminotransferase; ALB: albumin; 1U/1: international unit. *: Significant vs. control (G6).*

TABLE 8. Effect of Extract 2 on Serum kidney Function Parameters by control and treated rats.

Group	Dose (g/kg)	Urea		Creatinine	
		Male	Female	Male	Female
G1	1g/kg	43.13±11.57	50.50± 2.63	0.60±0.16	0.67±0.04
G2	0.5g/kg	40.99±9.10	43.43±1.40	0.57± 0.13	0.64±0.11
G3	0.25g/kg	30.50±12.18	60.13±18.11	0.43±0.17	0.54±0.16
G4	0.125g/kg	43.13±2.88	50.20±6.29	0.60±0.04	0.56±0.07
G5	0.625g/kg	45.75±0.0	40.33±30.96	0.64±0.0	0.68±0.15
G6	1mL/kg (dist.water)	45.75±3.22	56.70±16.07	0.64±0.05	1.03±0.54

Values are means ± S.D (for 3 determinations); Troponin I, CKMB: Creatine kinase myocardial band; LDH: lactate dehydrogenase test; v.w.pos.,: very weak positive. *: Significant vs. control (G6).

TABLE 9. Effect of Extract 2 on Serum Heart Function Parameters by control and treated male rats.

Group	Dose (g/kg)	CKMB		Troponin		LDH	
		Male	Female	Male	Female	Male	Female
G1	1g/kg	<3	<3	v.w.pos., *	Negative	3510.6±1635.19	2637.33±723.11
G2	0.5g/kg	<3	<3	v.w.pos., *	v.w.pos., *	3237.6±1534.8	3822.33±253.7 *
G3	0.25g/kg	<3	<3	v.w.pos., *	Negative	3361±1346.44	3460±2154.64
G4	0.125g/kg	<3	<3	Negative	Negative	3327.6±1534.09	5227±665.32 *
G5	0.625g/kg	<3	<3	Negative	Negative	1776.0±219.20	4600±2630.34
G6	1mL/kg (dist. water)	<3	<3	Negative	Negative	3211±1938.89	3066±282.42

Values are means ± S.D (for 3 determinations); mmol/L, Millimoles per Liter. *: Significant vs. control (G6).

TABLE 10. a. Effect of Extract 2 on Serum Lipid Profile by control and treated male rats.

Group	Dose (g/kg)	CHO Mg/dl	TG Mg/dl	HDL Mg/dl	VLDL Mg/dl	LDL Mg/dl
G1	1g/kg	65.87±24.39	41.33±6.11	40.67±9.29	8.27 ±1.22*	16.93±10.59
G2	0.5g/kg	57.20±0.72*	50.0 ±20.66	42.67 ±2.89	10.0 ±4.13	4.53 ±3.60
G3	0.25g/kg	51.93±14.51	45.87 ±20.65	36.67 ±16.44	9.17 ±4.13	6.09 ±2.24
G4	0.125g/kg	59.77±13.63	42.67 ±16.44	36.67 ±13.80	8.53 ±3.29	14.57±3.46*
G5	0.625g/kg	64.60 ±0.57	79.0 ±22.06*	48.50 ±7.78	15.80 ±4.41*	4.06 ±2.80
G6	1mL/kg (distilled water)	73.83 ±15.51	32.53 ±15.78	57.33 ±10.69	4.88 ±1.42	7.32 ±0.25

Values are means ± S.D.; No. of rats tested: = 3; CHO: Cholesterol; TG: Triglyceride; HDI: High-density lipoprotein; VLDL: very low-density lipoprotein; LDL: Low-density lipoprotein; *: Significant vs. control (G6).

TABLE 10. b. Effect of Extract 2 on Serum Lipid Profile by control and treated female rats.

Group	Dose (g/kg)	CHO Mg/dl	TG Mg/dl	HDL Mg/dl	VLDL Mg/dl	LDL Mg/dl
G1	1g/kg	69.07 ±5.90	145.00±64.71	51.67±10.97*	29.00±12.94	11.6±19.36
G2	0.5g/kg	60.40 ±5.11	41.03 ±22.53	50.67 ±3.51*	8.21 ±4.51	1.53 ±6.17
G3	0.25g/kg	70.87 ±10.77*	63.83 ±28.81	57.33 ±8.39*	12.77 ±5.76	0.77 ±5.60
G4	0.125g/kg	54.40 ±3.92	66.77 ±29.44	45.67 ±2.08*	13.35 ±5.89	4.62±7.51
G5	0.625g/kg	61.40 ±9.11	102.33±10.73	58.67±11.72*	20.47 ±2.15	17.73±7.70
G6	1mL/kg (dist.water)	51.57 ±4.75	79.97 ±28.75	27.43 ±6.28	15.99 ±5.75	8.14±12.26

Values are means ± S.D.; No. of rats tested: = 3; CHO: Cholesterol; TG: Triglyceride; HDI: High-density lipoprotein; VLDL: very low-density lipoprotein; LDL: Low-density lipoprotein; *: Significant vs. control (G6).

Discussion

As previously mentioned, medicinal plants have antimicrobial activities [30]. Pomegranate fruits are rich in polyphenols, such as ellagitannins (ETs), mainly including α and β isomers of punicalagin (PC), gallic acid (GA), ellagic acid (EA) and its glycosylated derivatives, and anthocyanins. Several studies showed similarities to the present study as demonstrated the antiviral potential of some classes of polyphenols against influenza virus and other viruses causing respiratory tract-related infections as revealed by Ganapathy [19], Tito, Colantuono, Pirone, Pedone, Intartaglia, Giamundo, Conte, Vitaglione and Apone [31], Reddy, Gupta, Jacob, Khan and Ferreira [32], Lin, Lin, Chen, Hsu, Liu, Hwang and Wan [33], Zang, Xie, Deng, Wu, Wang, Peng, Li, Ni, Luo and Liu [34]. Ganapathy [19], Maiti and Banerjee [20], Eggers, Jungke, Wolkinger, Bauer, Kessler and Frank [21], Tito, Colantuono, Pirone, Pedone, Intartaglia, Giamundo, Conte, Vitaglione and Apone [31], Frank, Conzelmann, Weil, Groß, Jungke, Eggers, Müller, Münch and Kessler [35]

The IR spectrum reading, demonstrated that the pomegranate extracts obtained many peaks. Several works, such as that of Magne [36], Abboud, Saffaj, Chagraoui, El Bouari, Brouzi, Tanane and Ihssane [37], have reported similar observations. Peaks were observed at 3408 cm^{-1}, 1717 cm^{-1}, and 1629 cm^{-1}. Agree with the hydroxyl, oxidated ester carbonyl, and conjugated carbonyl groups, respectively. Ascorbic acid (natural vitamin C), polyphenols, and other phytonutrients found in Extract 2, which has strong absorption bands bigger than P.J.L. or Extract 1, are principally necessary for the bioreduction process. This is because of the scavenging properties of their -OH groups. Polyphenolic substances' antioxidant properties are mainly due to their redox characteristic, enabling them to function as reducing agents [38].

The UV-Visible spectrum indicated the potential source of the absorptions was explored to provide a chemical explanation of the loadings. Particularly, the spectral regions between 250 and 300, which are, respectively, defined by loading values for Extract 2 that are higher than those for P.J.L. and Extract 1, may be connected to ellagitannins specific to the Punica botanical gender (such as ellagic acid, punicalagin isomers, etc.) and to a pattern of anthocyanins, which is characteristic of pomegranate (i.e., cyanidin, delphinidin, and pelargonidin 3-glucosides and 3,5-diglucosides). Polyphenols, which are abundant in fruit and are phenolic rings with many hydroxyl groups, are the primary class of pomegranate phytochemicals. Condensed tannins (proanthocyanidins), hydrolysable tannins (gallotannins and ellagitannins), and flavonoids (flavanols and anthocyanins) are all components of pomegranate polyphenols. A comparable occurrence was noted by Gil, Tomás-Barberán, Hess-Pierce, Holcroft and Kader [39], Barzinjy, Hamad, Esmaeel, Aydın and Hussain [40].

In the current study no mortality was recorded at the dose of 1g/kg body weight of Extract 2 (the maxmium soluble dose in 10ml/kg of body weight of rats). Therefore, the LD50 value for Extract 2 intraperitoneal toxicity is believed to be higher than this value. During the course of 15 days, we closely monitored the general behavior, as well as hematological and biochemical parameters, in rats that received eleven intraperitoneal doses of (Extract 2). It's important to note that none of the rats subjected to these doses, which ranged from 1 to 0.625g/kg body weight administered every 24 hours for 15 days, exhibited any signs of mortality. No apparent toxicity was found at any point over the entire testing period. The studied rats' outward physical structure did not change significantly. Our results showed no behavioral alterations, such as shivering, irritability, shocking, Laboured breathing, or convulsion, were seen in the investigated rats during the trial period. Compared to the control groups, there were no appreciable differences in body weights, food intake, or water intake between the groups treated with Extract 2. The liver and kidneys, heart, and brain weights were weighed at the end of the experiment (two weeks), and the results showed no appreciable differences between treated and controlled rats, according to statistical analysis.

Most of the hematological parameters evaluated Table 6 have shown the WBC, RBC, HGB, HCT, MCV, MCH, MCHC, PLT, LYM, MXD, and NEUT counts for male and female rats. Their hematological parameters analyzed did not differ noticeably from the control rats receiving distilled water.

The blood levels of biochemical indicators linked with liver function, namely ALT, AST, and ALB, were measured in each group of rats given the test compound. In addition, the amounts of urea and creatinine in the serum were measured to evaluate renal function, while troponin I, CKMB, and LDH were measured to evaluate cardiac function. In addition, the influence of the extract on the lipid profile of male and female rats was evaluated. There were no statistically significant changes in the serum function parameters affected by Extract 2 in untreated and treated rats.

Jacinto [41], Bhandary, Sharmila, Kumari and Bhat [42] revealed no behavior change or death in the treated groups. More than 2000 mg/kg of body weight was the LD50 value. No significant differences ($p<0.05$) were seen between the test groups regarding weight gain, food intake, or water intake. According to the current findings, neither the hematological, biochemical parameters nor organ

weights showed appreciable changes ($p<0.05$) between the treatment and control groups, as demonstrated by the present results.

Pomegranate toxicity tests in rats revealed LD50 of 217mg/kg body weight intraperitoneally and >5 g/kg body weight orally. Clinical observation, body weight, ocular examination, food consumption, and organ weight all had no statistically significant value. Differences from the control were seen in serum chemistry and hematology measures, but there was no evidence of a harmful effect on biological variation as presented by Lestari, Soemardji and Fidrianny [43], Patel, Dadhaniya, Hingorani and Soni [44].

According to the literature, the microencapsulated pomegranate juice (MPJ) was tested for toxicity in Wistar rats and CD-1 mice to see whether there were any fatalities or adverse side effects after large dosages of 5000 mg/kg were given orally to rats for 14 days, showing there was no subacute toxicity. Like this, CD-1 mice given 3000 mg/kg MPJ daily for 90 days did not exhibit subacute toxicity. MPJ prevented both rats and mice from gaining weight. Using the Alamar blue assay, MPJ had no cytotoxicity in epithelial cell culture. Additionally, the absence of toxicity in CD-1 mice, as shown by Álvarez-Cervantes, Izquierdo-Vega, Morán-León, Guerrero-Solano, García-Pérez, Cancino-Díaz, Belefant-Miller and Betanzos-Cabrera [45] was confirmed by histological examination of the kidney and liver. This agrees with the current research results; Extract 2 did not exhibit subacute toxicity, but it differed because it caused both rats and mice to gain less weight.

At levels typically employed in the current system of medicine, animal studies have failed to document any toxicities, as signified by Jacinto [41], Bhandari [46], Vidal, Fallarero, Peña, Medina, Gra, Rivera, Gutierrez and Vuorela [47].

Clinically, Aviram, Rosenblat, Gaitini, Nitecki, Hoffman, Dornfeld, Volkova, Presser, Attias and Liker [17], Bhandari [46] demonstrated that juice consumption (121 mg/LEA equivalents) for up to three years was shown by 10 patients with carotid artery stenosis to have no harmful effects on the blood chemistry analysis for kidney, liver, or heart function.

Numerous investigations confirmed similarities to the current study; there were no adverse changes in blood or urine test values in human trials utilizing doses of pomegranate fruit extracts up to 1,420 mg/day (870 mg gallic acid equivalents), for 28 days Jacinto [41], Heber, Seeram, Wyatt, Henning, Zhang, Ogden, Dreher and Hill [48].

The overall finding of this study suggests that Extract 2 from Punica granatum is safe up to 1g/kg body weight intraperitoneal administration and can be considered nontoxic.

Conclusion

The results of toxicity characteristics in vivo in male and female Sprague Dawley rats proved a safety dose of pomegranate extract by 1g/Kg. We conclude from the previous results that pomegranate extract is safe.

*Conflict of interest:*None

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Ethical approval

Animals were treated according to the national and international ethics guidelines. The study was approved by the ethics committee of NRC (Approval number: NRC-18040), and all procedures and experiments were performed according to the approved protocol.

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التقييم السمي لمستخلص فاكهة الرمان في ذكور وإناث الفئران

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غالبًا ما يتم استهلاك فاكهة الرمان (Punica granatum L.) كفاكهة طازجة وعصير. يعتبر الرمان "فاكهة رائعة" في الثقافات القديمة والحديثة بسبب فوائده الطبية المتنوعة في الطب التقليدي. تهدف الدراسة الحالية إلى تقييم ملامح السمية الحادة وتحت الحادة لمستخلص Punica granatum (L) في ذكور وإناث فئران سبراغ داولي باستخدام المبادئ التوجيهية للجنة الأخلاقية. في تحقيقات السمية الحادة، تم إعطاء الفئران ١ جرام/كجم من وزن الجسم من المستخلص داخل التجويف البريتوني. في تجربة السمية تحت الحادة، تم إعطاء الفئران المستخلص (١ جرام/كجم (وزن الجسم)) داخل التجويف البريتوني كل ٢٤ ساعة لمدة ١٥ يومًا، بينما تلقت المجموعة الضابطة ماء مقطر. تم تسجيل الإستهلاك اليومي من الطعام والشراب. لمدة أسبوعين، تم فحص وتوثيق التغيرات في وزن جسم الفئران بعناية. تم تشريح الحيوانات تحت التخدير في نهاية التجربة، وتم ملاحظة المظهر المورفولوجي للكلى والكبد والقلب والدماغ بعناية، وتم تسجيل أي سمات ملحوظة أو تغييرات عن الوضع الطبيعي. لاستكشاف التأثيرات السامة الرئيسية في الأنسجة، تم متابعة معايير أمراض الدم من خلال تحليل صورة الدم الكامل(CBC) ، وتم تحديد المعايير الكيميائية الحيوية في سيرا الدم من خلال تحاليل وظائف الكبد والقلب والكلى، وكذلك ملف الدهون. لم تكن هناك تغييرات سلوكية أو وفيات مسجلة في المجموعات المعالجة. كانت قيمة LD_{50} أكبر من ١ جرام/كجم (وزن الجسم). لم تكن هناك فروق ذات دلالة إحصائية ($P < 0.05$) في زيادة وزن الجسم والغذاء وتناول الماء في مجموعات الإختبار. لم تظهر معايير تحاليل الدم كذلك أوزان الأعضاء أي تغيرات كبيرة ($P < 0.05$) بين مجموعات العلاج والضابطة. تكشف النتيجة الإجمالية لهذه الدراسة أن مستخلص Punica granatum (L) آمن حتى ١ جرام/كجم (وزن الجسم) عند تناوله داخل التجويف البريتوني في ذكور وإناث فئران سبراغ داولي، ونستنتج من النتائج السابقة أن مستخلص الرمان آمن.

الكلمات الدالة: Punica granatum (L)، مستخلص الرمان، السمية الحادة، السمية تحت الحادة، السلامة.