Annona muricata L. suppress carbon tetra chloride effects through alleviating hematological disturbances in adult male rabbits

ABSTRACT

Background: *Annona muricata.* L has many of medicinal properties and has been used widely in traditional medicine for treatment various disorders. Carbon tetrachloride (CCl_4) is rapidly absorbed by any route of exposure in humans and animals. Once absorbed, it is widely distributed among tissues depending on exposure concentration or dose. This study was designed to find out possible role of *A. muricata* suppress CCl_4 toxicity through alleviating hematological parameters disturbances in adult male rabbits.

Materials and methods: Four rabbits per group were assigned to four groups: 0 mg *A. muricata* and 0 mg CCl_4/kg BW (control); 100 mg of *A. muricata* /kg BW; 0.5 mg CCl_4/kg BW; 0.5 mg CCl_4 plus 100 mg of *A. muricata* /kg BW. Rabbits were orally administered the respective doses every other day for 3 weeks.

Results: Treatment with *A. muricata* were not caused significant effect on values of red blood cells (RBCs), packed cell volume (PCV), hemoglobin (Hb), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC). On the other hand CCl_4 was caused increase on values of RBCs, Hb, MCV, and MCHC. Only MCV was significantly decreased with *A. muricata* alone. The presence of *A. muricata* with CCl_4 returned the values of the previous parameters close to the control values. CCl_4 caused high significant increased in mean number of WBCs compared with other three groups among three weeks of experiment. However, treatment with CCl_4 was not yielded significant deference in percentage value of neutrophil or lymphocytes compared to control. The presence of *A. muricata* with CCl4 returned mean number of WBCs close to control value. Current results also indicated that treatment with CCl_4 caused a significant decreased in number of PLT while *A. muricata* caused increased in the number of PLT compared with control group. Oral administration of CCl_4 induced profound alterations in the morphology of erythrocytes. In rabbit treated with CCl_4 the cells were showed alteration in normal shape to star shaped or sickle cells. The mentioned alterations were less pro-found in animals treated with CCl_4 plus *A. muricata*. The cells had nearly normal morphology and regular contour, only some erythrocytes (not normal) could be noticed.

Conclusion: Administration of *A. muricata* L. in combination with CCl_4 was able to minimize and alleviate the hazardous effect of CCl_4 on most of the measured parameters. So it can be concluded that the presence of *A. muricata* with CCl_4 counteracted the negative effects of CCl_4 on hematological parameters in male rabbit.

Keywords: A. muricata, CCl4, hematological parameters, and male rabbits

1. INTRODUCTION

Carbon tetrachloride (**CCL_4**) called also per chloromethane or tetra chloromethane, is a colorless, noninflammable volatile liquid with a distinct odour and immiscible with water, and is produced by chlorination of methane, ethane, propane, or propene. The molecular weight of this compound is 153.82 Da [1]. Although this compound is a haloalkane used in a variety of industrial and chemical applications. It has been widely used for its solvent properties, particularly in refrigerator fluids, as a propellant for aerosol cans, as a dry-cleaning agent in industry, as a household spot remover, as grain fumigant and as intermediate in the synthesis of chlorofluorocarbons. As a result of its widespread use, CCl_4 is a common contaminant of ground and surface waters where it persists for years. Therefore CCl_4 is now of greatest concern as an environmental contaminant [2]. It is known as hepatotoxic industrial solvent [3]. It is commonly used for free radical induced liver injury [4, 5]. Ozturk and Ucar [6] mention that liver is not the only target organ of CCl_4 but it also affects several organs of the body such as lungs, hearts, testes, kidneys and brain. Renal failure is associated with abnormalities affecting hematological parameters such as erythropoiesis, platelet function, thrombopoiesis, and immune function [7]. The result of a study made by Saba, Oyagbemi [8] pointed to the significant reduction in the packed cell volume (PCV), haemoglobin concentration (Hb), red blood cells (RBCs), platelet count (PLT), mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) values, while mean corpuscular haemoglobin concentration (MCHC) was higher in rats treated group intraperitoneally with a single dose of CCl_4. In contract the level of total white blood cells (WBCs) count was significant elevated which observed as a result of neutrophil lymphocyte, eosinohpil and monocytes increased. Sahreen and Khan [9] found that Dawley rats administration with CCl_4 (0.5 ml per kg body weight) intraperitoneally twice a week for eight weeks showed a significant increase in red blood cells and white blood cells level compared with control group. Treated rabbit group given orally a single dose of CCl_4 (1.25 ml per kg body weight) showed a non-significant increase in WBCs, RBCs, Hb and hematocrit (HCT) and non-significant decrease in PLT count compared with control group [10].

In study made by Al Mashhadani [11] suggested that a significant increase in the total count of WBCs, lymphocytes and platelet in male albino rats group after given intraperitoneally 1 ml per kg body weight of CCl_4 for four weeks. Albokhadai [12] found that treated group of albino rats given intraperitoneally injection with CCl_4 caused a significant reduction in the RBCs count and Hb concentration compared with normal control. In addition the total count of WBCs was increased significantly in rats exposed to CCl_4 when compared to the control. Another study reported that the cellular antioxidant action is reinforced by the presence of dietary antioxidants. Antioxidants and anti-inflammatory agents play a critical role against CCl4 intoxication by scavenging active oxygen and free radicals and neutralizing lipid peroxides [13, 14].

A. muricata (Linn.) commonly called soursop, Graviola or guanabana is an evergreen tree native to the tropical regions belonging to the Annonacea family [15]. It plays a crucial role in various traditional and alternative. All parts of this tree are extensively used as traditional medicines. The bark, leaves, roots, fruit and fruit seeds have their own respective use [16-18]. The leaves of *A. muricata* have been reported to contain several groups of substances collectively called annonaceous acetogenins [19], annopentocin A, B and C,(2,4-cis) -annomuricin-D-one, murihexocin A and B, (2,4-trans) -annomuricin-D-one, 4-acetyl gigantetrocin and cis-gigantrionin [20], muricatocin A, B, and C [21], and annohexocin [20]. The high potency, selectivity, wide chemical and biological diversity, and effectiveness of these compounds against microbial resistance could well make them the next class of useful natural antitumor and pesticidal agents [22] and other pharmacological effects .The leaves of *A. muricata* have essential oils with parasiticidal, antidiarrheal, rheumatological, and antineuralgic properties [23].

The boiled water infusion of the leaves have anti-plasmodic, astringent, and gastric properties, help treat diabetes and gastric upset [24], used in treating kidney aliments [26]. Arthur and Woode [27] indicate that *A. muricata* have a high annonaceous acetogenins content which have high antioxidant activity. Recent study found that leaf extract of *A. muricata* has and modulatory effects on hematopoietic of Male adults rabbits [28]. Despite extensive research into the antioxidant level and activity possessed by *A. muricata* and its effectiveness in treating disease, a comparative study of the antioxidant level and activity of *A. muricata* obtained from different locations has not been reported [29]. Nonetheless, previous studies have shown that there are different levels of antioxidant/phenolic content among plants of similar species [28, 30, 31]. This study was designed to find out possible role of *A. muricata* suppress CCL_4 toxicity through alleviating hematological parameters disturbances in adult male rabbits.

2. MATERIAL AND METHODS

CCl_4 was obtained from the chemistry department, faculty of science, Omar Al-Mukhtar University (0.5mg/ml). *A. muricata* leaf (powder) (maximum international company, Brasil) was purchased from local pharmacy. Each capsule contains 0.3125 g powder and the content of each capsule was dissolved in corn oil just before use. Mature male New Zealand White rabbits (age of 6 months and initial weight of (1.641 ± 27.2 Kg) were used. All animals were used following appropriate approval from the University of Omar Al-Mukhtar.

Experimental procedure: Sixteen mature male rabbits were randomly divided into four equal groups:

- Group I: The control group received an equivalent of 1 ml of the vehicle (corn oil) alone by oral gavage twice per week for 3 successive weeks.
- Group II: Rabbits were treated with *A. muricata* extract which was given twice per week by gavage at a dose of 100 mg/kg B.W [32] for 3 successive weeks.
- Group III: Rabbits were treated with CCl_4 of 0.5 mg/kg BW in 0.5 ml of corn oil by gavage twice per week [33].
- Group IV: Rabbits were given with CCl_4 twice a week at a dose of 0.5ml/kg BW by gavage like group III and given *A. muricata* extract concurrently twice per week at a dose of 100 mg/kg B.W. by gavage like group II for three successive weeks.

Hematological parameters: Blood samples were collected from the ear vein of all animals every week throughout the 3-weeks experimental period. Blood samples were obtained in the morning before accesses to feed and water. Values derived from complete blood count (CBC). All CBC tests were performed by automatic blood cell analyzer (XP-300). Automated Hematology Analyzer, Sysmex American, Inc [34, 34]. CBCs were performed on EDTA as anti-coagulated samples. Differential cell counts were performed manually using Dif-Quik-stained blood smears. At the end of the experimental period, all rabbits were weighed then sacrificed under ether anesthesia to prepare bone marrow smear.

Preparation of blood smears: Thin films, 3-5 cm in length, of the aspirated a drop of blood was made using a smooth-edged glass spreader of not more than 2 cm in width. And blood smears was prepared as method described previously [34, 35].

Preparation of bone marrow smears: Bone marrow cells were collected from rabbit's femora after cutting away the epiphyses and condyles. Bone marrow smears was prepared as method described previously [34, 35].

Statistical analysis: Where applicable, statistical analysis was carried out in Minitab software (version17)/ GraphPad prism8; statistical significance was assessed using ANOVA analysis

with Tukey multiple comparison test after detection normal distribution to the data and appropriate $P < 0.05$ consider significant.

3. RESULTS

All the rabbits (control and treated) were observed daily after every dosing for 3-5 hrs for clinical symptoms. CCl_4-fed rabbits showed varying degrees of clinical signs few minutes after dosing. The signs included disorientation, drowsiness, uncoordinated movements, mild tremor and diarrhea. Concerning symptom changes, CCl_4-treated rabbits showed hair loss whereas control animals did not display such change. Values derived from complete blood counts (CBC), including differential cell counts were recorded for each group and presented in Table 1. Treatment with *A. muricata* was caused non-significant effect on RBCs, PCV, HB concentration, MCH and MCHC. Only MCV was significantly decreased with *A. muricata*. On the other hand treatment with CCl_4 caused decrease in RBCs, HB, MCH, and MCHC. The presence of *A. muricata* plus CCl_4 returned values of the previous parameters close to the control values. The changes in these parameters were described separately among three weeks of experiments as shown in Figures 1 to 8. Treatment with CCl_4 caused significant increase in number of WBCs compared with other three groups. And this increasing started since first week till end of experiment as shown in Figure 8. The presence of *A. muricata* plus CCl_4 returned the mean number of WBCs close to control values. Treatment with CCl_4, and *A. muricata* was not yielded significant alterations in mean values of neutrophils and lymphocytes between first week and end of third week (Tables 2). Treatment with CCl_4 caused significant decreased in number of PLT comparison to control group.

Oral administration of CCl_4 induced profound alterations in the morphology of erythrocytes. In rabbit treated with CCl_4: morphology of erythrocytes showed alteration to star shape and sickle cells. The previously mentioned alterations were less pro-found in animals treated with CCl_4 plus *A. muricata*. The cells had nearly normal haemoglobin content and regular contour, only some erythrocytes (not normal) could be noticed (Figure 9).

All bone marrow smears are similar in terms of numbers and types of cells (Figure 10). These cell lines include myelomonocytic cells (LY) and erythroid cells (E). Megakaryocytes (MG) were apparent in these fields. Sizes of Nu, LY, E, and MG were similar in treated smear (*A. muricata* and *A. muricata* plus CCl_4) compared to control. In third group which treated with CCl_4 alone was showed alteration in E shape of E including irregular shape (at same magnification 40X).

Table 1. Mean values of hematological parameters in male rabbits treated with *A. muricata* , CCl_4 and their combination

Parameter	*Control* *Mean± SEM*	*A. muricata* *Mean± SEM*	*CCl_4* *Mean± SEM*	*A. muricata +CCl_4* *Mean± SEM*
RBC $\times 10^6$ (µl)	5.533± 0.135[a]	5.7400±0.07[a]	5.571±0.188[a]	4.947±0.352[a]
Hb (g/dl)	13.208±0.294[a]	12.892± 0.126[a]	13.558± 0.51[a]	12.054± 0.791[a]
PCV$\times 10^3$(µl)	40.250± 0.880[a]	39.042±0.411[a]	41.96±1.357[a]	36.48± 2.393[a]
MCV (fl)	72.91± 1.30[a]	68.133± 0.728[b]	74.31± 1.059[a]	71.98± 1.544[ab]
MCH (pg)	24.304± 0.358[a]	23.934± 0.420[a]	23.922± 0.41[a]	22.492± 0.193[b]

MCHC (dl)	32.690± 0.086^{b}	33.467±0.262^{a}	32.81± 0.06^{ab}	33.03 ± 0.23^{ab}
WBCs ×10^{3}(µl)	10.178 ±0.35^{b}	9.433 ± 0.78^{b}	36.06 ± 3.34^{a}	10.789 ± 0.65^{b}
PLT ×10^{3}(µl)	289.9 ± 21.97^{bc}	337.9 ± 26.33^{ab}	219.1 ± 20.12^{c}	412.5 ± 24.83^{a}

Values are expressed as means ± SEM; n = 4 for each treatment group. Mean values within a row not sharing a common superscript letters (a, b, c) were significantly different, p<0.05.

Table 2. Mean values of neutrophils/ lymphocytes in male rabbits treated with *A. muricata,* CCl4 and their combination.

	Neutrophils (%)		*Lymphocytes (%)*	
Groups/Weeks	1st Week	3rdWeek	1st Week	3rdWeek
	Mean± SEM	Mean± SEM	Mean± SEM	Mean± SEM
Control	34.33 ± 1.35^{a}	38.33 ± 3.97^{b}	55.67 ± 1.35^{a}	51.67 ± 3.97^{a}
A.muricata	42.00 ± 0.58^{a}	57.00 ± 2.65^{a}	46.0 ± 0.58^{a}	33.67 ± 4.64^{b}
CCl_4	35.67± 7.52^{a}	36.67 ± 4.42^{b}	53.33 ± 7.27^{a}	52.37 ± 4.42^{a}
A.muricata +CCl_4	43.67 ± 5.82^{a}	45.33 ± 2.03^{ab}	45.67 ± 6.37^{a}	42.0 ± 1.73^{ab}

Values are expressed as means ± SEM; n = 4 for each treatment group. Mean values within a row not sharing a common superscript letters (a, b, c) were significantly different, p<0.05.

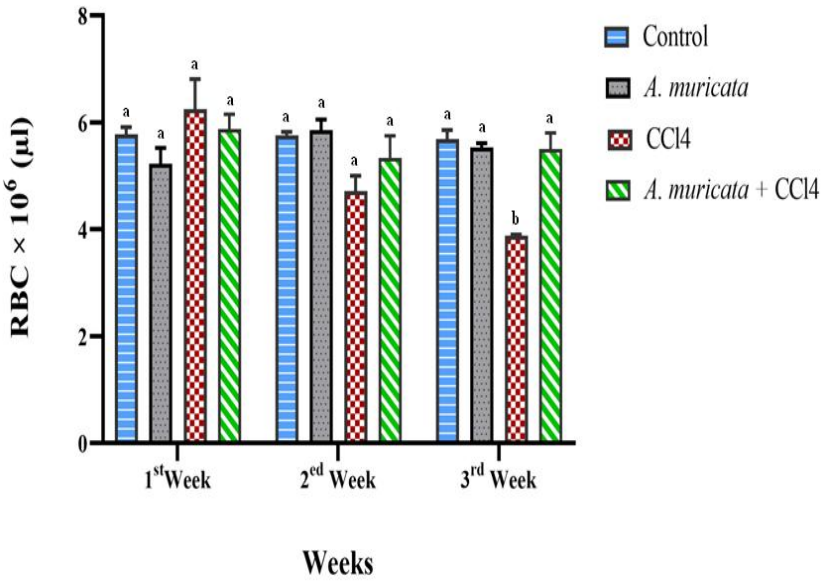

Fig. 1. Biweekly values of RBCs (Mean± SEM) in male rabbits treated with *A. muricata* , CCl_4 and their combination (*(a, b, c) were significantly different, p<0.05., Mean ± S.E.M = Mean values ± Standard error of means of six experiments)***

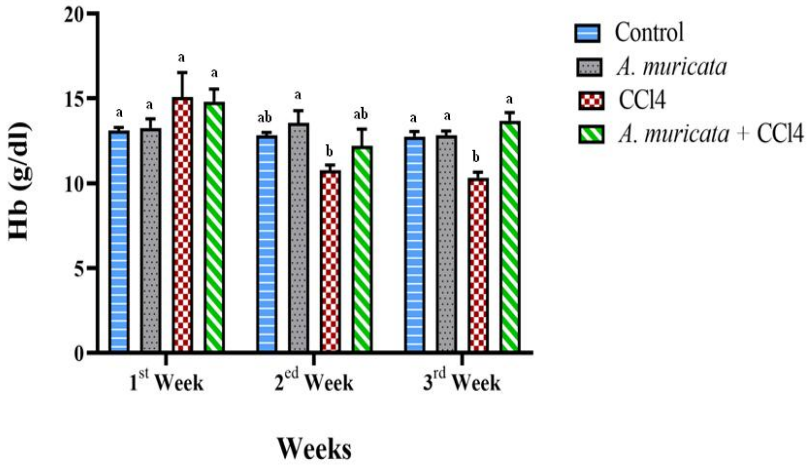

Fig. 2. Biweekly values of Hb (Mean± SEM) in male rabbits treated with *A. muricata*, CCl_4 and their combination (*(a, b, c) were significantly different, p<0.05., Mean ± S.E.M = Mean values ± Standard error of means of six experiments)***

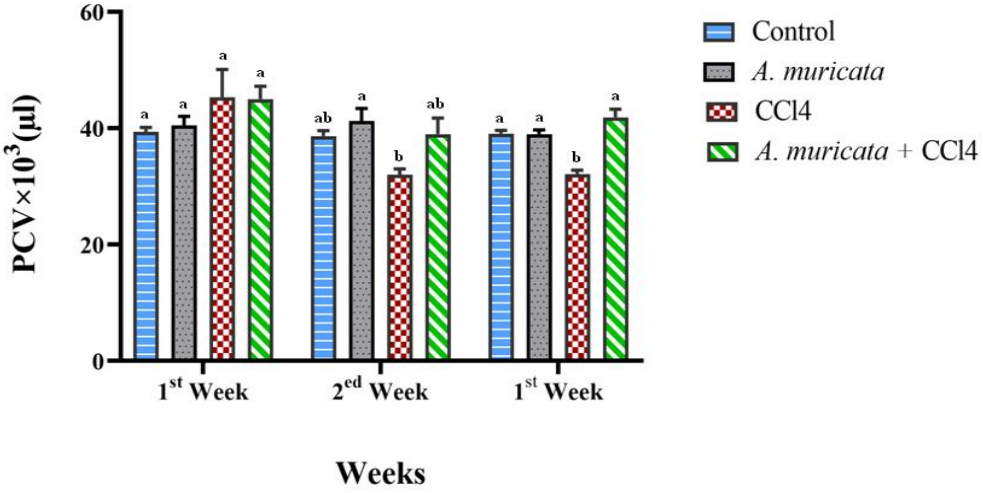

Fig. 3. Biweekly values of PCV (Mean± SEM) in male rabbits treated with *A. muricata* , CCl_4 and their combination (*(a, b, c) were significantly different, p<0.05., Mean ± S.E.M = Mean values ± Standard error of means of six experiments)***

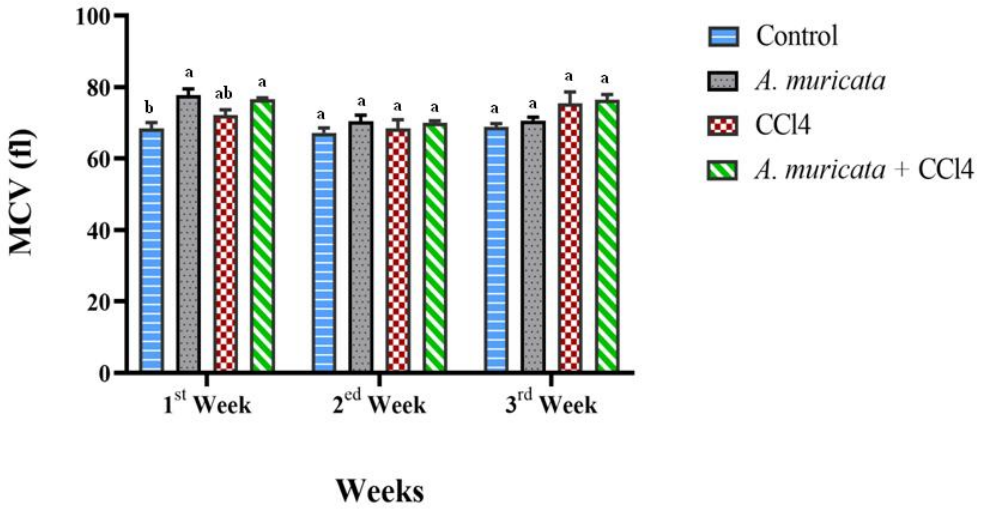

Fig. 4. Biweekly values of MCV (Mean± SEM) in male rabbits treated with *A. muricata* , CCl_4 and their combination ((*a, b, c) were significantly different, p<0.05., Mean ± S.E.M = Mean values ± Standard error of means of six experiments)***

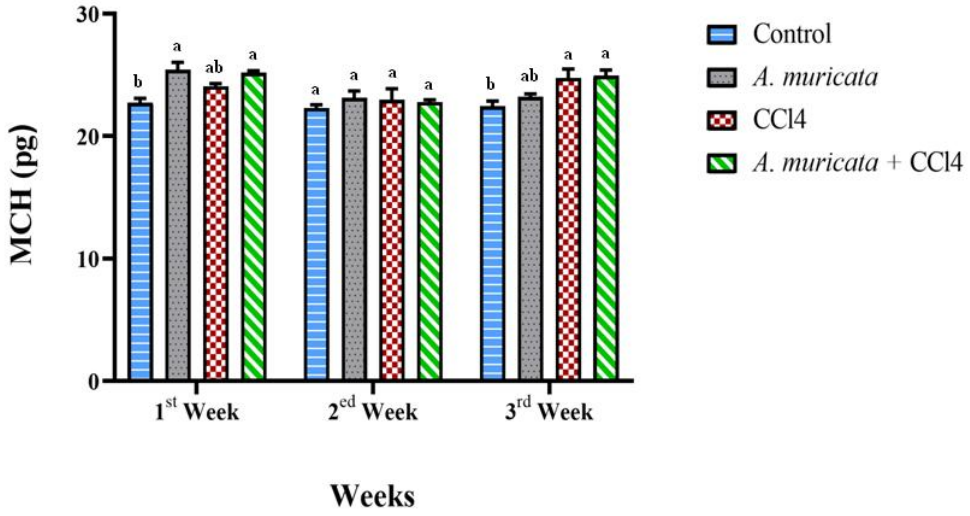

Fig. 5. Biweekly values of MCH (Mean± SEM) in male rabbits treated with *A. muricata* , CCl_4 and their combination ((*a, b, c) were significantly different, p<0.05., Mean ± S.E.M = Mean values ± Standard error of means of six experiments)***

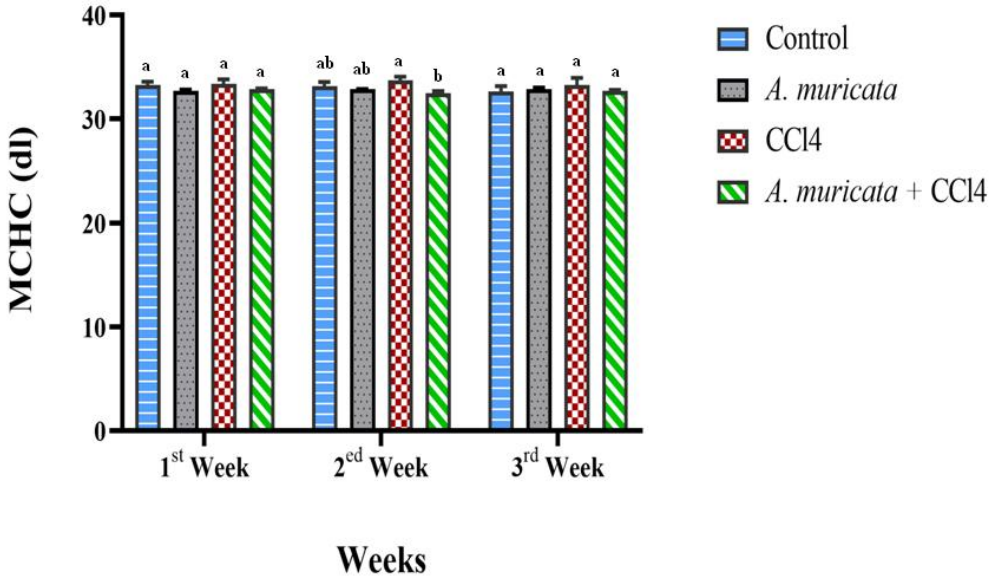

Fig. 6. Biweekly values of MCHC (Mean± SEM) in male rabbits treated with *A. muricata* , CCl_4 and their combination (*(a, b, c) were significantly different, p<0.05., Mean ± S.E.M = Mean values ± Standard error of means of six experiments)***

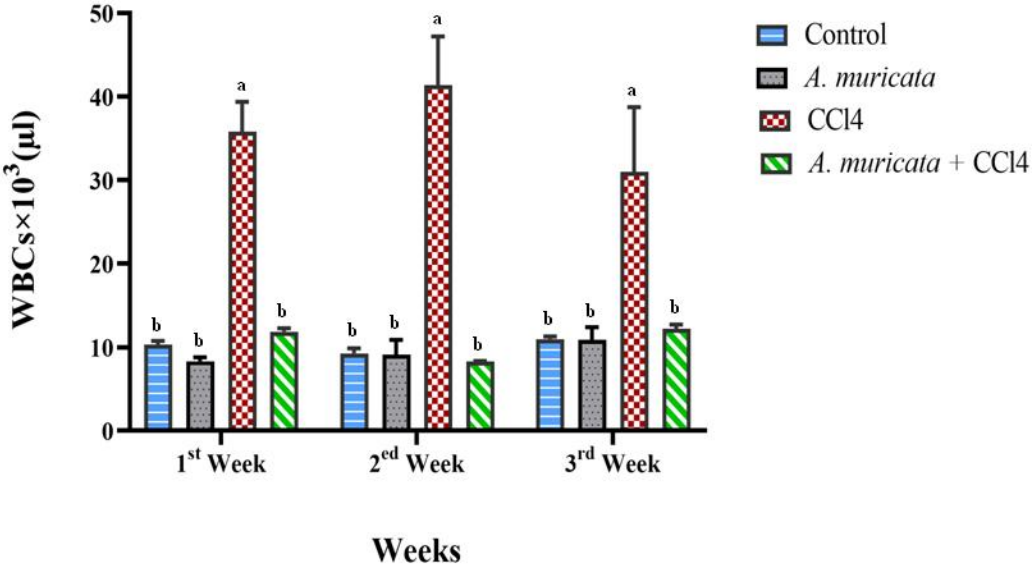

Fig. 7. Biweekly values of WBCs (Mean± SEM) in male rabbits treated with *A. muricata* , CCl_4 and their combination (*(a, b, c) were significantly different, p<0.05., Mean ± S.E.M = Mean values ± Standard error of means of six experiments)***

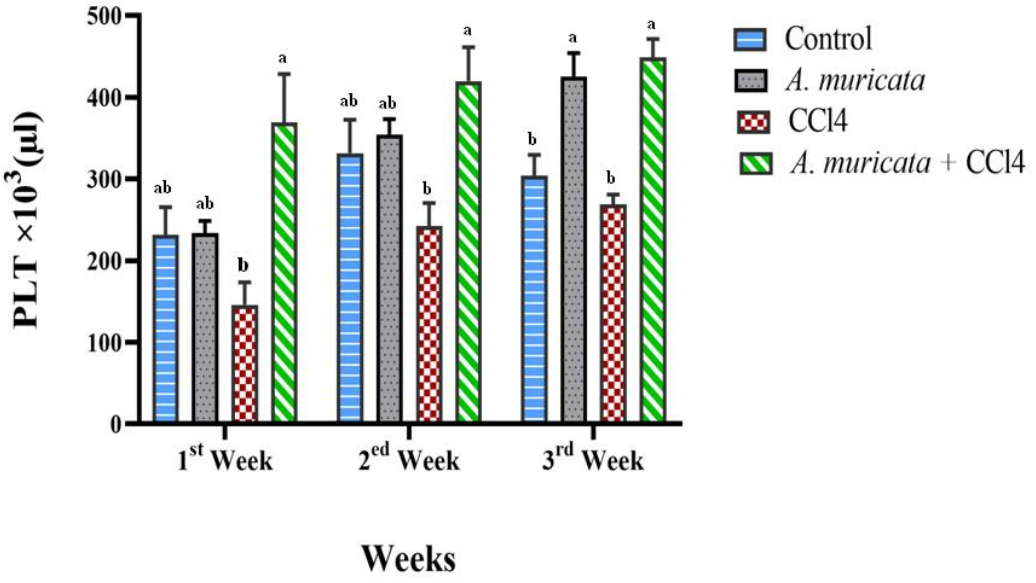

Fig. 8. Biweekly values of PLT (Mean± SEM) in male rabbits treated with *A. muricata* , CCl_4 and their combination (*(a, b, c) were significantly different, p<0.05., Mean ± S.E.M = Mean values ± Standard error of means of six experiments)***

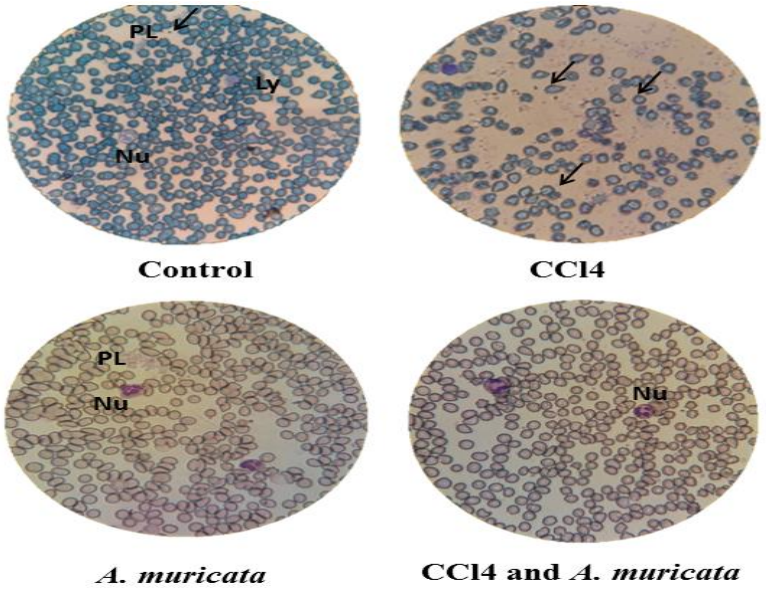

Fig. 9. Photomicrographs showing peripheral blood smears of rabbits in different groups: Control rabbit, arrow indicates blood platelets (PL), lymphocyte (Ly) and neutrophil (Nu) ; rabbit treated with CCl4, note spherocytes (arrow), sickle cells (arrows) and star shaped cells (arrow); rabbit treated with *A. muricata* note similarity to control and rabbit treated with CCl_4 with *A. muricata* erythrocytes have nearly normal haemoglobin content and regular contour. Giemsa's stained preparation of dry smear from EDTA anticoagulated blood. Magnification: 100X.

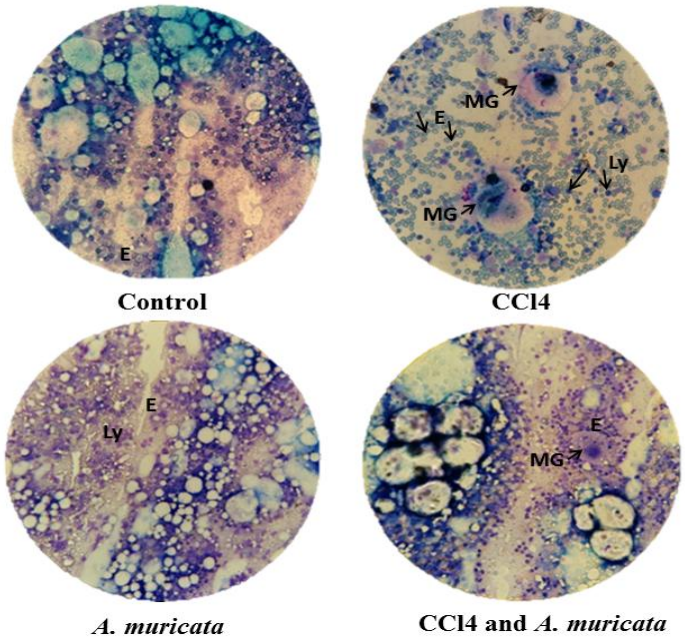

Fig. 10**. Different smears of bone marrow from four groups of treatment after three weeks of experiment.** Three major cell lines are represented. These cell lines include myelomonocytic cells (LY) and erythroid cells (E) and Megakaryocytes (MG) are apparent in these fields. (Magnification 40X).

4. DISCUSSION AND CONCLUSION

CCl_4 has been used for metal degreasing and as dry cleaning, fabric-spotting, and fire extinguisher fluids, grain fumigant and reaction medium [16]. Ozturk and Ucar [6] mention that CCl_4 affects several organs of the body such as lungs, hearts, testes, kidneys and brain. Recently, study found that leaf extract of A. muricata has modulatory effects on hematopoietic of male adult's rabbits [28]. The present study was undertaken to evaluate the potential protective effects of *A. muricata* against changing in hematological parameters of male rabbits induced by CCl_4.

In the present study, oral administration of CCl_4 affected not significantly in hematological parameters related to (RBCs, HB, PCV, MCV, MCH, and MCHC). Different alterations in these parameters have been previously described in mice treated with CCl_4 at dose 1.9 ml/kg/BW [36], which it was high compared to the dose in this study. The normal values in RBCs parameters recorded in the present work could be attributed to disturbed hematopoiesis, destruction/production of erythrocytes, and increasing in the rate of their formation and/or their enhanced removal from circulation. Previous study mentioned that the reduction in the values of blood parameters may be attributed to the hyperactivity of bone marrow, which leads to production of red blood cells with impaired integrity that are easily destroyed in the circulation [37].

This observation was constant with current results in blood smear and bone marrow smear that presented alteration in erythrocytes in term of shape. These alterations are in agreement with the result found that oral administration of CCl_4 induced morphological and ultrastructural alterations including both nucleus and cytoplasm of peripheral blood cells in mice [36]. Similar abnormalities in blood cells were observed in the peripheral blood of rats treated orally with CCl_4 [38]. According to Travlos, *et al.,* [39], the presence of altered red cells morphology is consistent with erythrocyte damage and is presumed to be related to

direct oxidative injury to the red cells by the chemicals or to the pitting function of the spleen. Only MCV was significantly decreased with *A. muricata* alone. The decrease in MCV indicates that older and healthier erythrocytes were destroyed [40], and this may due to the treatment induced cytotoxic stress erythrocyte membranes [41].

In this study, values indicated that CCl_4 caused high significant increase in number of WBCs compared with other three groups. At same time percentage values of neutrophil and lymphocyte was in normal values. In previous study found that injection of CCl_4 for six weeks increases the number of WBCs count in male rats. This may be attributed to the defensive mechanism of immune system [42]. Electron microscopy revealed various ultrastructural abnormalities in the leukocytes in the blood of mice treated with CCl_4 [36]. Neutrophils frequently appeared with irregular hyper-segmented nuclear lobes and destructed/vacuolated cytoplasm with indistinct contour. Lymphocytes displayed less electron dense cytoplasm with dense destructed mitochondria [36]. These alterations may explain presence of normal number for neutrophil and lymphocyte in current study (this study was not used techniques of Electron microscopy). Results in current study were disagreed to another study that found injection of rats with CCl_4 showed reduction of lymphocytes population in blood [36, 43].The presence of *A. muricata* plus CCl_4 returned the values of the previous parameters to near to the control values among three weeks of experiment. Other study found that *A. muricata* play role as bio-functionalized in maintaining erythrocyte membrane integrity and, consequently, decreasing the degree of haemolysis in male Wister rats at dose 100mg/kg body weight of leave extract [41]. Alkaloids, flavonoids and tannins present in the plant extracts have been considered to be responsible for the bio-reduction process [41].

Values indicated that CCl_4 caused high decrease in number of PLT compared with other three groups. Previous study found that PLT counts increased at 5-12 hr and then decreased from 24-120 hr after a single injection of CCl_4 (0.2ml/kg) in mice [44]. PLT level increased in the groups treated with *A.muricata* and combination *A. muricata* with CCl_4 suggesting enhanced cytoprotetive potential of *A. muricata* in which platelets can adhere to the walls of the blood vessels, release bioactive compounds, and aggregate to each other, resulting in increase in arterial thrombosis and atherogenesis [41, 45]. Study hypothesize that this activity may involve stimulating increases in bone marrow platelet production, increased mobilization, as well as direct modulatory interactions with biomolecules synthesized, store, or released by the platelets [46]. And this may explain presence of irregular MG in bone marrow smear of CCl_4 group.

Conclusion, the results of the present study convincingly demonstrated that CCl_4 exposure resulted in varying degree of changes in hematological parameters of rabbits and presence of *A. muricata* retuned these change to become close to control.

Ethical Approval:

As per international standard or university standard ethical approval has been collected and preserved by the authors.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the

advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors

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