Acute and Sub-acute Toxicity of Crataegus Aronia Syn. Azarolus (L.) Whole Plant Aqueous Extract in Wistar Rats

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Abstract: Problem statement: Hawthorn comprises more than 200 species worldwide but very few species have been used medicinally. In Mediterranean region, the predominant species of the genus Crataegus is C. aronia syn. Azarolus (L.). The extracts or tinctures prepared from the leaves, flowers and/or a fruit has been used traditionally for the treatment of different diseases including different cardiovascular problems. This species (C. aronia syn. Azarolus (L.)) has not been adequately studied. Thus, we aimed to investigate for the possible acute and sub-acute toxicity of C. aronia syn. Azarolus (L.) on Wistar albino rats. Approach: The phytochemical screening of the aqueous extract of C. aronia syn. Azarolus (L.) was determined. A thirty six Wistar rats of both sexes weighing 180-200 g. were divided randomly into sex groups of 6 rats each. The first group was the control group and fed with equal volume of distilled water, while the other 5 groups were given single daily dose of the aqueous extract per os at different doses (100, 200, 500, 1000 and 2000 mg kg\(^{-1}\)) for 28 days. The observation of acute toxicity and the sub-acute effects of the extract on the hematological, coagulation, liver function (LFT) and renal function parameters were reported. Results: The phytochemical screening of the aqueous extract of C. aronia syn. Azarolus (L.) indicates the presence of flavonoids, terpenes/sterols, saponins and tannins. There were no signs of acute toxicity and no fatality. There was significant increase of the Red Blood Cell Count (RBC) and Packed Cell Volume (PCV) in rats given the extract at dose of 200 mg kg\(^{-1}\) but not with other doses. Furthermore, the prothrombin (PT) and Activated Partial Thromboplastin Times (APTT) were significantly increased in rats given the extract at doses of 100-500 mg kg\(^{-1}\). There was no changes in the level of LFT, renal function and electrolytes. Conclusion: This study indicates that C. aronia syn. Azarolus (L.) whole plant aqueous extract has no acute or sub-acute adverse effects when administered under or equal to the dose of 2000 mg kg\(^{-1}\) body weight. The increase in the RBC, PCV as well as PT and APTT needs further studies.

Key words: Crataegus aronia, wistar rats sub-acute toxicity, crataegus aronia, whole plant, aqueous extract, hawthorn comprises, phytochemical screening, herbal remedies, laboratory animals, being listed

INTRODUCTION

Plants are one of the most important sources of active substances with therapeutic potential to cure a variety of diseases in humans (Gill et al., 2010; 2011). The evaluation of pharmacological effects can be used as a strategy for discovering new drugs of plant origin (Gill et al., 2011; Holloway et al., 2011). There is an ongoing world-wide revolution which is mainly premised on the belief that herbal remedies are safer and less damaging to the human body than synthetic drugs (Alam et al., 2011). According to World Health Organization about 80% of the world population relies on traditional medicine for primary health care and more than 30% of the plant species have been used medicinally. However, there is limited scientific evidence regarding the safety and efficacy to support the continued therapeutic application of these medicinal plants. Because of this renewed interest in herbal remedies and the increased use of plants extracts in food, cosmetics and pharmaceutical industries, there is a compelling need for thorough scientific safety evaluation of the medicinal plants (Ben-Arye et al., 2011; Haque and Haque, 2011).

Laboratory animals are sensitive to toxic substances occurring in plants. Hence, the administration of the extracts in increasing amounts enables the evaluation of the acute and sub-acute toxicity limits. Therefore, the test should be carried out for three doses and for both sexes, taking into account other factors such as age, weight, species, diet and environmental conditions (Silva et al., 2011).
Hawthorn is deciduous and a member of the rosaceae family. The Hawthorn is native to Mediterranean region, North Africa, Europe and Central Asia. Most species of Hawthorn have prominent, long, straight and sharp thorn, ranging from 1-5 inches. Hawthorn comprises more than 200 species in the world. In Mediterranean region, the predominant species of genus Crataegus is C. aronia syn. Azarolus (L) that populates the mountains of these areas.

The extracts or tinctures prepared from the leaves, flowers and/or fruits from the genus Crataegus (Rosacea) dates back to ancient times (Jayachandran et al., 2010; Kanyonga et al., 2011). Currently, there is increasing use of different hawthorn species in cardiovascular diseases and being listed as herbal drugs in pharmacopoeias of countries, such as Germany, France, China and England particularly for the treatment of mild form of heart failure (Jayachandran et al., 2010; Kanyonga et al., 2011; Swaminathan et al., 2010). Furthermore, several ethnobotanical and ethnomedicinal surveys on the therapeutic use of indigenous plants have revealed that the indigenous member of this genus, *Crataegus aronia* syn. *Azarolus* (L) (Rosaceae) is used in the traditional Arab medicine to treat cardiovascular diseases, as well as cancer, diabetes and sexual weakness (Nawash and Al-Horani 2011).

Despite the extensive use of the plant, *Crataegus aronia* syn. *Azarolus* (L), has not been subjected to adequate studies including its acute and sub-acute toxicity. Motivated by this, we aimed at present to report the acute and sub-acute effects of the aqueous extract of *Crataegus aronia* syn. *Azarolus* (L) whole plant on biochemical indices of liver and kidney functions as well as some hematological parameters in albino Wistar rats, which will be a guidance for the planned future studies of its effect on different systems particularly the cardiovascular system.

**MATERIALS AND METHODS**

**Preparation of the extract:** This study was performed in the Research labs of Medical School of King Khalid University at Abha, Saudi Arabia. *Crataegus aronia* syn. *Azarolus* (L) whole plant was purchased from a local market in Jordan (Middle-east). The plant was identified and extracted by the Department of Pharmacognosy of college of Pharmacy at King Khalid University.

The dried plant was ground to a powder and extracted by maceration in distilled water (1kg/1L, w/v) for 2 days at 37°C (Abdul et al., 2009). The extract was filtered and the excess water was evaporated under reduced pressure in a rotary evaporator. The resulting residue (28 g) called the aqueous extract was stored at 4°C. The residue was re-constituted in distilled water to obtain the various concentrations used in the study.

**Phytochemical screening tests (I-VII):** The phytochemical screening of the aqueous extract of the leaves *Crataegus aronia* was carried out according to the method of Evans (2000) and the method of Mogana et al., 2011). Qualitative analysis of alkaloids, flavonoids, glycosides, saponins and tannins were studied using freshly prepared ground samples of *Crataegus aronia* syn: *Azarolus* (L) whole plant extract.

**Test for flavonoids:** About 1.0 mL of 10% ferric chloride was added to 1.0 mL of extract. The formation of a greenish brown or black precipitate or color was positive test for phenolic nucleus. To 1.0 mL extract, 1.0 mL of dilute NaOH was added. Addition of 1.0 mL dilute NaOH to 1.0 mL extract gave a precipitate which shows presence of flavonoids.

**Test for saponins:** 1.0 mL of extract was boiled with 5.0 mL of distilled water for 5 min and decanted while still hot. The filtrate was used for the following tests:

- Frothing test: About 1.0 mL of the filtrate was diluted with 4.0 mL of distilled water shaken vigorously and observed on standing for stable froth which confirms the presence of saponins
- Emulsion test: About 2 drops of olive oil was added to 1.0 mL of filtrate. The solution was shaken and observed for formation of emulsion which confirms the presence of saponins

**Test for tannins:** About 5.0 mL of extract was added to 2.0 mL of 1% HCl. Deposition of a red precipitate was an evidence for the presence of phlobotannins.

**Test for terpenes/sterols:** Liebermann-Burchard’s Test: (200 mg plant material in 10 mL chloroform, filtered; 2 mL filtrate+2 mL acetic anhydride+1 mL of conc. H₂SO₄. A blue-green ring indicates the presence of terpenes/terols.

**Test for free anthraquinones:** The Bormtrger’s test for anthraquinones was used. 5mg of the plant extract was shaken with 10 mL of benzene, filtered and 5ml of 10% ammonia solution added to the filtrate and the mixture shaken.

**Combined anthraquinones:** 5mg of plant extract was boiled with 10 mL aqueous sulphuric acid and filtered while hot. The filtrate was shaken with 5 mL of benzene, the benzene layer separated and half its own volume of 10% ammonia solution added.
**Test for cardiac glycoside:** About 5.0 mL of extract was treated with 2.0 mL of glacial acetic acid containing 1 drop of 0.1% ferric chloride and then mixed with 1.0 mL concentrated sulphuric acid. A brown ring of the interface indicates a deoxysugar characteristic of cardenolides.

**Test for alkaloids:** About 1.0 mL of extract of the sample was added and shaken with 5.0 mL of 2% HCl on a steam bath and filtered. Five drops of Meyer’s reagent (potassium mercuric iodide solution) was then added to 1.0 mL of the filtrate and observed for cream colored precipitate which is a positive test for alkaloids.

**Experimental animals:** About 36 Wistar strain rats of both sexes weighing between 50.7-60.9 g were procured from a random bred colony in the animal house of College of Medicine of King Khalid University, Abha, Saudi Arabia. The rats were caged in plastic cages (6/cage) in a controlled environment (ambient temperature, 27.0 ± 2.0°C and with a 12 h light/darkness cycle). They were allowed to acclimatize for 10 weeks during which the weight gained was between 180-200 g. During this period the rats were fed on rats-chow diets and water ad-libitum. The experiments performed complied with the rules of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council and approved by the Ethical Committee of the King Khalid University.

**Acute toxicity study:** Acute toxicity bioassay was conducted according to the World Health Organization guideline for the evaluation of safety and efficacy of herbal medicines (WHO, 1993). The rats were divided into six groups of six animals each. The aqueous extract of *Crataegus aronia* was administrated per os to rats of groups 2-6 in a single dose/day of 100, 200, 500, 1000, 2000 mg kg⁻¹ body weight (bwt) respectively by intra gastric gavages using a feeding needle. The control group (group 1) received an equal volume of distilled water as vehicle.

Observations of acute toxic symptoms were made and recorded systematically 1, 2, 4, 6 and 24 h. after administration of the extract. The number of rats that survived were noted after 24 h and then maintained for the further 3 days with daily observations. This visual observation included skin changes, mobility and aggressiveness, sensitivity to sound and pain, as well as respiratory movements. The acute toxic effects of the extract were assessed on the basis of mortality, which was expressed as LD50. Lethal dose (LD 50) was calculated using the following equation (Turner and Hebborn, 1965):

\[
LD50 = \frac{\sum (a \times b)}{N}
\]

**Sub-acute toxicity study:** The extract was administered in a manner similar to that used in acute toxicity study with same doses to the animals for 28 days (Ozolua et al., 2010).

**Collection and analyses of blood:** At the end of the experiment, all animals were anaesthetized with diethylether vapor. Blood samples were collected by cardiac puncture into three sets of plain, EDTA treated and sodium citrate tubes. The blood in the plain and sodium citrate tubes were allowed to clot. The clotted blood samples in the plain tubes were spun in a bench top centrifuge (3000 rpm for 10 min) to obtain sera. The separated serum samples from the plain tubes were stored in the refrigerator until required for the biochemical analyses. The serum biomarkers analyzed include: Alanine Amino Transferase (ALT), aspartate amino transferase (AST), total bilirubin, total proteins, Urea, Creatinine, potassium (K+), sodium (Na+) and chloride (Cl-). These tests were assayed using commercial available kits according to the manufacture’s instruction. Blood sample collected in EDTA treated tubes were used for Full Blood Count (FBC), which included: Total Erythrocytes Count (TEC), Hgb, PCV, MCV, MCH, MCHC, Platelets Count (PLC) and Total Leucocytes Count (T LC). The FBC was analyzed using Automated Hematology System (Sysmex Hematology-Coagulation Systems®, Model KX-21N, Sysmex Incorporation, Kobe, Japan).

**Determination of clotting time:** The blood collected directly from the heart to avoid contamination with tissue thromboplastin (200 µL mL from each rat). At every time, the blood was delivered into four glass test tubes that had previously been warmed and maintained at 37°C and the tubes immediately placed in a 37°C water bath to mimic the temperature of the internal environment. The glass test tubes were continually tilted at 10s intervals until blood stopped flowing when tilted at an angle of 90°. The period in seconds was recorded from the time the blood is delivered into the glass test tubes until it stops flowing when tilted at an angle of 90° using stopwatch.
Determination of Prothrombin Time (PT): Blood was collected into sample vials containing 3.2% sodium citrate (as specified in the Prothrombin Time (PT) test kit used) in the ratio 1:9 with the blood sample. The blood was then centrifuged at 1000 g for 15 min. to obtain platelet poor plasma. Thromboplastin PT-S was placed in a water bath at 37°C and 0.1 mL of test plasma was also put into a test tube and placed in the water bath to prewarm to 37°C. A 0.2 mL of warmed thromboplastin PT-S was then forcibly added to the test plasma and the stopwatch was started. The tube was tilted repeatedly until a clot was formed and the time taken for clot to form was noted. Precaution was taken to perform the test within 3 h of blood collection.

Determination of Activated Partial Thromboplastin Time (APTT): A mixture of 0.1 mL of plasma with 0.1 mL of APTT reagent containing Cephalin-Kaolin suspension was incubated at 37°C for 5 min, followed by the addition of 0.1 mL of 0.025 M CaCl$_2$ solution. APTT was taken as the interval between the addition of CaCl$_2$ and the moment when the fibrin clot was visually detected. The blood tests were done by an expert technicians in the fields and were unaware of the detailed treatment groups.

Statistical analysis: The results were expressed as the mean value ± SD. Statistical differences between groups were assessed using the SPSS software, version 16 and the Student’s t-test was used to determine the difference between groups and p<0.05 denoted statistical significance.

RESULTS

The results of this study are shown in Table 1-5. In this study, the oral administration of the aqueous extract of Crataegus aronia at all given doses (up to 2000 mg kg$^{-1}$) did not produce any visible sign of acute toxicity or instant death in rats tested during the period of observation.

Preliminary phytochemical screening of the Crataegus aronia aqueous extract indicates the presence of flavonoids, terpenes/sterols, saponins and tannins, while anthraquinones (free and combined), glycosides and alkaloids were not detected (Table 2).

The effect of C. aronia aqueous whole plant extract on hematological parameters is depicted in (Table 3). The Red Blood Cells count (RBC’s) and hematocrit value increased significantly (p<0.05) on administration of the extract to both male and female rats only at dose of 200 mg kg$^{-1}$. However, there was no reduction in any other hematological parameters with the use of higher doses of the extract.

Table 1: LD50 determination by arithmetic method of Karbar

| Groups         | Number of rats | No. of animals dead | Dose difference (a) | Mean mortality (b) | Probit (a×b) |
|---------------|----------------|---------------------|---------------------|--------------------|-------------|
| Control group | 6              | 0                   | ----                | ------             | -----       |
| C. aronia 100 mg kg$^{-1}$ | 6              | 0                   | 100                 | ------             | -----       |
| C. aronia 200 mg kg$^{-1}$ | 6              | 0                   | 100                 | ------             | -----       |
| C. aronia 500 mg kg$^{-1}$ | 6              | 0                   | 400                 | ------             | -----       |
| C. aronia 1000 mg kg$^{-1}$ | 6              | 0                   | 500                 | ------             | -----       |
| C. aronia 2000 mg kg$^{-1}$ | 6              | 0                   | 1000                | ------             | -----       |

Table 2: Phytochemical screening of Crataegus aronia syn. Azarolus (L) aqueous extract

| Chemical compound | Observations |
|-------------------|--------------|
| Flavonoids        | A reddish color was observed |
| Saponins          | A stable froth was observed |
| 1- Frothing test  | An emulsion is formed |
| 2- Emulsion test  | Blue black color was observed |
| Tannins           | A reddish brown ring was observed at the interface |
| Terpenes/sterol   | Gold color was not observed on the ammoniacal phase |
| Free anthraquinones | No pink, red or violet color was observed on the ammoniacal phase |
| Combined anthraquinones | No reddish brown ring was observed at the interface |
| Cardiac glycosides | A cream colored precipitate was not observed |

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**DISCUSSION**

Investigation of the acute toxicity is the first step in the toxicological investigation of an unknown substance. The index for the acute toxicity is the LD50. The results in this study showed that the acute administration of the aqueous extract of *Crataegus aronia* at all given doses (up to 2000 mg kg⁻¹) did not produce any sign of acute toxicity or instant death in rats tested during the period of observation. However, suggest that the extract has no acute toxicity when administered orally and the lethal dose if any is above 2000 mg kg⁻¹. Clarke *et al.* (1975) showed that substances with LD50 of 1000 mg kg⁻¹ body weight given orally are considered safe or of low toxicity. Similarly, the chemical labeling and classification of acute systemic toxicity based on oral LD50 values recommended by the Organization for Economic Cooperation and Development (OECD, Paris, France) (Walum, 1998) are as follow: very toxic, <5 mg kg⁻¹; toxic, >5<50 mg kg⁻¹; harmful, >50<500 mg kg⁻¹ and no label, >500<2000 mg kg⁻¹. Therefore, the expected high LD50 (>2000 mg kg⁻¹ body weight) of the aqueous extract *Crataegus aronia*, is an indication that the extract could be considered relatively safe especially when administered orally where absorption may not be complete due to inherent factors limiting absorption in the gastro intestinal tract. Although, obtaining a lethal dose in animals may not predict the human lethal dose of a drug or acute poisoning overdose (Chapman et al., 2010). However, it is usually used to provide a guideline for selecting doses for the sub-acute dosage for future clinical relevance.

Preliminary phytochemical screening carried out in this study indicated that *Crataegus aronia* syn: *Azarolus* (L) contain mainly flavonoids, tannins, saponins, Trepens and sterols in its aqueous extract. These phytochemicals are known to perform several (Vanclay, 1994).
general and specific functions in plants and may exhibit different biochemical and pharmacological actions in different species of animals when ingested. These actions range from cell toxicity to cell protective effects. Similar phytochemical findings are present in other *Crataegus* species like *C. monogyna* and *C. laevigata* (Barros et al., 2011; Jayachandran et al., 2010).

The various biochemical and hematological parameters investigated in this study are useful indices of evaluating the toxicity of plant extract in animals. Assessment of hematological parameters can be used to determine the extent of deleterious effect of foreign compounds including plant extracts on the blood constituents of an animal. It can also be used to explain blood relating functions of chemical compounds and plant extracts (Soa et al., 2011). Such analysis is relevant to risk evaluation as the change in hematological system has higher predictive value for human toxicity, when the data are translated from animal studies.

The various blood cells (erythrocytes, leucocytes and platelets) produced at a turnover rate of about 1 to 3 million per second in a healthy human adult and this value could be altered in certain physiological or pathological states (Guyton and Hall, 2000). Certain drugs including alkylating cytotoxic agents affect blood formation rate and the normal range of hematological parameters (Zuk et al., 2011).

The results of the hematological parameters of the present study did not show any worrisome results since all the changes were within the normal expected range for the rat species used in this study. However, there was an important observation which might be interesting. The RBC and PCV values were significantly higher among the group of rats treated with the extract at dose of 200 mg kg\(^{-1}\) compared to the other groups treated with different doses. These values were above the well established reference ranges for Wistar rats (Cameron and Watson, 1949). The reason for this change with this dose (200 mg kg\(^{-1}\)) is not clear, however, this could be due to variations in the absorption, bioavailability and metabolism of the antioxidants (flavonoids) present in the extract (Prochazkova et al., 2011; Amran et al., 2010). Hawthorn extract is well known rich source for flavonoids which include: hyperoside, isoquercitrin, epicatechin, chlorogenic acid, quercetin, rutin and protocatechuic acid (Barros et al., 2011). In the present study, the phytochemical screening confirmed the presence of flavonoids in the aqueous extract *C. aronia*. Thus, the possible mechanism of the increase in RBC and PCV values involves the effect of flavonoids on maintaining the cell membrane through inhibition of peroxidation of polyunsaturated fatty acids (Wang et al., 2009; Hasan et al., 2009). Also, flavonoids are capable to inhibit the formation of superoxide ions and hydroxy radicals, which are two strong peroxidation agents that are produced in the body under normal conditions and cause destruction of cells (Esmaeili and Sonboli, 2010). Therefore, flavonoids may protect both the hematopoietic committed stem cells and the formed blood cells from the attack of the reactive free radicals. Furthermore, the antioxidant activity of flavonoids may maintains the haeme iron in its ferrous state and this could enhance erythropoiesis. Whether the aqueous extract of *C. aronia* has any effects on the level of erythropoietin remains to be established.

The aqueous extract of *C. aronia* exhibited anticoagulant activities by increasing clotting times, PT and APTT. APTT and PT are a qualitative measurement of factors involved in the intrinsic and extrinsic pathways respectively (Salawu et al., 2008; Bamidele et al., 2010; Cipil et al., 2009). The increase in APTT and/or PT could be due to, a deficiency of, or an inhibitor to, any of the clotting factors involved in either pathways or in the final step of the clotting pathway (Abdullah et al., 2010). The observed increase in clotting time, PT and APTT occurred at a doses between 100-500 mg kg\(^{-1}\)but not with higher doses indicate that this effect is probably not a toxicity and may have a therapeutic potential as anticoagulant and warrant further investigation.

Many xenobiotics are capable of causing some degree of liver injury (Omiecinski et al., 2011). The liver is prone to xenobiotic-induced injury because of its central role in xenobiotic metabolism, its portal location within the circulation and its anatomic and physiologic structure. Generally, analysis of the activities of some basic liver enzymes in the plasma or serum can be used to indirectly assess the integrity of tissues after being exposed to certain pharmacological agents (s) (Al-Hashem, 2009). These enzymes are the usual liver markers whose plasma concentrations above the homeostatic limits could be associated with various forms of disorders which affect the functional integrity of the liver tissue. In the assessment of liver damage by drugs or any other hepatotoxin, the determination of enzyme levels such as ALT and AST is largely used (Ramaiah, 2011). Necrosis or membrane damage releases the enzyme into circulation; therefore, it can be measured in the serum. High levels of AST indicate liver damage. ALT catalyses the conversion of alanine to pyruvate and glutamate and is released in a similar manner. Therefore, ALT is more specific to the liver and is
thus a better parameter for detecting liver injury (Ozer et al., 2008). The results of the liver enzymes (ALT and AST) as well as bilirubin suggest that Crataegus aronia is not a hepatotoxic at all doses used in our study.

Bilirubin is formed by the breakdown of hemoglobin in the liver, spleen and bone marrow. An increase in tissue or serum bilirubin concentrations occurs as a result of increased breakdown of RBC (haemolysis) or liver damage e.g., hepatitis or bile duct obstruction. The normal levels of serum bilirubin concentrations at all doses of the extract used in this study are indicative of non-adverse effects of the extract on haemoglobin metabolism pathways.

The Kidneys are highly susceptible to toxicants for two reasons: A high volume of blood flows through it and its ability to filter large amounts of toxins which is not a hepatotoxic at all doses used in our study.

Bilirubin can concentrate in the kidney tubules. It can result in obstruction. The normal levels of serum bilirubin concentrations at all doses of the aqueous extract of Crataegus aronia are not toxic under a dose less than or equal to 2000 mg kg\(^{-1}\). The extract may possess hematopoietic and anticoagulant potentials at a doses between 100-500 mg kg\(^{-1}\) and warrants further investigations.

CONCLUSION

The Data of the present study do suggest that the whole plant aqueous extract of Crataegus monogyna syn: Azarolus (L) is not toxic under a dose less than or equal to 2000 mg kg\(^{-1}\). The extract may possess hematopoietic and anticoagulant potentials at a doses between 100-500 mg kg\(^{-1}\) and warrants further investigations.

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