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Consumption of *Citrullus colocynthis* Fruit Extract Causes Histological and Immunological Alterations in Mice

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Original article

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Traditionally, the usage of *Citrullus colocynthis* (CCT) causes severe side effects. The side effects of CCT fruit extract administered orally at different doses related to gastric tissues and circulating cytokines profiles were reported. Thirty-five adult male albino mice were divided into 4 groups, a control group (G1) and three experimental groups. They orally received aqueous fruit extract over 20 days at different doses; 100, 200, and 300 mg/kg of body weight/day. Total body weight, stomach tissue, peripheral blood, anti-inflammatory, and pro-inflammatory cytokines were evaluated. Body weight significantly decreased in groups 2 and 3 over four weeks. Neutrophils, lymphocytes, monocytes, and basophils were significantly ($p<0.05$) elevated in group 4; while hemoglobin and mean corpuscular hemoglobin concentration (MCHC) were significantly decreased ($p<0.05$). Interleukin-8 (IL-8) level was significantly elevated in groups 3 and 4 versus the control group ($p<0.05$). Interleukin-6 (IL-6) levels showed a significant ($p<0.05$) increase in group 4 only. Anti-inflammatory cytokines showed a significant ($p<0.05$) decrease in all treated groups. The stomach tissues revealed that the extract induced a superficial focal loss of the surface mucous protective epithelium, the atrophy of peptic cells, and a thickening of mucosal connective tissue. The submucosa showed vascular congestion and inflammatory cell infiltration. Severe histological changes were reported in group 4. Using the extract for 20 days led to the elevation of the differential white blood cell (WBC) count as well as the destruction of the gastric mucosal lining at high doses. This could be due to an increase in pro-inflammatory and declining anti-inflammatory cytokines. It is not recommended to use CCT in high doses or for long periods.

Key words: *Citrullus colocynthis*, extract, mice, oral route, stomach, cytokines.

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Citrullus colocynthis (L.) *Schrad* (CCT) is a profitable cucurbit plant belonging to the Cucurbitaceae family. Some common individuals of the family are melon, cucumber, pumpkin, and bitter apple. CCT is a little scarbid perennial inching plant that has either a harsh or precise stem, having smooth circular fruits that are colored green when unripe, turning yellow once ripe (PRAVIN *et al.* 2013). CCT fruit is well known in Saudi Arabia as Handal or Sherry (AL-YAHYA *et al*. 2000). It's also called Colocynth/Bitter Apple, bitter-cucumber, colocynth, wild gourd, vine-of-sodom. The plant grows in Saudi Arabia, Kuwait, Iraq, Jordan, Turkey, India, China, Pakistan, and other Asian, African, and European countries (AL-SNAFI 2016). Different CCT plant parts such as the seeds, leaves, fruit, stem, and root, are utilized as oil or aqueous extracts, dried or fresh, and are believed to have anti-diabetic (RAHIMI *et al*. 2012), anti-hyperlipidemic (RAHBAR and NABIPOUR 2010), laxative (MARZOUK *et al*. 2010), anti-inflammatory (MARZOUK *et al*. 2010), analgesic (MARZOUK *et al*. 2010), hair-growing (DHANOTIA *et al*. 2011), vermifuge (RAHIMI *et al*. 2012), antifungal (MARZOUK *et al*. 2009), antibacterial (MARZOUK *et al*. 2009), and anti-oxidant properties (TANNIN-SPITZ *et al*. 2007).

Behind most of these therapeutic potentials are cucurbitacin, flavonoids, alkaloids, and phenolic acids (HUSSEIN *et al*. 2017; ZHENG *et al*. 2020). A phytochemical analysis of CCT fruit revealed the presence of amino acids, carbohydrates, tannins, proteins,

saponins, flavonoids, terpenoids, phenolics, anthranol, alkaloids, steroids, J, L, caffeic acid, Cucurbitacin A, B, C, D, and E (α-elaterin), and cardiac glycoloids (HUSSAIN *et al*. 2014; AL-SNAFI 2016; BASHA *et al.* 2019). Despite the medicinal properties and long therapeutic history of CCT (ZHENG *et al*. 2020), it has serious side effects that seem related to either over-consumption, patient allergy, or an extended period of usage. It can cause gastrointestinal upset as intestinal damage and rectorrhagia. Studies regarding CCT are quite different, mainly in respect to fruit parts being examined for toxic and physiological properties, the utilized solvent for the extraction from plant parts, doses given with lyophilized extract, route of administration, and acute or chronic extract effects. Despite being motivated by delineating plant treatment actions, these differences restrict the possibilities for comparing different results (SANADGOL *et al*. 2011). The toxicity complication effects of CCT ethanolic extract on the kidneys, heart, liver, spleen, stomach, and intestines, with varied severity, was definitive evidence and dose-dependent (MARZOUK *et al*. 2010; HUSSAIN *et al*. 2014; SHOKRZADEH *et al*. 2013; AL-SNAFI 2016). The acute median lethal dose (LD50) of the extract was found to be 1311.45 mg/kg (at a 95% confidence limit of 1037.80 to 1657.27 mg/kg) for male rats. The LD16 and LD84 were 825.61 mg/Kg and 2083.19 mg/Kg, respectively (SOUFANE *et al*. 2013). We used an aqueous extract according to (MUHSEN & ALALI 2010) which seems more close to reality than ethanol extract, and alternative medicine usually advises the patient to take CCT with water. Because of this, the current study follows these extraction methods and selected doses to avoid mortality, 00-300 mg/ kg/ day for 20 consecutive days. The current study aimed to evaluate the possible side effects of ingesting various CCT fruit extract doses on mouse stomachs and possible mechanisms.

Material and Methods

Aqueous extract preparation

The matured fruit of CCT was collected from local open farmland between Jeddah and Mecca. The entire fruit was thoroughly washed, dried with tissue paper, and ground to a powder. The powder (18 g) was then mixed in 600 ml of hot distilled water on a magnetic stirrer for 1 hour at room temperature and then left overnight. Next the solution was filtered through surgical gauze and then Whitman No.1 filter paper to obtain the aqueous extract which was used in the experiment. Extract preparation and the given doses (100, 200 and 300 mg) were determined according to previously published protocols (MUHSEN & ALALI 2010).

Experimental animals

All experimental procedures are approved by the Unit of Biomedical Ethics Research committee at King Abdulaziz University (KAU) and proceed with its guidelines (Reference No. 585-18). Thirty-five albino male mice of SWR strain aged eight weeks and weighing 35-40 grams were enrolled in this study. Mice were obtained from the house of an animal unit at King Fahad Medical Research Centre (KFMRC), KAU, Jeddah, Saudi Arabia. The animals were acclimatized to the laboratory environment for one week before experiment initiation. The mice were put in plastic cages (5 mice/ cage) and kept in controlled laboratory conditions, light: dark cycle (12:12 h), temperature (20±1ºC), and humidity (65%) and fed *ad libitum* with astandard diet containing 11.7% fat calories (20% soybean, 8% concentrated proteins, 50% wheat, 21% corn, and 1% vitamins & salts) and had free access to tap water.

Experimental design

Mice were randomly divided into 4 groups as follows: a control group (n =5)and the treated groups; group 2, group 3, and group 4 (10/each). The treated groups 2,3, and 4, were administrated aqueous CCT fruit extract in doses of 100, 200 & 300 mg/kg of body weight/day via oral gavage for 20 consecutive days. The animals were kept under observation after dosing to check for any morphological symptoms or behavioral changes. The animals appeared normal in behavior and no interesting symptoms were observed. The weight of all animals was measured weekly till the end of the experiment, without any animals dying. All blood samples were collected over a period using the retro-orbital vein method on EDTA – or without. At the end of the experiment, the animals were anesthetized using Isoflurane inhalation. Their blood was collected from the jugular vein, and the stomachs were collected and weighed using thestandard protocol (ANTAL *et al.* 2007; VALENTIM *et al.* 2008).

Hematological data

Complete blood count (CBC) was measured by an automated analyzer for the determined number of each cell type. Neutrophil, lymphocyte, and monocyte indexes were calculated (NFAMBI *et al*. 2015).

Determination of pro-inflammatory and anti-inflammatory cytokines

Serum samples were obtained by centrifuging (2000 rpm for 5 min) the blood samples, aliquoting, and then storing them at -20ºC till used.The anti-inflammatory cytokines (IL-10) & pro-inflammatory (IL-8 & IL-6) concentration profile was estimated in all serum samples using commercially available ELISA kits, according to the kit instructions.

Histological data analysis

Immediately after blood sampling, general anatomy was performed and the stomach was isolated from all animal groups. The collected stomachs were dissected along the greater curvature, washed with nor-

mal saline to eradicate stomach remnants and clots of blood and then inspected via a 10× magnifier lens for ulcers. Midline strips along the stomachs' lesser curvature were fixed in 10% formalin neutral, buffered, processed, & embedded in paraffin. 4 µm sections were dissectedand stained with hematoxylin and eosin (H&E) according to the standard protocol (CARDIFF *et al*. 2014).The normal histological procedure was applied on the lining mucosa to study the possible histopathological alterations of the gastric mucosa cells.

Statistical analysis

Data were analyzed by IBM SPSS Statistical program for Windows, version 23 (IBM SPSS, IBM Corp., Armonk, N.Y., USA). Data expressed as mean ± standard deviation ($\bar{x}$±SD). Percentage changes of total body weight were calculated as group weight minus control weight divided by control weight then multiplied by 100 (E.weight-C.weight/C.weight ×100). Differences between the groups were made using the One Way ANOVA test followed by the least significant test (LSD) for normally distributed parameters. * p-value <0.05, ** p-value<0.01 was significant and *** p-value<0.001 was highly significant.

Results

Animal weights

The animals total body weights were recorded weekly. Although there were fluctuations in weight in some animals, average weights were increasing in all the experimental groups as shown below due to an increase in age. Total body weight was significantly decreased in groups 2 and 3 versus the control group on the 1st day ($p<0.05$), 8th day ($p<0.05$), 15th day ($p<0.05$) and 20th day ($p<0.05$). Meanwhile there were insignificant changes regarding body weight between group 4 and the control group on the 1st day ($p<0.05$), 8th day ($p<0.05$), 15th day ($p<0.05$), and 20th day ($p<0.05$). While the percentage of the change in the body weight was detectably decreased in groups 2, 3, and 4 versus the control group at different durations (Figs 1 and 2).

Hematological analysis

Blood samples were being collected from all animals for CBC. In group 4, total WBC counts were significantly higher than in any other group. However, the WBC differential counts were highly significant ($p<0.001$), elevated for neutrophils, lymphocytes, and monocytes, and significantly increased for basophils ($p<0.05$). The animals in both groups 2 and 3 showed a total and differential WBC count comparable to the control group. There were insignificant changes to any CBC measured parameters between groups 2 and 3 compared to the control group. Neutrophil, lymphocytes, and monocytes indexes as calculated (under material and methods) were significantly ($p<0.001$) high in group 4. The leucocytes index was presented as the average of each cell type. The lymphocyte index value wasthe highest followed by monocytes and the neutrophil index, especially in the animals of group 4 (Fig. 3). The hemoglobin &

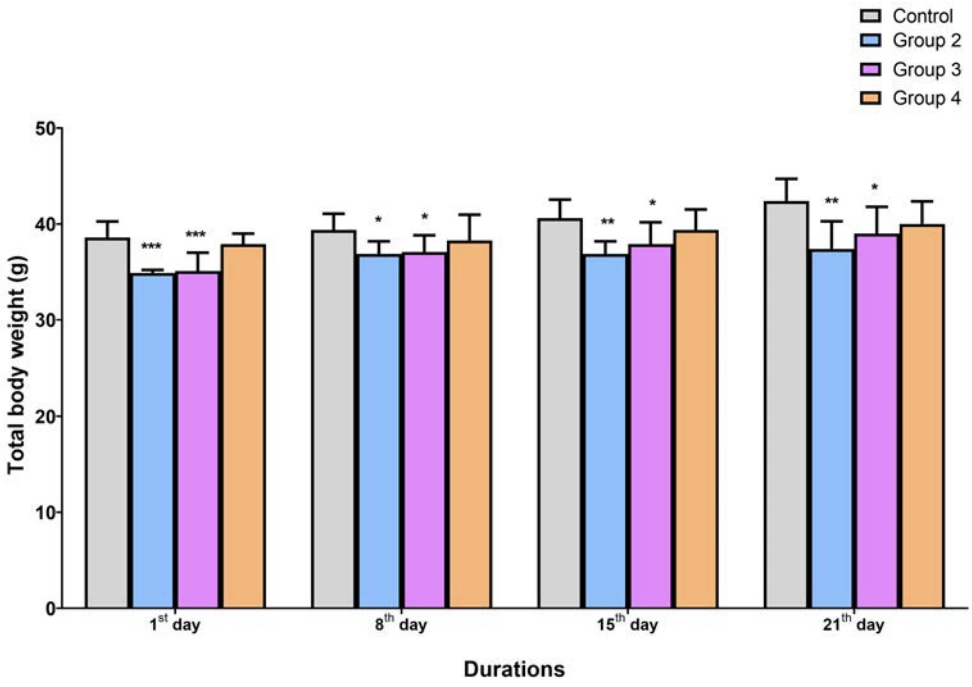

Fig. 1. Aqueous CCT extract effects. The diagram shows the effect of the aqueous extract of CCT fruit pulp on the bodyweight of albino mice at different durations (astricts indicate if the values were significant *, more significant **, or highly significant***). The bodyweight of each group is presented as average ± standard deviation (x±SD) .

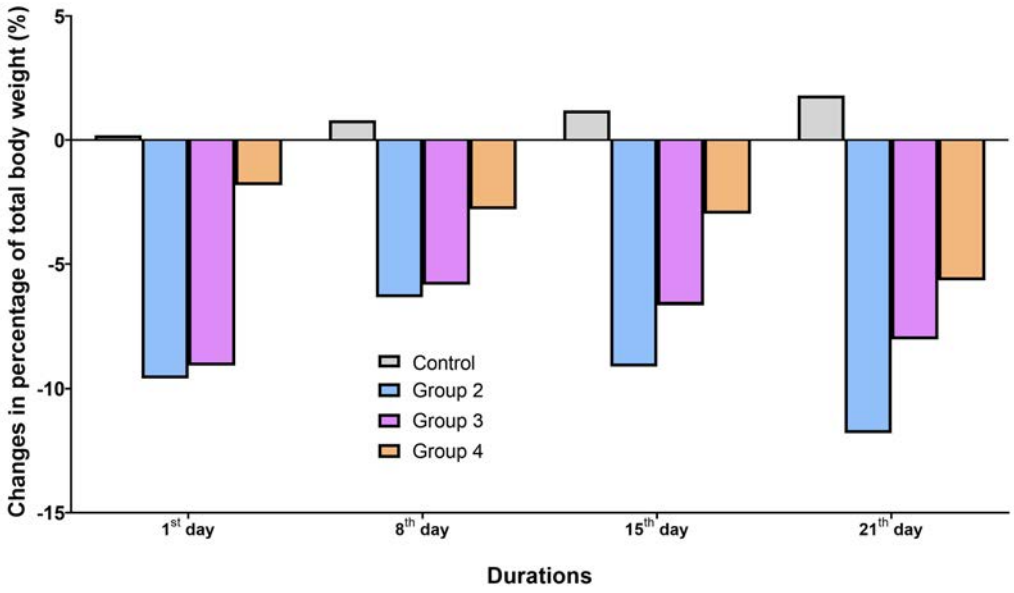

Fig. 2. Aqueous CCT extract effects. The diagram shows the percentage of change in the body weight of albino mice at different durations. The presented values are the average body weight of each group.

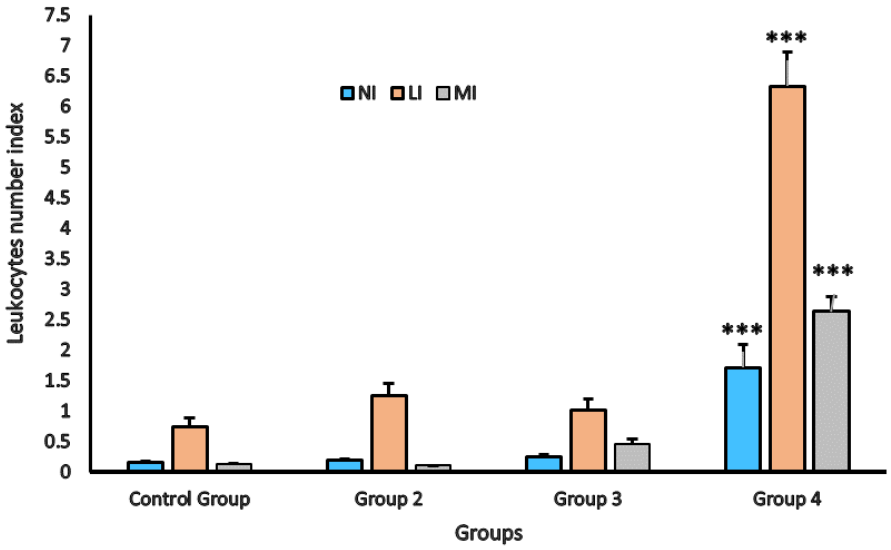

Fig. 3. Neutrophil (NI), Lymphocyte (LI), and Monocyte (MI) distribution indexes within the control and experimental groups. The presented values are the average ± standard deviation ($\bar{x}$±SD) of the total number of each cell type/each animal group, (***) indicate if the values were highly significant.

MCHC were significantly ($p<0.05$) decreased in the animals of group 4, lower than the control group (Figs 4, 5 and 6).

Determinationof pro-inflammatory/anti-inflammatory cytokines profile

To study CCT extract effects on the cytokine's serum levels, both pro-inflammatory (IL-8 & IL-6) and anti-inflammatory (IL-10) cytokines were measured (Fig. 7). At the experimental end, the serum levels of IL-8 were significantly and exponentially elevated from group 3 ($p<0.05$) to group 4 ($p<0.001$) in comparison to the control group, while the second pro-inflammatory cytokine (IL-6) level was significantly increased in only group 4 in comparison to the control ($p<0.05$) group. Meanwhile, the anti-inflammatory cytokine (IL-10) level was exponentially decreased in groups 2, 3, and group 4 serum levels. This threefold decline between the control and group 4 was highly significant ($p<0.001$).

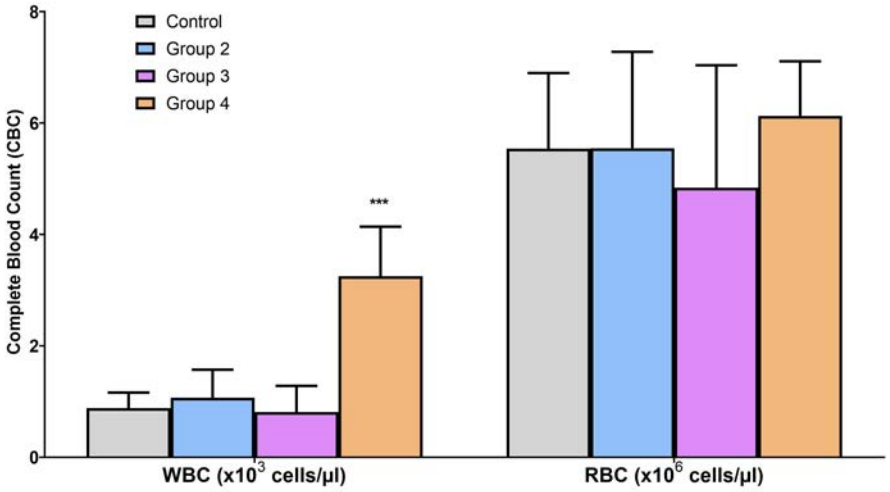

Fig. 4. Aqueous CCT extract effects. The diagram shows the effect of aqueous CCT fruit pulp extract on RBCand WBC counts in albino mice. The presented values are the average ± standard deviation ($\bar{x}$±SD) of the calculated numbers of the WBC and RBC /each animal group, (***) indicate that the values were highly significant.

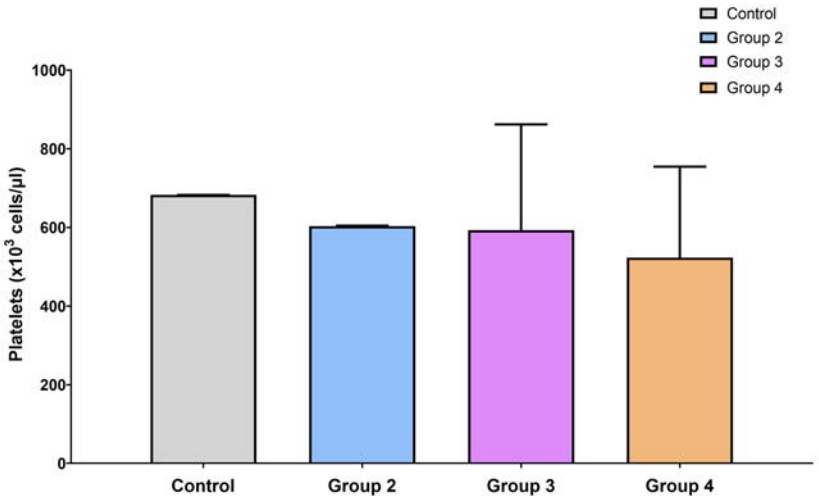

Fig. 5. Aqueous CCT extract effects. The diagram shows the effect of aqueousCCT fruit pulp extract on platelet count in albino mice. The presented values are the average ± standard deviation ($\bar{x}$±SD) of the platelet count/each animal group.

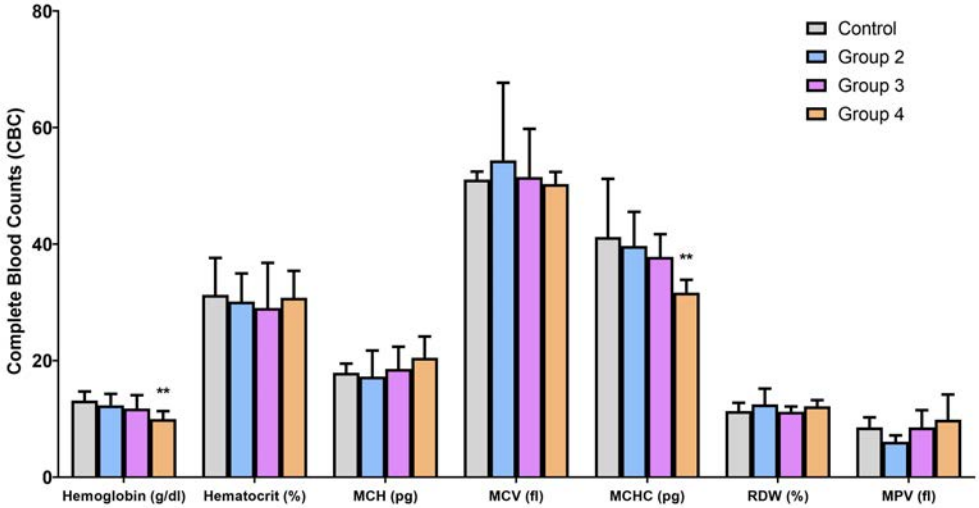

Fig. 6. Aqueous CCT extract effects. The diagram shows the effect of aqueous CCT fruit pulp extract on the concentration of Hemoglobin, Hematocrit, MCH, MCV, MCHC, RDW, and MPV in mice. The presented values are the average ± standard deviation ($\bar{x}$±SD) of the hemoglobulin (g/dl), hematocrit (%), MHC (pg), MCV (fl), MCHC (pg), RDW (%), and MPV (fl) count/each animal group, (**) indicate that the values were significant.

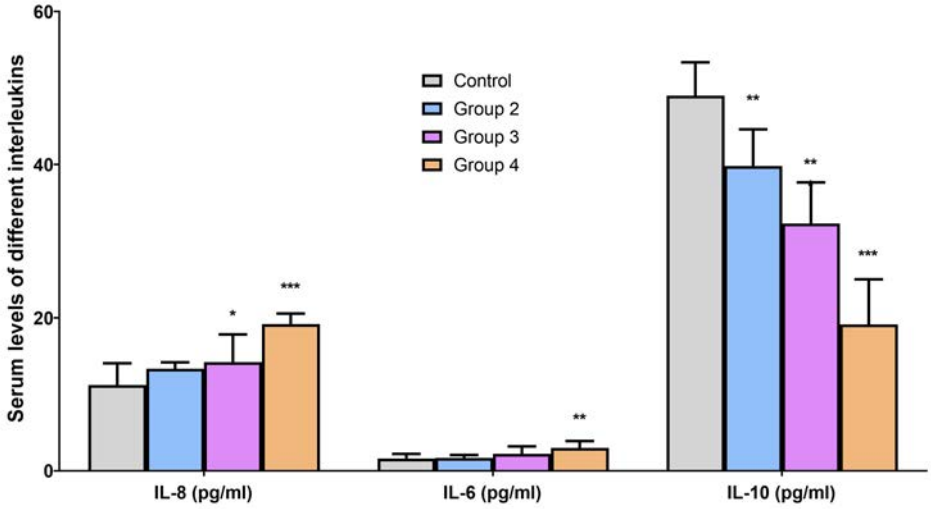

Fig. 7. Aqueous CCT extract effects. The diagram shows the effect of aqueous CCT fruit pulp extract on serum levels of interleukin (IL-8), (IL-6), and (IL-10) in albino mice. The presented values are the average ± standard deviation ($\bar{x}$±SD) of the circulating concentration of interleukin-8 (IL-8), interleukin-6 (IL-6), and interleukin-10 (IL-10) in each animal group, the asterists indicate that a value was significant.

Histological analysis

Macroscopic examination of the stomachs did not show any noticeable morphological changes in the treated animals in comparison to those of the control group. The histological examination of the control group tissues showed normal stomach wall layers, mucosa, submucosa muscle layers, andouter connective tissue layers. Higher magnification revealed the intact surface epithelium which secretesa protective mucous. Both HCL-secreting oxyntic cells and Chief peptic cells were in normal density population and integrity with no features of hypertrophy or degeneration (Fig. 8).

Histological changes were observedin the animals that received 100 mg/kg of fruit extract. A slight super facial ulceration was observed, with a marked enlargement of HCl-secreting cells, while the lower mucosa showed prominent peptic cells secreting gastric enzyme pepsin (Fig. 9). The animals in group 2 who

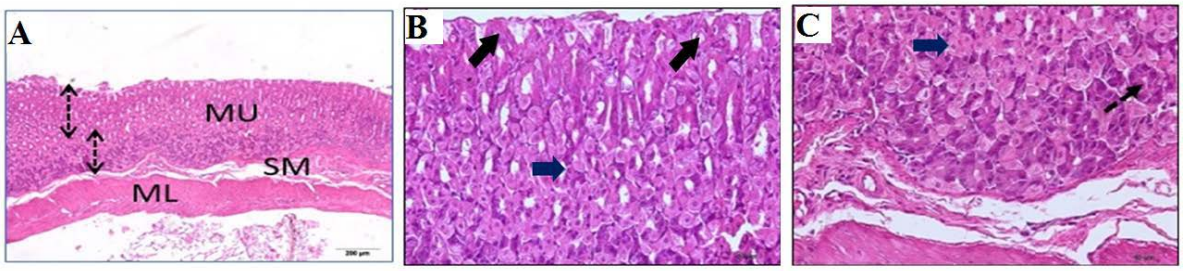

Fig. 8. Sections from the control group's stomachs (fundic region) stained by H&E. Low power ×40 (A): all stomach wall layers' mucosa (MU) with upper and lower layers (double head arrows), submucosa (SM), muscle layer (ML). Upper mucosa ×400 (B) showing intact normal surface epithelium (black arrows) normal population of HCL-secreting cells (oxyntic cells) with a acidophilic pink cytoplasm and rounded active nuclei (black arrow). Lower mucosa ×400 (C) showing peptic cells (dark blue stained basophilic cells (dotted arrow)).

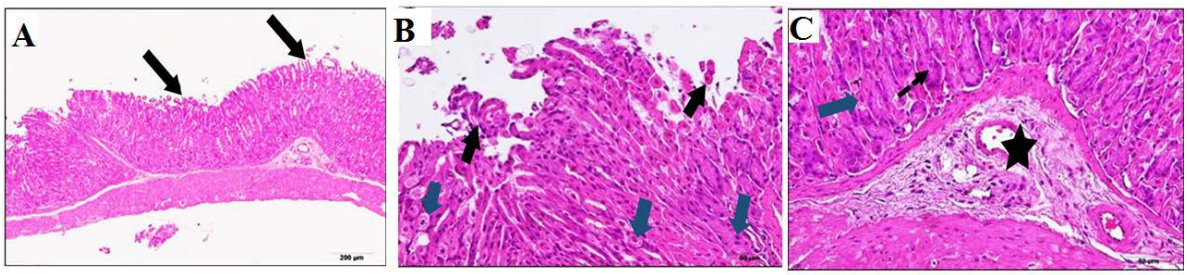

Fig. 9. Sections from group 2's stomachs (100 mg/kg) (fundic region) stained by H&E to show the effects of aqueous CCT extract on stomach mucosa. Low power ×40 (A) showing surface ulceration (black arrows) and deep mucosa degeneration and increase of HCl-secreting cells. Upper mucosa ×400 (B), showing superficial slight surface epithelium ulceration (black arrows) and atrophy of mucous neck cells. An increased population of HCL-secreting cells (oxyntic cells) with dark stained or degenerated cytoplasms and rounded active nuclei (black arrow). Lower mucosa ×400 (C), showing atrophy of peptic cells (dark blue stained basophilic cells (dotted arrow) and enlarged oxyntic cells (black arrows)). Submucosa shows congested vessels (star).

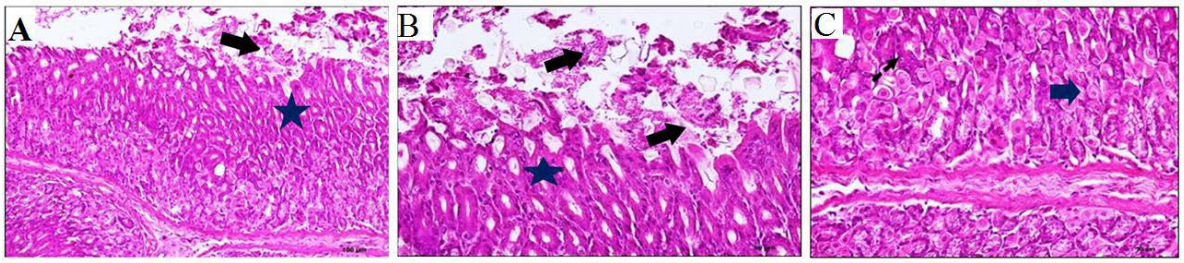

Fig. 10. Sections from group 3's stomachs (200 mg/kg) (fundic region) stained by H&E to show the effects of aqueous CCT extract on stomach mucosa. Low power ×40 (A), showing more surface ulceration (black arrows) and deep mucosa degeneration of HCl-secreting cells. Upper mucosa ×400 (B): showing superficial slight surface epithelium ulceration (black arrows), atrophy of mucous neck cells, and HCL-secreting cells (star). Lower mucosa ×400 (C): showing atrophy of peptic cells (dark blue stained basophilic cells (dotted arrow) and enlarged oxyntic cells (black arrow)).

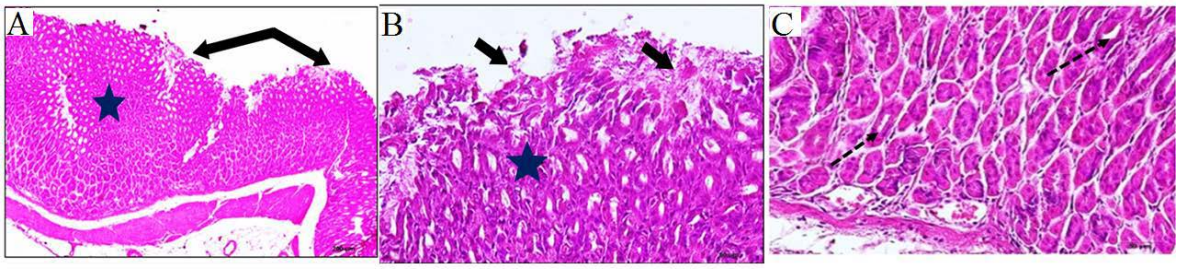

Fig. 11. Sections from group 4's stomachs (300 mg/kg) (fundic region) stained by H&E to show the effects of aqueousCCT extract on stomach mucosa. Low power ×40 (A), showing more patches of surface ulceration (black arrows) and deep mucosa degeneration glandular cells (black star). Upper mucosa ×400 (B), showing superficial marked surface epithelium ulcerations (black arrows), atrophy of mucous neck cells and other glandular cells (black star). Lower mucosa ×400 (C) showing marked atrophy and shrinkage of glandular elements widely spaced out (dotted arrows).

received 200 mg/kg showed an increase in the histological features of superficial ulceration with slight atrophy of the glandular cells (Fig. 10). Most histopathological changes were observed in group 4 which received 300 mg/kg of fruit extract. Frequent patches of ulceration were commonly observed, glandular epithelium naps were staved andwere shrunken and degenerative in appearance (Fig. 11).

Discussion

The goal of this research was to shed light on the side effects of various concentrations of aqueous CCTfruit extract on hematological parameters, the stomach tissue structure, and the immunological changes accompanied. The current results revealed that the total body weights of the mice in the different experimental groups were increased over different weeks compared to the 1st week which could be contributed to an increase in animal age. Meanwhile, the percentage changes of the total body weight of the different experimental groups decreasedcompared to the control. The weights of the 2nd and 3rd groups were significantly lower than the control group which might be due to the toxic effects of CCT onthe gastrointestinal tract as revealed by microscopic examination of the stomach tissues. The first sign was the change of the experimental animal's body masses. Body mass was decreased in groups 2, 3, and 4 due to the unhealthy status of animals which was directly affected by the extract. We believe that severe damage may have occurred at the physiological level which induces a kidney or liver function disorder. While the body mass of group 4 increased with the highest dose, this may be due tothe subjects retaining too much water.

Generally, the hematological pictures reflect the immune response of the host to external agents. Differential leucocytes such as lymphocytes and neutrophils are produced by erythrocyte tissue and are responsible for the induction of the immune response with monocytes (JANEWAY *et al*. 1999). The results of the complete blood count showed that the animals in group 4, which received a higher the dose of aqueous CCTfruit extract, had significantly higher total WBC and differential leukocyte counts; while hemoglobin and MCHC were significantly lower than in the control group.The observed increase in WBC and differential leukocytescould have been due to increased inflammation as proven by our histological results of the stomach and a significant increase of pro-inflammatory circulating signals (IL-8 and IL-6). The decline inhemoglobin and MCHC could be explained by decreased iron absorption due to the damage to the stomach mucosa as the stomach is responsible for the ionization of iron. In this respect, (SHAH *et al*. 1989) mentioned that CCThad significant hematological dysfunction effects. Meanwhile, (ELGERWI *et al*. 2013) reported a significant increase

in RBC count, hemoglobin concentration, and hematocrit after CCT administration in albino rats. The authors explained these effects as a CCT dehydrating effect as the animals used in this experiment had diarrhea. Also, there was a significant elevation in the total WBC and differential leucocyte counts. These observed results were similar to previous studies performed elsewhere (ELAWAD *et al*. 1984 and ATOLE *et al*. 2009).

The phytochemical analysis of CCT fruit demonstrates that, in addition to alkaloids, tannins, saponins, flavonoids, terpenoids, phenolic, anthranol, steroids, J, L, caffeic acid, Cucurbitacin A, B, C, D, E (α-elaterin), and cardiac glycoloids, it contains amino acids, proteins, lipids, and carbohydrate nutrients (RAHIMI*et al*. 2012; MIAO *et al*. 2012 and AL-SNAFI 2016). A dose-dependent observed increase in WBC and differential leucocytes counts could be also attributed to these micronutrients in the CCT fruit extract. Extracted Cucurbitacin E glucoside escalating inhibits the testosterone-induced experimental BPH (Benign prostatic hyperplasia) in mice via, at least partly, its antiproliferative, antioxidant, anti-inflammatory, and antifibrotic effects (BASHA *et al.* 2019). The Cucurbitacin E glucoside seems to exhibit anti-inflammatory activity as it decreased cyclooxygenase-2 and interleukin-1β protein expression in prostatic tissues (BASHA *et al.* 2019). While, synthesized Cucurbitacin E glucoside (1) and its derivatives (2 and 3) inhibited the elevation of proinflammatory cytokines (TNF-α, IL-6, and IL-23) in the livers of t-BHP-treated rat models (HUSSEIN *et al*. 2017).

These compounds are fundamental for the development and maturation of the immune system, specifically the cellular components of hemopoiesis (MAGGINI *et al*. 2007). Neutrophils are not only one of the main components of innate immunity but are considered a Cinderella of the innate immune system (KUMAR & SHARMA 2010). They have always been considered tissue-destructive cells responsible for inflammatory tissue damage occurring during acute responses (KUMAR & SHARMA 2010). They also contribute to the clearance of foreign bodies by recognition and migration to the foreign body, phagocytosis, and destroying these foreign agents (JANEWAY *et al*. 1999). Our depicted results showed a dose-dependent increment in the neutrophils index which may indicate an acute activation and migration. It is important to note that this is the first reported link of the neutrophil activation index with CCT fruit extract consumption. The significant elevation of both the lymphocytes and monocytes indexes also supports the above finding. The vigorous inflammatory signals elicited by CCT fruit extract could therefore be used when the immune system is immunocompromised to recovery and improve the cell-mediated immune response as this extract potentiates immune responses (SHOKRZADEH *et al*. 2013). They do not exclude the possibility that some CCT fruit extract metabolites were absorbed and transported from the treated mice'sgastrointestinal region into their livers, where Kupffer cells (the liver is the main source of monocytes) are nourished (JANEWAY *et al*. 1999). This may explain and substantiate the higher monocyte index in the treated groups especially group 4, which could beconsidered a defense mechanism exerted by the animals.

Pro-inflammatory cytokines contain Th1-type cytokines [interferon (IFN) -γ and tumor necrosis factor (TNF) -α] and others [interleukin (IL)-1β and IL- 6], whereas anti-inflammatory cytokines are Th2-type cytokines [IL-4 and IL-10] and others [transforming growth factor (TGF)-β1] (WILCZYŃSKI 2005). The results of this study revealed that oral administration of aqueousCCT fruitextract induced a significant elevation in the serum levels of IL-8 in groups 3 and 4 which received 200 and 300 mg/kg of body weight/day and in IL-6 in group 4 only. On the other hand, IL-10 was significantly decreased in all the treated groups. In a similar line, it was reported that thegastric mucosal levels of IL-6 increased in *H. pylori*-accompanied gastritis (SUGIMOTO *et al*. 2010) and *H. pylori*-positive individuals with gastric carcinoma (MEJÍAS-LUQUE *et al*. 2008). Also, a high expression of IL-8 was shownin gastric mucosa infected with *H. pylori* (KUSUGAMI *et al*. 1997). IL-8 leads to chemotaxis and inflammatory cell activation in gastric mucosa infected with *H. pylori* (NAITO *et al*. 2010). Meanwhile, IL-10 downregulates cell-mediated immune actions and cytotoxic inflammatory actions (KIM *et al*. 2012). Interleukin-10 suppresses pro-inflammatory cytokines (TNF-α & IL-6) released via human macrophage/monocyte in action for polymethyl methacrylate (PMMA, spherical 1-10 μm), the challenge of a particle *in vitro* (TRINDADE *et al*. 2001). However, others reported that aqueous CCT fruitextract (4 mg/kg) resulted in an anti-inflammatory action upon using a carrageenan-made edema of paw assay in rats (MARZOUK *et al*., 2010). *In vivo,*the anti-inflammatory actions of various components of CCT extract were found utilizing a carrageenan-made edema of paw assay in albino rats (ALY & NADDAF 2006). Also using a carrageenan-made edema of paw assay in rats, research about CCT crude extract from immature fruitsfrom South of Tunisia revealed an anti-inflammatory action (MARZOUK *et al*. 2011). A previous study reported that an oral intake of 50 mg/kg of CCT extract for 6 weeks resulted in anti-inflammatory action by decreasing both IL-6 and TNF- α levels, while not affecting the IL-10 steady-state in obese mice (SANADGOL *et al*. 2011).

The results of this study revealed that the macroscopic examination of the stomachs *in situ* did not show any morphological changes in the treated animals in different treated groups compared with control mice. Microscopic examination however revealed that CCT resulted in the superficial focal loss of the

surface mucous protective epithelium, the atrophy of peptic cells secreting enzymes, and a thickening of sum mucosal connective tissues. The submucosa showed vascular congestion and inflammatory cells in the treated animals which recieved 300 mg/kg of body weight/day. Surface degeneration was more frequent and deep in the high dose group (group 4), with marked atrophy of both oxyntic and peptic cells. The severity of damage to the stomach mucosa reported in this study was positively proportional with the increasingthe CCT doses, this may indicate that the toxicity was due to toxic components such as saponin and alkaloids that lead to toxicity according to the dose used. The detected lesions of the stomach presented in this study point out the direct local irritant action of CCTextract on the mucosae. CCT toxicity at high doses was reported in human cases and in experimental animals (BAKHIET & ADAM 1995; AL-YAHYA *et al*. 2000; BARTH *et al*. 2002), but the fruit extract,also has significant anti-proliferative potential against MCF-7 and AGS cell lines of gastric adenocarcinoma and breast cancer. These cytotoxicity effects were dose-dependent and probably induced by apoptosis (REZAI *et al*. 2018). Systemic CCT use causes adverse side effects such as acute rectorrhagia, abortifacient, liver intoxication, cardio-suppression, and vomiting (KUMAR *et al*. 2008; AKHZARI *et al*. 2015; ALHAWITI 2018). In 1989, 3 cases of acute toxic colitis after admiration of CCTwererecorded by (GOLDFAIN *et al.* 1989). The main symptom was dysenteric diarrhea. Colonoscopiesrevealed hyperemia and congestion with abundant exudates of the mucosa that faded within 14 days after the ceasing CCT administration (GOLDFAIN *et al*. 1989). CONTRERAS *et al*. (1996) recorded that poisoning by colocynth isa rare cause of acute diarrhea. KHAN *et al*. (2003) observed that five cases of toxicity caused by colocynth intake in Saudi Arabia for a period of more than two yearsresultedin acute severe bloody diarrhea. Four cases of colocynth intoxication were symptomatized with acute rectorrhagia after mucosal diarrhea associated with tenesmus, that progressed to rectorrhagia and bloody diarrhea in 3 to 4 hours. Colonoscopies showed ulcers on the mucosa that had completely disappearedby the next colonoscopy 14 days later (JAVADZADEH *et al*. 2013).

The membranolytic actions of different CCTconstituents were blamed for the destruction of the intestine due to CCT's cucurbitacin glycoside content (JAVADZADEH *et al*. 2013). The fruit extract of CCThad teratogenic effects if taken in early pregnancy in rats (ELGERWI *et al*. 2013). In a study where rabbits administered with 100 or 200 mg/kg of body weight/day of CCT seed or extract for 30days, all rabbits treated with 200 mg/kg of body weight/day of extract died. The rabbits that were administrated 100 mg/kg of body weight/day of extract showed massive damageto their kidneys, liver, and small intestine (SHAFAEI *et al*. 2012). A single daily dose of CCT ethanol extract (50, 100, 200, and 400 mg/kg of body weight) given intraperitoneally, for 14 days, induced hepatocyte necrosis and liver fibrosis in rats, in a dose-dependent pattern (DEHGHAN & PANJEH 2006). Meanwhile, ICKERT (1980) observed that extract from dried CCT fruits had no toxicological action in Wister rats when administered subcutaneously for four weeks and advised the administration of the extract as a cathartic purgative.

The proper therapeutic dose of CCT fruit varied from 0.6 to 1.75 grams/day and 0.1 to 0.4 grams/day according to Traditional Iranian Medicine (TIM) and modern phytotherapy. CCT seed must be given at 120 to 300 mg/day (maximum: 600 mg), and root powder at 0.2 to 0.4 grams/day. If the fruit is given with its correctives as a gum of Arabic, side effects are decreased and larger doses are allowed. The toxic dosage of CCT reported was 600-1000 mg and may lead to hematochezia, abdominal colic, diarrhea, nephrosis of the kidneys, and frequent vomiting. Fatal doses ($\geq$2 g) led to paralysis, fits, and death due to the collapse of the circulation system (RAHIMI, *et al*. 2012). However, SACHIN & ARCHANA (2009) examined (aqueous and alcoholic extracts) of CCT for their anti-ulcer actions in a pylorus ligation induced ulcer model in male Wistar rats. They reported that aqueous and alcoholic extracts had significant anti-ulcer actions due to flavonoids, tannins, alkaloids, saponins glycosides, and phenolic substances. These active compounds enhance bicarbonate, mucus, and prostaglandin secretions and antagonized the deteriorating actions of reactive oxidants in the lumen of the gastrointestinal tract.

Conclusion

Traditional, natural herbal medicine would offer an advantage over conventional treatments but with the appropriate dose and duration. Current results provide biological evidence for the histological and immunological activities of aqueous CCT extract in experimental mice and may suggest and support the limitation of the traditional use of this plant. A point of interest is the side effects accompanied bya higher dose. At higher doses, CCT is suspected to induce injury to the mucosal layers of the stomach (300 mg/kg of body weight/day) and shouldn't be taken for more than 20 consecutive days to avoid possible toxicity, through immunological inflammatory signals, which is congruent with TIM's recommendation. Our study's goal was to investigate the long-term actions and toxicity of CCT to prevent unexpected life-threatening complications. Our findings may suggest that the identification and characterization of different components of CCT fruit extract followed by theirconversion to synthesized medicines will not only be helpful for determining an accurate estimation of the harmful effects of CCT but the permitted and toxic dosages as well.

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Author Contributions

Research concept and design: M.O.A.; Collection and/or assembly of data: R.S.B.; Data analysis and interpretation: R.S.B., E.M.R.; Writing the article: R.S.B.; Critical revision of the article: M.O.A., E.M.R.; Final approval of article: M.O.A., E.M.R.

Conflict of Interest

The authors declare no conflict of interest.

References

AKHZARI M., MIRGHIASI S., VASSAF M., BIDGOLI M., TARI Z. 2015. The effect of *Citrullus colocynthis* on the reduction of inflammatory agents in osteoarthritis. Mol. Biol. **14**: 147-155. https://doi.org/10.4172/2168-9547.1000147

ANTAL C., TELETIN M., WENDLING O., DGHEEM M., AUWERX J., MARK M. 2007. Tissue collection for systematic phenotyping in the mouse. Curr. Protoc. Mol. Biol. **29**: 1-29.A.4.23 https://doi.org/10.1002/0471142727.mb29a04s80

AL-SNAFI A.E. 2016. Chemical constituents and pharmacological effects of *Citrullus colocynthis*-A review. IOSR J. Pharm. **6**: 57-67.

AL-YAHYA M., AL-FARHAN A., ADAM S. 2000. Preliminary toxicity study on the individual and combined effects of *Citrullus colocynthis* and Nerium oleander in rats. Fitoterapia **71**: 385-391. https://doi.org/10.1016/s0367-326x(00)00135-0

ALHAWITI N.M. 2018. Antiplatelets and profibrinolytic activity of *Citrullus colocynthis* in control and high-fat diet-induced obese rats: Mechanisms of action. Arch. Phys. Biochem. **124**: 156-166. https://doi.org/10.1080/13813455.2017.1369999

ALY A.M., NADDAF A. 2006. Anti-inflammatory activities of Colocynth topical gel. J. Med. Sci. **6**: 216-221. https://doi.org/10.3923/jms.2006.216.221

ATOLE S., JANGDE C., PHILIP P., REKHE D., AGHAV D., WAGHODE H., CHOUGULE A. 2009. Safety evaluation studies of *Citrullus colocynthis* for diabetes in rats. Vet. World **2**: 423-425.

BAKHIET A., ADAM S. 1995. An estimation of *Citrullus colocynthis* toxicity for chicks. Veterinary Hum. Toxicol. **37**: 356-358.

BARTH A., MÜLLER D., DÜRRLING K. 2002. In vitro investigation of a standardized dried extract of *Citrullus colocynthis* on liver toxicity in adult rats. Exp. Toxicol. Pathol. **54**: 223-230. https://doi.org/10.1078/0940-2993-00252

CARDIFF R.D., MILLER C.H., MUNN R.J. 2014. Manual hematoxylin and eosin staining of mouse tissue sections. Cold Spring Harbor Protocols **6**: prot073411. https://doi.org/10.1101/pdb.prot073411

BASHA S.Z., MOHAMED G.A., ABDEL-NAIM A.B., HASAN A., ABDEL-LATEFF A. 2019. Cucurbitacin E glucoside from *Citrullus colocynthis* inhibits testosterone-induced benign prostatic hyperplasia in mice. Drug Chem. Toxicol. **12**: 1-11. https://doi.org/10.1080/01480545.2019.1635149

CONTRERAS M., GALLARDO A., GARCIA F., RODRIGUEZ F. 1996. Poisoning by coloquintide, an infrequent cause of acute diarrhoea syndrome. Med. Clin. (Barc) **106**: 599-599.

DEHGHAN F., PANJEHS, HAHIN M.R. 2006. The Toxic Effect of Alcoholic Extract of *Citrullus Colocynthis* on Rat Liver. Iranian J. Pharmacol. Therapeutics **5**: 117-119.

DHANOTIA R., CHAUHAN N.S., SARAF D.K., DIXIT V.K. 2011. Effect of *Citrullus colocynthis* Schrad fruits on testosterone-induced alopecia. Nat. Prod. Res. **25**: 1432-1443. https://doi.org/10.1080/14786410802632820

ELAWAD A., ABDEL E.B., MAHMOUD O., ADAM S. 1984. The effect of *Citrullus colocynthis* on sheep. Vet. Hum. Toxicol. **26**: 481-485.

ELGERWI A., BENZEKRI Z., AWAIDAT S., EL-MAGDOUB A., ABUSNINA A., EL-MAHMOUDY A. 2013. Subchronic haemotoxicity and histotoxicity of *Citrullus colocynthis*. J. Am. Sci. **9**: 79-87.

ELGERWI A.A., BENZEKRI Z., EL-MAGDOUB A., EL-MAHMOUDY A. 2013. Qualitative identification of the active principles in *Citrullus colocynthis* and evaluation of its teratogenic effects in albino rats. Int. J. Basic & Clin Pharmacol. **2**: 438-445. https://doi.org/10.5455/2319-2003.ijbcp20130818

GOLDFAIN D., LAVERGNE A., GALIAN A., CHAUVEINC L., PRUDHOMME F. 1989.Peculiar acute toxic colitis after ingestion of colocynth: a clinicopathological study of three cases. Gut **30**: 1412-1418.

HUSSAIN A.I., RATHORE H.A., SATTAR M.Z., CHATHA S.A., SARKER S.D., GILANI A.H. 2014. *Citrullus colocynthis* (L.) Schrad (bitter apple fruit): A review of its phytochemistry, pharmacology, traditional uses and nutritional potential. J. Ethnopharmacol. **155**: 54-66. https://doi.org/10.1016/j.jep.2014.06.011

HUSSAIN A.I., RATHORE H.A., SATTAR M.Z., CHATHA S.A., UDDIN AHMAD F., AHMAD A., JOHNS E.J. 2013. Phenolic profile and antioxidant activity of various extracts from *Citrullus colocynthis* (L.) from the Pakistani flora. Ind. Crop. Prod. **45**: 416-422. https://doi.org/10.1016/j.indcrop.2013.01.002

HUSSEIN M.A., EL-GIZAWY H.A., GOBBA N.A.E.K., MOSAAD Y.O. 2017. Synthesis of Cinnamyl and Caffeoyl Derivatives of Cucurbitacin-Eglycoside Isolated from Citrullus colocynthis Fruits and their Structures Antioxidant and Anti-inflammatory Activities Relationship. Curr. Pharm. Biotechnol. **18**: 677-693. https://doi.org/10.2174/1389201018666171004144615

ICKERT G. 1980. Toxicology of Colocynth. Zentralbl Pharma. Pharmakother. Laboratoriums Diag. 118p.

JANEWAY C.A., TRAVERS P., WALPORT M., SHLOMCHIK M. 1999. The Immune System in Health and Desease. (In: Immunobiology. Garland Science, New York, 4th ed.): 471-500.

JAVADZADEH H.R., DAVOUDI A., DAVOUDI F., VALIZADEGAN G., GOODARZI H., MAHMOODI S., FARAJI M. 2013. *Citrullus colocynthis* as the cause of acute rectorrhagia. Case Reports Emerg. Med. ID 652192. https://doi.org/10.1155/2013/652192

KHAN S.A., SHELLEH H.H., BHAT A.R., BHAT K.S. 2003. Colocynth toxicity. A possible cause of bloody diarrhea. Saudi Med. J. **24**: 904-906.

KIM J., CHO Y.A., CHOI I.J., LEE Y.S., KIM S.Y., SHIN A., CHO S.J., KOOK M.C., NAM J.H., RYO K.W., LEE J.H. 2012. Effects of interleukin-10 polymorphisms, *Helicobacter pylori* infection, and smoking on the risk of noncardia gastric cancer. PloS One **7**: e29643. https://doi.org/10.1371/journal.pone.0029643

KUMAR S., KUMAR D., JUSHA M., SAROHA K., SINGH N., VASHISHTA B. 2008. Antioxidant and free radical scavenging potential of *Citrullus colocynthis* (L.) Schrad. methanolic fruit extract. Acta Pharm. **58**: 215-220. https://doi.org/10.2478/v10007-008-0008-1

KUMAR V., SHARMA A. 2010. Neutrophils: Cinderella of innate immune system. Int. Immunopharmacol. **10**: 1325-1334. https://doi.org/10.1016/j.intimp.2010.08.012

KUSUGAMI K., ANDO T., OHSUGA M., IMADA A., SHINODA M., KONAGAYA T., ICHIYAMA S. 1997. Mucosal Chemokine Activity in *Helicobacter pylori* Infection. J. Clin. Gastroenterol. **25**: S203-S210.

MAGGINI S., WINTERGERST E.S., BEVERIDGE S., HORNIG D.H. 2007. Selected vitamins and trace elements support immune function by strengthening epithelial barriers and cellular and humoral immune responses. Br. J. Nutr. **98** (Suppl 1): 29-35. https://doi.org/10.1017/s0007114507832971

MARZOUK B., MARZOUK Z., DÉCOR R. 2009. Antibacterial and anticandidal screening of Tunisian *Citrullus colocynthis* Schrad. from Medenine. J. Ethnopharmacol. **125**: 344-349. https://doi.org/10.1016/j.jep.2009.04.025

MARZOUK B., MARZOUK Z., HALOUI E., FENINA N., BOURAOUI A., AOUNI M. 2010. Screening of analgesic and anti-inflammatory activities of *Citrullus colocynthis* from southern Tunisia. J. Ethnopharmacol. **128**: 15-19. https://doi.org/10.1016/j.jep.2009.11.027

MARZOUK B., MARZOUK Z., HALOUI E., TURKI M., BOURAOUI A., AOUNI M., FENINA N. 2011. Anti-inflammatory evaluation of immature fruit and seed aqueous extracts from several populations of Tunisian *Citrullus colocynthis* Schrad. Afr. J. Biotechnol. **10**: 4217-4225.

MEJÍAS-LUQUE R., PEIRÓ S., VINCENT A., VAN SEUNINGEN I., DE BOLÓS C. 2008. IL-6 induces MUC4 expression through gp130/STAT3 pathway in gastric cancer cell lines. Bioch. Bioph. Acta (BBA)-Mol. Cell Res. **1783**: 1728-1736. https://doi.org/10.1016/j.bbamcr.2008.05.020

MIAO J., ZHANG J., DENG S.-M., DAI B. 2012. A new flavone C-glycoside from *Citrullus colocynthis*. Chinese Her. Med. **4**: 1-3. https://doi.org/10.3969/j.issn.1674-6384.2012.01.001

MUHSEN U., ALALI Z. 2010. Effect of hot aqueous extract of *Citrullus colocynthis* L. fruit on some biochemical and haematological parameters in alloxan – induced diabetic rat. Kuphah Univeirty J. **2**: (2), 1-8.

NAITO M., EGUCHI H., GOTO Y., KONDO T., NISHIO K., ISHIDA Y., KAWAI S., OKADA R., HISHIDA A., WAKAI K., HAMAJIMA N. 2010. Associations of plasma IL-8 levels with *Helicobacter pylori* seropositivity, gastric atrophy, and IL-8 T-251A genotypes. Epidemiol. & Infect. **138**: 512-518. https://doi.org/10.1017/s0950268809990677

NFAMBI J., BBOSA G.S., SEMBAJWE L.F., GAKUNGA J., KASOLO J.N. 2015. Immunomodulatory activity of methanolic leaf extract of *Moringa oleifera* in Wistar albino rats. J. Basic Clin. Physiol. Pharmacol. **26**: 603-611. https://doi.org/10.1515/jbcpp-2014-0104

PRAVIN B., TUSHAR D., VIJAY P., KISHANCHNAD K. 2013. Review on *Citrullus colocynthis*. Int. J. Res. Pharm. Chem. **3**: 46-53.

RAHBAR A.R., NABIPOUR I. 2010. The hypolipidemic effect of *Citrullus colocynthis* on patients with hyperlipidemia. Pakistan J. Biol. Sci. **13**: 1202. https://doi.org/10.3923/pjbs.2010.1202.1207

RAHIMI R., AMIN G., ARDEKANI M.R.S. 2012. A review on *Citrullus colocynthis* Schrad.: from traditional Iranian medicine to modern phytotherapy. J. Altern. Compl. Med. **18**: 551-554. https://doi.org/10.1089/acm.2011.0297

REZAI M., DAVOODI A., ASORI M., AZADBAKHT M. 2017. Cytotoxic activity of *Citrullus colocynthis* (L.) schrad fruit extract on gastric adenocarcinoma and breast cancer cell lines. Int. J. Pharm. Sci. Rev. Res. **45**: 175-178.

SACHIN S.S., ARCHANA R.J. 2009. Antiulcer activity of methanol extract of *Erythrina indica* Lam. leaves in experimental animals. Pharmacog. Res. **1**: 396-401. http://www.phcogres.com/text.asp?2009/1/6/396/58026

SANADGOL N., NAJAFI S., GHASEMI L.V., MOTALLEB G., ESTAKHR J. 2011. A study of the inhibitory effects of *Citrullus colocynthis* (CCT) using hydro-alcoholic extract on the expression of cytokines: TNF-and IL-6 in high fat diet-fed mice towards a cure for diabetes mellitus. J. Pharmacog. Phytother. **3**: 81-88.

SHAFAEI H., ESMAEILI A., RAD J.S., DELAZAR A., BEHJATI M. 2012. *Citrullus colocynthis* as a medicinal or poisonous plant: a revised fact. J. Med. Plan. Res. **6**: 4922-4927.

SHAH A., QURESHI S., TARIQ M., AGEEL A. 1989. Toxicity studies on six plants used in the traditional Arab system of medicine. Phytother. Res. **3**: 25-29.

SHOKRZADEH M., CHABRA A., NAGHSHVAR F., AHMADI A. 2013. The mitigating effect of *Citrullus colocynthis* (L.) fruit extract against genotoxicity induced by cyclophosphamide in mice bone marrow cells. Sci. World J. 980480. https://doi.org/10.1155/2013/980480

SOUFANE S., BEDDA A., MAHDEB N., BOUZIDI A. 2013. Acute Toxicity study on *Citrullus colocynthis* fruit methanol extract in Albino rats. J. Appl. Pharm. Sci. **3**: 88-93. https://doi.org/10.7324/JAPS.2013.3614

SUGIMOTO M., YAMAOKA Y., FURUTA T. 2010. Influence of interleukin polymorphisms on development of gastric cancer and peptic ulcer. World J. Gastroenterol. **16**: 1188. https://doi.org/10.3748/wjg.v16.i10.1188

TANNIN-SPITZ T., BERGMAN M., GROSSMAN S. 2007. Cucurbitacin glucosides: antioxidant and free-radical scavenging activities. Biochem. Biophys. Res. Commun. **364**: 181-186. https://doi.org/10.1016/j.bbrc.2007.09.075

TRINDADE M.C., LIND M., NAKASHIMA Y., SUN D., GOODMAN S.B., SCHURMAN D.J., SMITH R.L. 2001. Interleukin-10 inhibits polymethylmethacrylate particle induced interleukin-6 and tumor necrosis factor-α release by human monocyte/macrophages *in vitro*. Biomat **22**: 2067-2073. https://doi.org/10.1016/S0142-9612(00)00376-8

WILCZYŃSKI J.R. 2005. Th1/Th2 cytokines balance – yin and yang of reproductive immunology. Eur. J. Obstet. Gyn. R. B. **122**: 136-143. https://doi.org/10.1016/j.ejogrb.2005.03.008

VALENTIM A.M., ALVES H.C., OLSSON I.A.S., ANTUNES L.M. 2008. The effects of depth of isoflurane anesthesia on the performance of mice in a simple spatial learning task. J. Am. Assoc. Lab. Anim. Sci. **47**: 16-19.

ZHENG M.S., LIU Y.S., YUAN T., LIU L.Y., LI Z Y., HUANG X.L. 2020. Research progress on chemical constituents of *Citrullus colocynthis* and their pharmacological effects Zhongguo Zhong Yao Za Zhi. (Chinese Journal of Traditional Chinese Medicine). **45**: 816-824. (In Chinese). https://doi.org/10.19540/j.cnki.cjcmm.20191104.201