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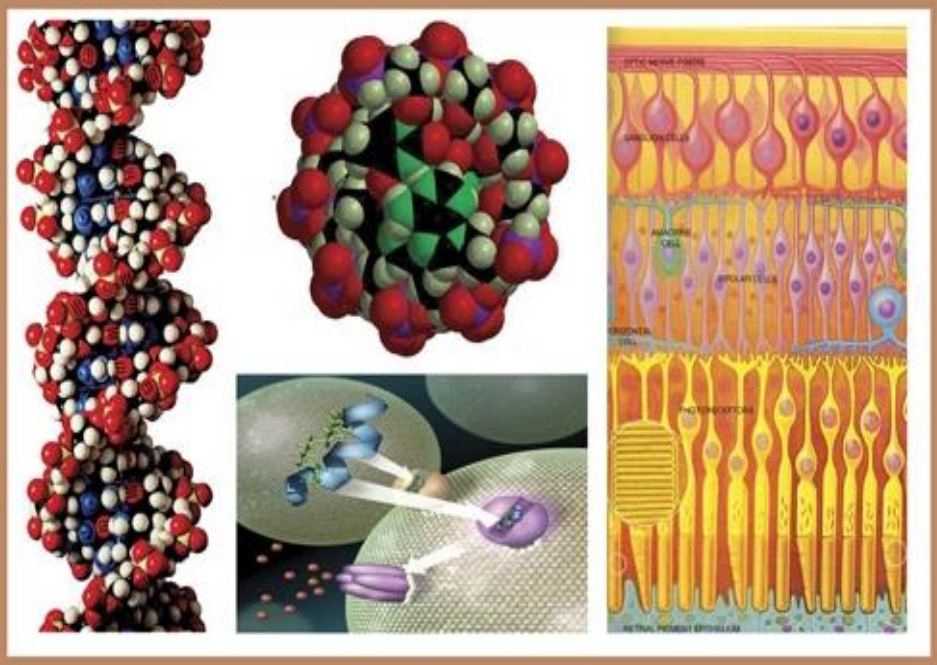

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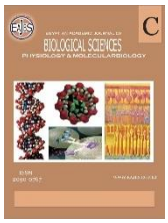

Micronucleus and Comet Assay as An Index for Carbon Tetrachloride Genotoxicity in Rats

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ABSTRACT

Many studies have reported that green coffee extract (GCE) has many benefits such as anti-diabetic, antioxidant, antihypertensive, and anti-obesity effects. The hepatoprotective effect of Green Coffee Extract (GCE) and olive oil in liver rats were studied as a result of the treatment with Carbon Tetrachloride (CCL4) compared to that of control. There was a sharp increase in tail DNA% from the GCE group to the CCL_4 group, which reached the beak in the CCL_4 group (24.67$\pm$5.09) ($P<0.001$), after being treated with GCE with CCL_4, the chart declined to (14.67$\pm$2.68). CCL_4 group induced a very high frequency of MN (14.00$\pm$1.67) compared to the control one (1.83+0.75). While animals treated with GCE at the same time with the CCL_4 group induced a significant reduction in the number of MN, 38/6000 (6.33$\pm$1.21). The results of this trial illustrated the protentional antioxidant effects of Green Coffee Extract and Olive Oil together or apart on the hepatotoxicity and cancerous effects of Carbon Tetrachloride in the liver and bone marrow of male albino rats. So, it was concluded that the GCE and olive oil minimizes the genotoxic role of CCL4 in rats.

INTRODUCTION

Cytoprotective, as well as cytotoxic compounds with potential properties, were detected in extracts from olive oil such as oleuropein glucoside, and tyrosol, besides hydroxytyrosol and caffeic acid (Habibi et al., 2021 and García-Martínez *et al.*, 2016). Green coffee is rich in chlorogenic acid, gallic acid, caffeine, protocatechuic, hydroxybenzoic, caffeic acid, ferulic acid, sinapic acid, and vanillic acid (Rizk *et al.*, 2021).

Many studies have reported that green coffee extract (GCE) has many benefits such as anti-diabetic, antioxidant, antihypertensive, and anti-obesity effects Revuelta-Iniesta and Al-Dujaili (2014) and Onakpoya *et al.* (2010). However, many studies have reported side effects of GCE if consumed in large amounts, causes leaching of minerals from the body Hutachok *et al.* (2021) and irritation of the stomach (Cano-Marquina *et al.*, 2013). The main polyphenol compound in green coffee is caffeine and chlorogenic acid which possess great therapeutic potential (Garg *et al.* 2021). Many investigators proved the antioxidant activities of chlorogenic acid and its inhibitory effects on chemical-induced carcinogenesis in vitro and in vivo (Villota *et al.*, 2021). Many studies and articles put more focus on caffeine, while others focus on beneficial compounds such as polyphenols and the Chlorogenic acid in coffee (Perdani and Pranowo 2019).

Carbon tetrachloride (CCL_4) is an ozone-depleting poly-chlorinated hydrocarbon that has been used as an alternate substance as a liquid solvent for years (Sayed *et al.,* 2021) and that evaporates easily in the surrounding environment (Fahmy *et al.,* 2018). CCL_4 is a famous compound known as hepatotoxin that is used in scientific experiments for the induction of hepatotoxicity in animals (El Rabey *et al.,* 2021). Liver injuries are associated with the release of Reactive oxygen species (ROS) or free radicals (Xiao *et al.* 2012). The main target of free radicals in the cell membrane (phospholipid molecules) commanding oxidative stress, lipid peroxidation, and reactive aldehyde causing a block of intracellular DNA and proteins (Weber *et el.,* 2003). In addition, ROS alters the structure and function of cellular and intracellular membranes producing hepatotoxicity and genotoxicity (Ingawale *et al.,* 2014 and Lettéron *et al.,* 1990). CCL_4 is bioactivated to an extremely reactive trichloromethyl radical CCL3-, and dichloromethyl CHCl2-, these two free radicals are very reactive and can bind to macromolecules like nucleic acids, proteins, and lipids causing mutation (Boll *et al.,* 2001).

The objective goal of this study was to investigate the hepatoprotective effect of green coffee extract prepared at home in a normal way beside the same role for the olive oil against liver damage induced by CCL_4. It is worthy to note that the doses were determined to be equivalent to the daily consumption of a human. The role of the study is to see the effect of GCE besides olive oil which amplifies or enhances the end result benefits.

MATERIALS AND METHODS

Experimental Animals:

Nine-week-old Male rats Rattus norvegicus (40-50 gm) were used in the study. The animals involved in the study were maintained and used in accordance with the guide to the care and use of laboratory animals prepared by Ain Shams University. The study's experimental procedures were approved by the medical Faculty Experimentation Ethics Committee. Animals were housed in the departmental animal facility for approximately 5 days after receipt prior to the experiment (21 C, 50% humidity, and 12 h, light per day).

The Applied Drugs:

Carbon tetrachloride CCL_4 was obtained from sigma company while pure olive oil and Green Coffee bean were purchased from the local market. All items were sourced in Egypt. The Green coffee extract (GCE) was prepared daily and adapted for animals to be equivalent to human consumption.

Experimental Design:

Thirty male rats were used in five groups of six. Group one served as control, group two is the olive oil (1 ml/kg), group three received GCE (0.2 ml/kg), group four received CCL_4 (1ml/kg) dissolved in olive oil (1: 1), Hanafi (2012) and group 5 with CCl4 in olive oil and GCE 3 on alternate days thrice a week.

The bone marrow micronucleus test was done according to the modified method by Schmid (1976) and Heddle et al. (1983). The animals were slaughtered, and both femurs were separated. The proximal ends of the femurs were cut to reveal the bone marrow and then aspirated with fetal bovine serum into a centrifuge tube. Centrifugation was used to collect the cells, and slides were prepared. The slides were air-dried, methanol fixed, and stained with Giemsa. Micronucleated polychromatic erythrocytes (PCE) were counted in 1000 PCEs from each animal. In addition, 100 of both types, Micronucleated polychromatic erythrocytes (PCE) and Micronucleated normochromatic erythrocytes (NCE) were randomly counted from each animal to determine the PCEs/NCEs ratio.

The Comet assay was searched,

as the liver was removed and frozen at 80 Co until use. The DNA fragments migration patterns of six animals for each group were evaluated. 1g of crushed liver samples were transferred to 1ml ice-cold PBS. This suspension was stirred for 5 mins and filtered. Cell suspension (100 ul) was mixed with 600ul of low-melting agarose (0.8% in PBS). 100ul of this mixture was spread on pre-coated slides. The coated slides were immersed in lysing buffer (0.045M TBE, pH 8.4, containing 2.5% SDS) for 15 mins. In the electrophoresis chamber containing the same TBE buffer, the slides were mounted, but devoid of SDS. The Conditions for electrophoresis were 2 V/cm for 2 mins. Staining with ethidium bromide 20ug/ml. Kinetic Imaging, Ltd. created the Comet 5 image analysis software (Liverpool, UK) By measuring the length of DNA migration, a microscope linked to a CCD camera was used to assess the quantitative and qualitative extent of DNA damage in cells and the percentage of migrated DNA. The Comet's tails extents were measured from the mid of the nucleus to the end of the tail with a 40x objective for the total and measure of the size of the Comet (Olive et al., 1990).

Statistically, significance was evaluated using an ANOVA (one-way) test with the help of Sigma Plot 14.0. Ink. In cases where ANOVA showed significant differences, post hoc analysis Tukey HSD was performed. P values of 0.05 or less were well-thought-out statistically significant.

RESULTS

Physical Appearance:

CCL_4 group showed weight loss and lazy animals compared to the rest of the groups, while the weight of liver in CCL_4 was the highest compared to the other groups (data not scored) on one hand, and the animals' fur of CCL_4 dissolved in olive oil turned to pale yellow color, on another hand.

Comet Assay:

Comet tail DNA % in rats treated with a dose of 0.1 ml/kg of the CCL_4 was very highly significantly increased compared to the control group. While GCE significantly reduced the effect of CCL_4 on the liver cells, Table (1) and Fig. (1). There was a sharp increase in tail DNA% from the GCE group to the CCL_4 group, which reached the beak in the CCL_4 group (24.67 $\pm$ 5.09) ($P<0.001$) that indicating the genotoxicity of CCL_4, after being treated with GCE with CCL_4, the chart declined to (14.67 $\pm$ 2.68), Fig. (2). Furthermore, when compared to the control group (1.50 $\pm$ 0.38) both GEC and olive oil had no significant effect on tail DNA percent (1.67$\pm$0.19 and 2.40$\pm$0.76 respectively), which implies the protection of both.

Table (I): Rates of Comets tail DNA % in liver of rats treated orally for 4 weeks with CCL4, olive oil and GCE groups apart or together compared to that of control.

Groups	No. of examined animals	No. of analyzed cells / 6 animals	Mean $\pm$ S.D.
control	6	600	1.50 $\pm$ 0.38
Olive Oil	6	600	2.40 $\pm$ 0.76
GCE	6	600	1.67 $\pm$ 0.19
CCL_4+ Olive Oil	6	600	24.67 $\pm$ 5.09**
CCL_4+GCE&Olive Oil	6	600	14.67 $\pm$ 2.68**

N.B.: 600 hepatocytes were examined per each group.
** = $P<0.001$: very highly significant.
* = $P<0.01$: highly significant

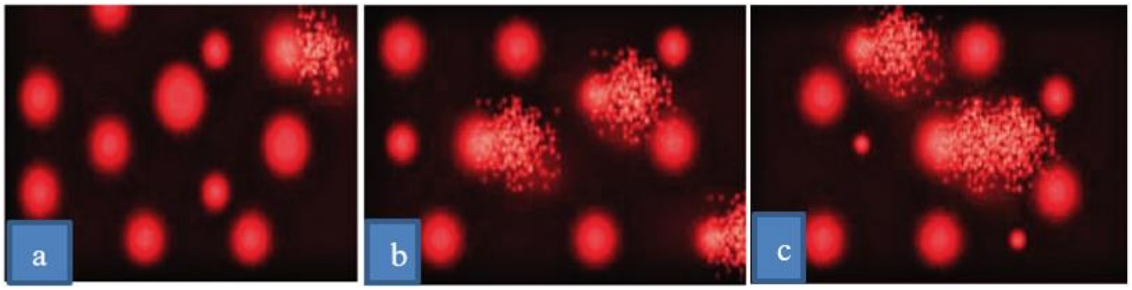

Fig.1: Comet photomicrographs showing the DNA migration pattern in hepatocytes of rats. **a**) unaffected cells. **b**) and **c**) are the different forms of the affected cells.

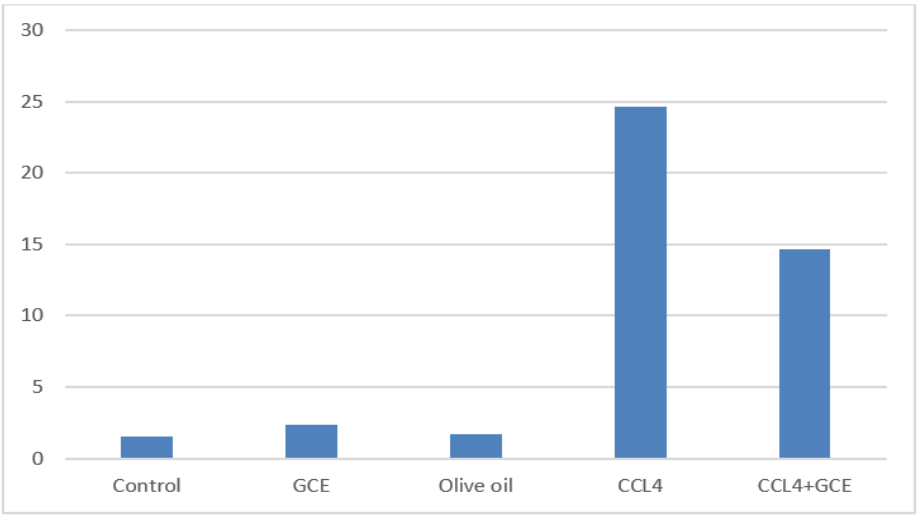

Fig. 2: The mean of tail DNA % in the liver of male rats treated orally with CCL4, olive oil and GCE apart or together for 4 weeks.

Micronucleus Test:

In this study, the diversity in shape and number of MN was recorded. The cells were categorized as single micronucleus and more than one micronucleus. There was a very high frequency of MN induced in the CCL_4 group (14.00±1.67) compared to the control one (1.83±0.75), which indicates the toxicity of CCL_4. However, animals treated GCE at the same time with CCL_4 group induced a significant reduction in the number of MN, 38/6000 (6.33±1.21), Table (2). The micronuclei were varied in size, some micronuclei were small and the others were large. The PCEs frequency of the CCL_4 animals was 3.6 times the GCE group (Figs. 3 & 4). As illustrated in Table (3), the PCEs were increased after treatment with CCL_4, which signified the cytotoxicity of CCL_4 (0.57+0.05) and the evident decline in such ratio after treatment GCE at the same time with CCL4 (0.79+0.03).

Table 2: Induction of micronuclei by CCL4, olive oil and GCE groups combined or apart as compared to control group in bone marrow cells of male rats.

Groups Serial number	Control	Olive Oil	GCE	CCL_4+Olive Oil	CCL_4+GCE & Olive Oil
1	2	2	4	12	5
2	1	3	5	15	7
3	3	4	4	16	6
4	2	3	3	15	7
5	1	2	4	14	8
6	2	3	3	12	5
total	11	17	23	84	38
Mean+ SD	1.83±0.75	2.83± 0.75	3.83± 0.75*	14.00±1.67**	6.33± 1.21**

N.B.: 600 hepatocytes were examined per each group.
** = P<0.001: very highly significant.
* = P<0.01: highly significant

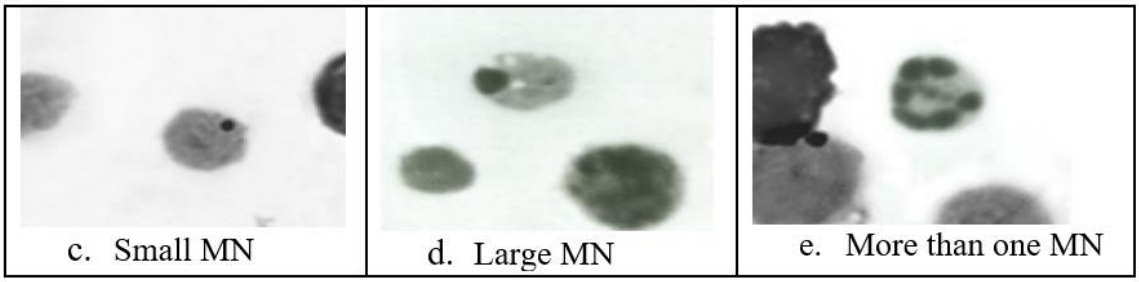

Fig.3: Micronucleated polychromatic erythrocytes in rat bone marrow after oral administration of CCL4, olive oil and GCE apart or together for 4 weeks.

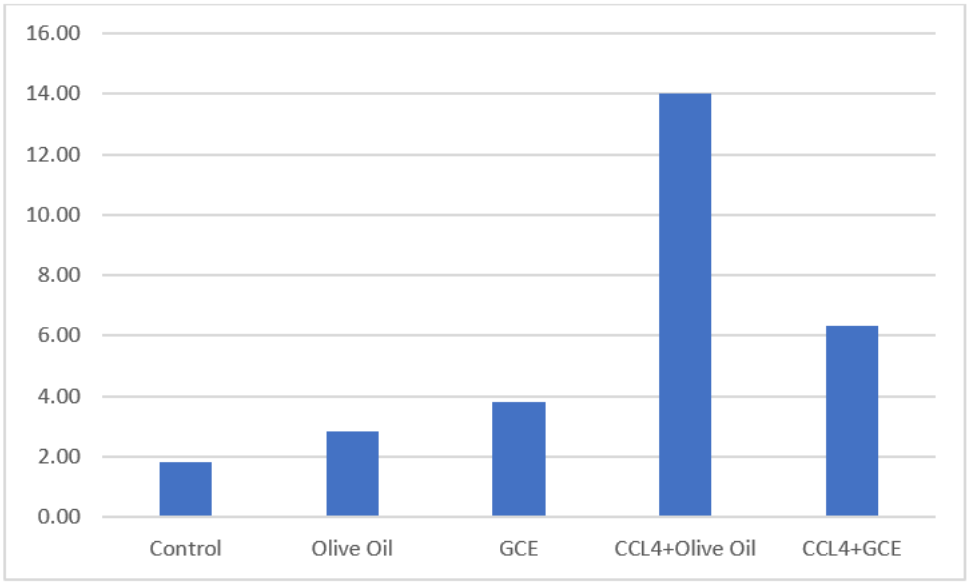

Fig. 4: The mean of frequencies of micronucleated polychromatic erythrocytes in bone marrow cells of male rats following treatment with CCL4, olive oil and GCE apart or together for 4 weeks.

Table 3: Ratio of polychromatic erythrocytes (PCE) to normochromatic erythrocytes (NCE) in rats treated orally for 4 weeks with CCL4, olive oil and GCE combined or apart compared to that of control group.

Groups Serial number	Control	Olive Oil	GCE	CCL_4+Olive Oil	CCL_4+GCE & Olive Oil
Total	5.57	5.57	5.3	3.44	4.76
Mean+ SD	0.93±0.03	0.88± 0.04	0.93± 0.03	0.57±0.05**	0.79± 0.03**

N.B.: 600 hepatocytes were examined per each group.
** = P<0.001: very highly significant.
* = P<0.01: highly significant.

DISCUSSION

CCL_4 group presented weight loss in the liver and animals' body weight. Therefore, this increase combined with the reduction of animals' activity-induced liver stress compared to the other groups. These findings align with Naz *et al.* (2020) and Mesalam *et al.* (2021), who recorded the lowest body weight and liver damage in the CCL_4-treated group.

Many studies demonstrated as aforementioned the effect of CCL_4-induced oxidative stress and it is one of the main mechanisms underlying CCL_4 hepatotoxicity (Xiao *et al.,* 2012). This in turn affected the DNA directly or indirectly, Alkreathy *et al.*, 2014. It was demonstrated that CCL_4 induced toxicity increased lipid peroxidation, IL-6, kidney function parameters, liver function enzymes, total cholesterol, triglycerides, and low-density lipoproteins, and decreased irisin, antioxidants, CYP450, and high-density lipoprotein levels (El Rabey *et al.,* 2021).

This study indicated that the ability of CCL_4 to induce DNA breakage - compared to the control group as indicated by Comet assay in liver cells - suggested that CCL_4 enhanced oxidative stress by releasing free radicals. CCL_4 itself is non-toxic, but its metabolite ·CCl3 and ·OOCCl3 formed by cytochrome P450-dependent monooxygenases cause hepatotoxicity Lee et al. (2019). These free radicals caused lipid peroxidation which in turn led to liver injury Shah *et al.,* 2017 and decreased the efficiency of the liver to overcome oxidative stress ending up in the change of antioxidant enzyme activities Mesalam *et al.,* 2021. In addition, at a high level of O2, CCL_4 is metabolized to trichloromethyl peroxy radical CCL3OO- running to peroxidation of lipid to trigger the cell to steatosis or apoptosis Boll *et al.,* 2001. Furthermore, at the molecular level CCL_4 stimulated tumor necrosis factor (TNF)-alpha, nitric oxide (NO), and transformed growth factors (TGF)-alpha and -beta in the cell, which directed the cell primarily to self-destruct or fibrosis Weber et al., 2003. The same author mentioned that these findings may be interfered with using antioxidants and mitogens by restoring cellular methylation. Antioxidant molecule's activity can scavenge ROS before these molecules cause tissue damage, especially in the genetic material Adewale et al., 2004 and Sheweita *et al.,* 2001.

Chlorogenic acid (CGA), a phenolic compound found in both olive oil and green coffee, has antioxidant activity and the ability to trap and neutralize superoxide anions or hydroxyl radicals Fazel Nabavi *et al.,* 2017. GCE and olive oil indicated a great ability to overcome the oxidative stress of CCL_4 on hepatocytes by increasing the antioxidant to counteract ROS, as an antagonist to the toxicity effect of CCL_4 on both cytotoxic and molecular effects. Therefore, the hypotensive effect of caffeic acid in spontaneously hypertensive rats might be mediated via the muscarinic acetylcholine

receptors Suzuki *et al.*, 2002.

The protective effect of GCE and olive oil against the damage that occurred by CCL_4 on the liver and bone marrow cells of rats clearly appears in this study. The study outcome is in the same line as Singh, *et al.* (2021) who examined the effect of chlorogenic acid on metabolizing enzymes in vitro and diabetic complications in vivo. Their results showed important enhancement of body weight, HDL-cholesterol, total protein, and albumin levels leading to improvement in atherogenic keys related to diabetes-associated cardiovascular risks. GCE and olive oil may suppress the formation of free radicals released by CCL_4 which is concurrent with Venkatakrishna *et al.* (2021) who concluded the safety of green coffee bean extract. Furthermore, Hsu *et al.* (2022) showed CGA efficiency hepatoprotective consequences for CCL_4-incited liver injuries in mice by the elevation of the activities of antioxidant enzymes and hindrance of lipid peroxidation.

Detection of micronuclei is a simple, inexpensive, and relatively minimally invasive technique commonly used to evaluate chemical genotoxicity but rarely applied to assess wildlife genotoxic effects Sandoval-Herrera *et al.*, 2021. The Micronucleus test (MN) has become one of the most popular methods to assess the genotoxicity of different chemical and physical factors, including ionizing radiation-induced DNA damage Sommer *et al.*, 2020. Although many tests classified as "in vivo biomonitoring" are available, a micronucleus test (MN) is one of the most popular and widely used to test genotoxicity in vitro OECD (2010).

Micronucleus assay can detect spindle poisons (Thomson and Perry 1988) leading to the appearance of large MN (Yamamoto and Yasumoto, 1980 and Högstedt and Karlsson, 1985). The present findings showed MN size ranged from small to large which may be part of a chromosome, deletion, break, or acentric fragment as a result of disrepair of DNA double-strand or DNA break (Lin *et al.*, 2021 and Mosallam 2020). The findings of the study revealed that CCL_4 had a strong effect on the induction of MN, which reflected its cytotoxic impact throughout the anaphase period throughout the deterioration route of the erythrocyte nucleus (Moras *et al.*, 2017). The results are in line with El-Shorbagy (2017) who stated that CCL_4 increased the frequency of MN in the bone marrow of mice. Moreover, Abdou *et al.* (2012) detected a high frequency of chromosomal aberrations in the bone marrow and liver cells of CCL_4-treated male mice at a dose level of 1 ml/kg/b.wt., which is in harmony with these results. Recently, Pegoraro *et al.* (2021) studied the *Bidens pilosa* species of herbaceous, against toxicity of CCL_4 on the liver, kidney, intestine, and bone marrow through MN test and Comet assay, the finding showed that CCL_4 exerted a mutagenic effect on rats, which manipulated by *Bidens pilosa* herb. Once again, the present consequences of the treatment with CCL_4 revealed a statistical decrease in MN-PCEs when compared with control animals, indicating the cytotoxic effects of CCL_4 which disrupted the normal bone marrow cell proliferation. Alshahrani *et al.*, 2021 and Tripathi *et al.*, 2012, mentioned that, when normal bone marrow cell proliferation is disrupted by a toxic agent, the number of immature erythrocytes (i.e., PCEs) decreases in comparison to mature erythrocytes (i.e., NCEs), resulting in a decrease in the PCE/NCE ratio.

The findings revealed the significant influence of GCE and olive oil in reducing the cytogenetic effects of CCL_4 on bone marrow cells. In addition, Ansari *et al.* (2021), examined the radioprotective effect of three herbal extracts including green coffee on bone marrow cells of mice after exposure to irradiation (3 Gy gamma-rays of Co-60). The study outcomes signified the appearance of

large MN in the CCL_4 treated group which is considered as an index of toxicity of CCL_4 on spindle fibers, and the ability of green coffee to decrease its effect. Green coffee methanolic extract, silymarin, and their combination succeeded in protecting the male rats against CCL_4 hepatotoxicity due to their antioxidant activity (El Rabey *et al.*, 2021). The degree of roasting affects the antioxidant and anti-inflammatory effects of coffee extracts, the lightly roasted coffee is the richest antioxidant and anti-inflammatory effects (Choi *et al.*, 2018). Nevertheless, Masek *et al.*, 2020, stated that both ethanol green coffee extract and aqueous solutions have strong antioxidant properties. Finally, it can be concluded that Green Coffee and olive oil showed a great potency of antioxidant and anticancer effects which antagonist the hepatotoxicity of CCL_4. The positive results obtained in the two tests revealed the genotoxic potential of CCL_4. The present study has several strengths including 1- the large size of samples, 2- The usage of two markers, MN as a carcinogenic marker and Comet assay as a DNA breakage marker, 3- the usage GCE equivalent to the daily human consumption.

Conclusion

In conclusion, the results of this trial illustrated the protentional antioxidant effects of Green Coffee Extract and Olive Oil together or apart on the hepatotoxicity and cancerous effects of Carbon Tetrachloride in the liver and bone marrow of male albino rats.

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