Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Amelioration of Hepatotoxicity Accompanied to Cyclophosphamide Therapy by Citrus aurantium L. Peel in Wistar Rats

Halaby M. S.[a], Badawy I. H.[b], Maghraby A. S.[c, d], Mohammed F. E-Z. S.[b], Mahmoud M. H.[b]*

a. Nutrition and Food Sci. Dept., Faculty of Home Economics, Helwan University.,Cairo, Egypt.

b. Nutrition and Food Sciences Department, Institute of Food Industries & Nutrition Research, National Research Centre, 33 El Bohouth St. (former El Tahrir St.), Dokk, Cairo, Egypt, P.O. 12622.

c . Research Group Immune- and Bio-markers for Infection, the Center of Excellence for Advanced Sciences, National Research Centre, 33 El Bohouth St. (former El Tahrir St.), Dokk, Cairo, Egypt, P.O. 12622.

d. Department of Therapeutic Chemistry, Institute of Pharmaceutical and Drug Industries Research, National Research Centre, 33 El Bohouth St. (former El Tahrir St.), Dokk, Cairo, Egypt, P.O. 12622.

Abstract

The use of cyclophosphamide as a chemotherapy drug for treatment of many malignancies is mostly accompanied by hepatotoxicity. So, the present study aimed to investigate the potential ameliorative impact of the Egyptian bitter orange peel (BOP) or *Citrus aurantium* L. against hepatotoxicity of cyclophosphamide in rats. Total and differential polyphenolic content by HPLC of BOP as well as its antioxidant activity were determined. Then, a biological experiment was done including four groups of male adult Wistar rats. A control negative group injected with saline, a group that was injected with saline and fed on basal diet supplemented with 20% BOP and two groups that were injected intraperitoneally by 40 mg/kg body weight of cyclophosphamide for four successive days to induce hepatotoxicity; one of them was control positive fed on basal diet and the other was fed on 20% bitter orange peel to evaluate its hepatic ameliorative effect. Results revealed the presence of seventeen polyphenolic compounds in the BOP methanolic extract and also, that it has a relatively high antioxidant activity. An elevation in the serum ALT and AST activities and in hepatic MDA and TNF-α concentrations, while a reduction in the serum total protein, albumin, A/G ratio and in hepatic GSH was detected for the cyclophosphamide-injected group compared to the control negative. All these alterations were restored to near the normal values of the control negative group in the cyclophosphamide-injected group that received BOP. Histopathological examination confirmed the biochemical findings. In conclusion, bitter orange peel has a potent antioxidant activity that enable it to counteract hepatotoxicity accompanied to cyclophosphamide therapy.

Key words: *Bitter orange peel; Cyclophosphamide; Polyphenols; Hepatotoxicity.*

1. Introduction

Cyclophosphamide (CP) is a prominent member in the family of chemotherapy drugs for cancer treatment [1, 2]. Cancer is among the leading causes for death worldwide. According to the estimates ofthe GLOBOCAN in 2020 for the cancer incidence and mortality in the world reported by the International Agency for Research on Cancer, about 19.3 million new cancer cases and almost about 10 million deaths were detected in the year 2020 [3]. Cyclophosphamide is used excessively as a broad-spectrum treatment for cancer in many chemotherapy protocols because of its high curative impact [4]. It is used in the treatment of many solid and hematologic neoplasms like different myelomas, leukemias, lymphomas, ovary adenocarcinoma, breast cancer, and prostate carcinoma [2, 5]. It is one of the oldest and most widely prescribed synthetic nitrogen

*Corresponding author e-mail: mahahanafymmm@yahoo.com, (Maha Hanafy Mahmoud).
Receive Date: 30 November 2021, **Revise Date:** 18 December 2021, **Accept Date:** 21 December 2021
DOI: 10.21608/EJCHEM.2021.108506.4964

mustard alkylating cytostatic medicine. Alkylating drugs are one of the oldest well-established classes of anticancer drugs [6]. In general, the alkyl carbon groups of alkylating drugs act directly on the DNA double strand which alters the DNA helical structure by affecting the binding of chromatin protein and inducing DNA strand breaks, followed by genotoxic effects and tumor cell death [7, 8]. However, normal growing cells could also be affected through the same mode of action by its cytotoxic and genotoxic effects [6, 9, 10]. Thus, as well as killing tumor cells, CP damages a series of quickly proliferating normal cells, for instance, hepatocytes, bone marrow cells and gastrointestinal mucosal cells resulting in many side effects such as hepatotoxicity, decreasing immune function and inducing damage of the gastrointestinal mucosal barrier, respectively [4]. Also, other severe side effects were detected such as nausea, vomiting, hair loss, anemia, leukocytopenia, and thrombocytopenia, gonadal toxicity which leads to infertility [2]. Hepatotoxicity was reported to be among the most prominent side effects of cyclophosphamide chemotherapy [11].

The liver has a major and outstanding role in the metabolism and the excretion of various drugs and toxic chemicals from the human body. That's why this high-powered engine is considered as the main target organ for many toxins [11, 12]. Hepatotoxicity is damage of liver caused by toxic chemicals and drugs [13]. In European, American and Asian countries acute liver failure is mostly due to acute liver damage that is caused by drugs [14]. Liver injury induced by drugs accounts for approximately half of all acute hospitalized liver failure cases. In particular, the hepatotoxicity due to some cancer chemotherapeutic drugs is common among cancer patients. Up to 85% of cancer patients under chemotherapy may develop hepatic steatosis since most drugs are lipophilic agents. This steatosis increases the vulnerability of hepatocytes which may finally induces irreversible hepatocellular damage [11]. Maor & Malnick [15] added that the potential mechanism for hepatic injury induced by chemotherapy may be secondary to the reactive oxygen species production that is supposed to induce apoptosis of tumor cells.

Today, the role of herbal medications is highlighted as they receive an increasing attention in many medical applications due to their high efficiency, low toxicity and low-cost. They are used as alternative or complementary therapeutic agents [16] for many metabolic disorders as hyperglycemia [17], nephrological disorders [18], and liver toxicity [19]. Also, they were trialed to improve the athletic performance [20]. Citrus plant is one of these plants of therapeutic benefits. In recent years, citrus peel has been utilized as environment friendly, inexpensive and efficient platform for the synthesis of novel nutraceuticals because of its high content of bioactive polyphenols, easy availability and low cost [21, 22].

Citrus aurantium L. which belongs to the Rutaceae family and known as bitter orange or sour orange, is a popular and famous fruit that is mostly originating from the world tropical areas and subtropical areas [23]. In traditional Chinese Folk Medicine, the bitter orange fruit is also known as zhi shi as reported by Firenzuoli et al., [24] and it is now included in the Pharmacopoeia of China due to its actual wide clinical applications [14]. Although the *Citrus aurantium* L. fruit possesses a bitter taste that renders it to be not attractive and not the fruit of choice for the human consumption, yet, the peel which represents almost one half of the fruit mass, contains the highest concentration of flavonoids in the citrus fruits and is used for the production of jam [25]. These polyphenolic flavonoid compounds include mainly naringin, hesperidin, and neohesperidin [26]. The volatile oils of the citrus peel in general have wide applications. The full characterization of orange peel oil is illustrated by Liu et al., [27]. In particular, the bitter orange peel was reported to have many health benefits based on its antioxidant activity such as anti-inflammatory, anticancer and antiviral effects [28].

To the extent of our knowledge, although many studies dealing with polyphenolic compounds' characterization and quantification of different parts of the bitter orange; like fruits, leaves, flowers and seeds were found, yet, very rare studies highlighted the identification and characterization of the polyphenolic compounds of the bitter orange peel but none was found concerning the Egyptian variety. Also, studies dealing with the hepato-ameliorative impact of bitter orange are very rare. In particular, the studies on the ameliorative effect of the bitter orange peel on hepatotoxicity were not found and specially the hepatotoxicity that was recorded as one of the side effects for cyclophosphamide which is one of the most famous and widely used chemotherapy drugs for many malignancies. Hence, the present study was designed in a trial to evaluate the ameliorative impact of bitter orange peel on the hepatotoxicity induced by

cyclophosphamide injection in Wistar rats as well as characterization and quantification of the Egyptian bitter orange peel polyphenolic compounds.

2. Material and methods

Materials

Bitter orange (Citrus aurantium L) fruits were collected from the local market during their ripening season. Cyclophosphamide (CP) with a Cat. No of "PHR1404-1G", that was used for hepatotoxicity induction, was purchased from Sigma-Aldrich Chemical Co. (St. Louis, Mo, 63103, USA). Regarding the ingredients used to formulate the experimental diet, they were as follows; Casein was obtained from Al-Ahram Laboratory Chemicals (Egypt) while, cellulose was purchased from the Laboratory of Rasayan, Fine Chemical Limited, Mumbai, India. On the other hand, ingredients used to constitute the mixtures of salt and vitamin were purchased from either BDH (England) or Fluka (Germany), respectively. Most of the other constituents of the experimental diet, rather than the aforementioned ones, were purchased from the local market.

ELISA kits that were used for determination of the concentration of malondialdehyde (MDA), reduced glutathione (GSH) and tumor necrosis factor alpha (TNF-α) in the liver tissue homogenate were purchased from Elabsceince Co., (China) with a Cat. No. of "E-EL-0060", Sunlong Biotech Co. (China) with a Cat No. of "SL1410Ra" and Elabsceince Co., (China) with a Cat. No. of "E-EL-R0019", respectively.

Diagnostic kits used for spectrophotometric determination of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein (TP) and albumin (AL) in serum were obtained from Salucea Co., Netherlands. Methanol and ethanol were obtained from Al-Nasr Pharmaceutical Company, Egypt. Folin & Ciocalteu's phenol reagent that was used for determination of total polyphenolic content of the bitter orange peel was obtained from LOBA Chemie Company (UN: 3264), India while, DPPH (d 1,1-diphenyl-2-picrylhydrazyl) reagent that was used to assess the antioxidant activity of the bitter orange peel was purchased from Sigma-Aldrich Chemical Co. (St. Louis, Mo, 63103, USA).

Male albino adult Wistar rats used for the biological evaluation were purchased from the Central Animal House, National Research Centre, Dokki, Egypt. The study protocol was approved by the Scientific Committee at the National Research Centre (NRC, Dokki, Egypt). The experiment was conducted according to the guidelines of the Institutional Animal Care and Ethics Committee of the NRC with an approval No. of "18-106".

Methods

Preparation of bitter orange peel

After cleaning the fresh fruits of bitter orange carefully, the external peel was removed manually. Then peel of all fruits was allowed to dry in an air ventilated oven at 40°C until complete dryness. Then, the dried peels were grinded into a fine powder using electric grinder (Mienta, Egypt). The obtained powder was divided into two portions. The first portion was subjected to extraction by either ethanol or methanol for determination of total phenolic compounds, differential phenolic compounds by HPLC and antioxidant activity. On the other hand, the second portion was kept at -20°C for further use in the formulation of the experimental diet for the biological evaluation.

Extraction of bitter orange peel

One gm of the powder was put in 10 ml of 70% ethanol followed by sonication for 1 hour on 37°C. Then, the suspension was centrifuged at 3000 rpm for 0.5 hour followed by filtration. The volume of the filtrate was adjusted to 25 ml in a volumetric measuring flask by adding appropriate amount of ethanol. This solution was used to determine the total polyphenolic content (TPC), the differential polyphenolic compounds and the antioxidant activity as represented by radical scavenging activity (RSC). The same extraction steps were repeated by replacing ethanol with methanol in order to evaluate the effect of solvent on extraction capacity [29].

Determination of total phenolic compounds

The total phenolic compounds (TPC) were determined in both extracts, methanol and ethanol using the Folin-Ciocalteu assay according to Aboelsoued et al., [30]. A volume of 0.25 ml of each extract was diluted with 3.5 ml of distilled water followed by 0.25 ml of Folin-Ciocalteu reagent. After that, 0.25 ml of 20% sodium carbonate solution was added. All samples (triplicates) were vortexed, and then incubated in a water-bath at 40 °C for 20 minutes. The absorbance of the blue color formed was read against, the blank standard at a wave length of 765 nm. The TPC were calculated with respect to

gallic acid (concentration range: 0-12 µg/ml). Results were expressed as mg of gallic acid per plant material. Blank was prepared using 0.25 ml of 80% ethanol or methanol instead of plant extract for both ethanol and methanol extracts, respectively.

Determination of the radical scavenging activity

Radical scavenging activity of both extracts; the ethanol extract and the methanol extract was determined using the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical assay according to Hayat et al., [29] with some modifications. An aliquot of 0.5 ml of either extracts was mixed with 1 ml of methanolic solution of DPPH (0.2mM). The reaction mixture was incubated for 30 min in the darkness at room temperature. The absorbance of the resulting solution was measured at 517 nm using a spectrophotometer of Shimadzu model, UV-2401 PC (Australia). For the control, the assay was conducted in the same manner without the extract but instead the solvent only was used. DPPH scavenging capacity of the tested samples was measured as a decrease in the absorbance and was calculated by using the following equation:

$$\text{Scavenging activity (\%)} = \frac{Ac - As}{Ac} \times 10$$

Where, Ac and As are the absorbance at 517 nm of the control and the sample, respectively.

Characterization and quantification of differential polyphenolic content of bitter orange peel methanolic extract by HPLC.

HPLC analysis was carried out using an Agilent 1260 series. The separation was carried out using Eclipse C18 column (4.6 mm x 250 mm i.d., 5 µm). The mobile phase consisted of water (A) and 0.05 % trifluoroacetic acid in acetonitrile (B) at a flow rate 1 ml/min. The mobile phase was programmed consecutively in a linear gradient as follows: 0 min (82% A); 0–5 min (80% A); 5-8 min (60% A); 8-12 min (60% A); 12-15 min (82% A); 15-16 min (82% A) and 16-20 (82%A). The multi-wavelength detector was monitored at 280 nm. The injection volume was 5 µl for each of the sample solutions. The column temperature was maintained at 40 °C. The concentration of the obtained peaks for the polyphenolic compounds of the extract was then calculated by comparing them to their corresponding peaks of the standards.

Animal experiment

The basal control diet was prepared according to the method of Reeves et al. [31]. Dried bitter orange peel (BOP) was added to the diet of the selected groups on the expense of starch as 200 g BOP / Kg diet as described previously [32].

Twenty-four adult male albino Wistar rats of a mean body weight of 200 ± 20 g were included in this study. The rats were housed individually in separate stainless-steel cages at controlled temperature of 25 ± 1 °C and a light-dark cycle of 12 hours for one week prior to starting the experiment as an adaptation period during which food and water were allowed *ad libitum* to animals. All the aforementioned conditions were continued for the whole experimental period. Then, animals were divided into four groups each of six rats. Two groups out of the four were injected intraperitoneally by cyclophosphamide (CP) to induce hepatotoxicity with a dose of 40 mg/kg body weight for four successive days, according to Zhu et al. [33] with some modifications. The other two groups were injected with saline with similar volume and duration of the first two groups. Then, the feeding experiment was started including four experimental groups as follows:

Group I: including rats pre-injected with saline and fed on control basal diet to serve as a control negative group.

Group II: including hepatotoxicity-induced rats (by pre-injection with cyclophosphamide (CP) with a dose of 40 mg / kg for 4 successive days as explained above) fed on control basal diet to serve as a control positive group.

Group III: including rats pre-injected with saline and fed on control basal diet + BOP) as 200 g / kg diet.

Group IV: including hepatotoxicity-induced rats (by pre-injection with CP with a dose of 40 mg / kg / 4 successive days) fed on control basal diet + BOP as 200 g / kg diet.

The experiment lasted for 4 weeks during which the body weight was recorded once a week and feed intake of each rat was observed and recorded daily.

Calculation of growth parameters

At the end of the experiment, the body weight gain was calculated according to Mahmoud et al. [34] as:

BWG = final body weight - initial body weight

Also, total food intake (FI) was calculated as the sum of daily food intake for each rat. Then, the food efficiency ratio (FER) for each rat was calculated as:

FER = Gain in body weight / total food intake [34].

Estimation of biochemical parameters

Fasting blood samples were withdrawn from the suborbital vein of each rat under slight xylazine/ ketamine anesthesia. The blood samples were delivered into dry clean tubes and let stand until clotting, then they were centrifuged for 15 min at 4000 rpm to separate the serum. Then, all samples were kept in deep freezer at – 80 °C for further biochemical analysis. The liver was separated from each rat, washed carefully with saline, dried on a filter paper and weighed, then each liver was divided into two portions. The first portion of the liver was kept in deep freezer at – 80 °C until further homogenization for biochemical analysis. The second portion was immersed in 10% formalin solution prepared in saline for further histopathological examination.

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined as described by Young [35]. Serum total protein and albumin were determined according to Koller [36] and Doumas [37], respectively. Then, the A/G ratio or the ratio of albumin to globulin was calculated as follows: A/G = albumin concentration / (total protein concentration – albumin concentration).

All the previously mentioned parameters were measured spectrophotometrically using a Shimadzu UV-2401 PC (Australia).

Liver tissue was homogenized in 50 mM phosphate buffer saline (PBS) of a pH of 7.4 as 250 mg liver tissue in 2500 µL of the PBS (10 %) according to Li et al. [38]. Then, the concentration of each of malondialdehyde (MDA), reduced glutathione (GSH) and tumor necrosis factor alpha (TNF-α) in liver homogenate were assessed by ELISA technique according to manufacturer's instructions by using ELISA Reader with a model of Sunrise, Tecan Austria GmbH 5082 Grödig (Austria).

Histopathological examination

Specimens from liver was examined histopathologically after being cleared in xylol, embedded in paraffin, sectioned at 4-6 micrometer thickness and stained with Heamatoxylin and Eosin according to the method of Carleton [39]. Finally, liver was examined under microscope. An Olympus (U.TV0.5C-3) light microscope with Olympus digital camera for photographing of slides was used.

Statistical analysis

Results were analyzed statistically using the computerized program SPSS software, version "25" for Windows (SPSS Inc. Chicago, IL, USA) and GraphPad Prism statistical program, version "7.0" (GraphPad Software, Inc., San Diego, CA, USA). The one-way analysis of variance "ANOVA" test followed by Duncan post hoc test was done for comparison among multiple groups. However, independent T-test was used for analysis of the obtained data that compare between two groups. All data was represented as mean ± SE. Significance was considered at $p<0.05$, otherwise was considered as non-significant.

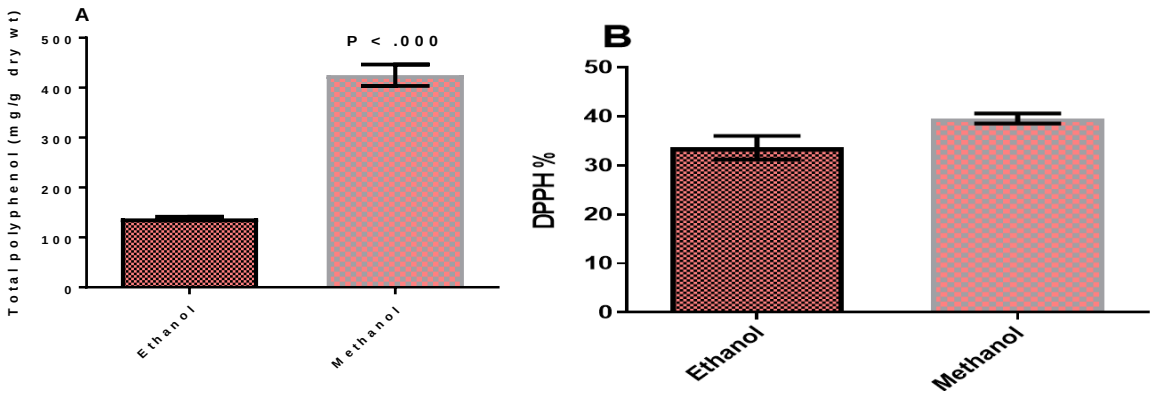

Fig. 1: A- Total polyphenolic content of BOP for ethanolic and methanolic extracts. B- Radical scavenging activity of BOP for ethanolic and methanolic extracts ($p < 0.000$).

3. Results and discussion

Citrus aurantium L., or sour orange or bitter orange belongs to the Citrus genus which is known for its an anti-inflammatory and anticarcinogenic effect has been used in many parts of the world as a folk medicine [40]. Citrus peels are famous for their bioactive compounds [22] and known to be rich in secondary metabolites like essential oils and phenolic compounds [40]. Polyphenols are considered as one important class of the most popular antioxidants. They possess health-related benefits, which are attributed to their antioxidant potency including anti-inflammatory, antiviral, anticancer activities [28] As shown in fig. (1), which illustrates the total polyphenols and the antioxidant activity of the ethanolic and methanolic extracts of BOP, the methanolic extract for both total polyphenolic content and the antioxidant activity shows higher values compared to its corresponding ethanolic extract, but this increase was significant only in case of the total polyphenols with $p < 0.000$. The obtained value of the total polyphenolic content of BOP is considered as relatively high which is in agreement with what was reported previously about the high polyphenolic content of the citrus fruits [40]. The antioxidant potency of the citrus fruits in general was attributed to their high content of polyphenols, vitamin c and carotenoids [22].

The food and generally, the agricultural processing industries produce considerably large amounts of by-products which are phenolics-rich. They could be valuable from the economic point of view, since being wastes of natural sources and containing large amounts of antioxidants [28]. The concentration of the individual polyphenolic compounds of BOP as analyzed by HPLC which was recorded in table (1) illustrates the presence of seventeen polyphenolic compounds with varied concentrations. Their concentrations in descending order are: naringenin (9050 µg/g dwt), ellagic acid (16383 µg/g dwt), gallic acid (1252 µg/g dwt), chlorogenic acid (1246 µg/g dwt) in addition to other thirteen compounds with considerable concentrations. In fact, it was reported previously that the citrus genus is rich in naringenin, and also, it was confirmed that the highest flavonoids' concentration in citrus fruits in general was found in the peel [28]. It is worth mentioning that the peel represents about 50% of the total fruit mass [22]. Also, rutin derivatives and hesperidin were detected in the peel of the bitter orange. Moreover, Karoui & Marzouk [41] identified the presence of fifteen polyphenolic compounds in the peel of the Tunisian bitter orange which were: gallic, vanillic, hydroxybenzoic, ferulic, syringic, p-coumaric, rosmarinic, trans-cinnamic, trans-2-hydroxycinnamic, naringin, rutin, catechin, epicatechin and flavone. These findings seem to be in accordance with our findings to some extent. Also, Suntar et al. [42] and Suryawanshi [43] confirmed the presence of hesperitin, naringenin and rutin as bioactive compounds in the BOP. Hesperidin was reported to revive vitamin C after it has quenched a free radical thus, it strengthens and amplifies the effect of vitamin C in our bodies [43].

Table 1: Characterization and quantification of polyphenolic content of BOP by HPLC.

Polyphenolic compound	µg/g dry wt.
Gallic acid	1252
Chlorogenic acid	1246
Methyl gallate	10.75
Coffeic acid	44.25
Pyro catechol	20.25
Rutin	204.5
Ellagic acid	16383
Coumaric acid	123.25
Ferulic acid	53.75
Naringenin	9050
Quercetin	35
Cinnamic acid	3.25
Kaempferol	16.25
Hesperidin	7

Food intake, body weight gain, food efficiency ratio and hepatosomatic index (percent of liver weight / final boy weight) of all groups are illustrated in table (2). As shown in the table; there is no significant difference among all groups in case of food intake, body weight gain and hepatosomatic index. However, there is a slight but remarkable rise in food intake of the control positive group with a concomitant decrease in body weight gain although non-significant but this phenomenon seems to be strange meaning that the utilization of the food is decreased due to the overall toxicity of the cyclophosphamide that affects every function of the body among which absorption and utilization of the food components [22]. In addition, CP was reported before to induce intestinal mucosal damage [44],

Table 2: Food intake, body weight gain, food efficiency ratio and hepatosomatic index of all groups.

parameters	Groups			
	Control negative	**Control positive**	**Control + BOP**	**BOP + CP**
FI (g)	420.80 ± 31.55^{a}	463.17 ± 29.09^{a}	425.50 ± 1.90^{a}	421.60 ± 34.88^{a}
BWG (g)	58.68 ± 3.44^{a}	43.50 ± 6.91^{a}	52.00 ± 1.81^{a}	47.68 ± 7.55^{a}
FER	0.14 ± 0.01^{b}	0.09 ± 0.01^{a}	0.12 ± 0.004^{ab}	0.11 ± 0.01^{ab}
HI	2.83 ± 0.08^{a}	2.93 ± 0.32^{a}	3.38 ± 0.04^{a}	3.18 ± 0.14^{a}

FI: food intake, BWG: body weight gain, FER: food efficiency ratio, HI: hepatosomatic index which is the percent of liver weight to final body weight. Values are represented as mean ± SE and $P < 0.05$ was considered as the level of significance. Values sharing the same letters at the same raw are non-significant, while values sharing different letters at the same raw are significant.

which in turn affects negatively the function of absorption of the intestine. This phenomenon was confirmed by the significant decrease in the food efficiency ratio for the control positive group compared to the control negative group. Anyway, this decrease was improved and was more or less restored in the cyclophosphamide injected group that received bitter orange peel with its diet to the extent that FER became non-significant with the control negative group. This finding reflects the general improvement in all body functions exerted by the BOP and indicates the ability of the BOP to detoxify the harmful effect of cyclophosphamide. This detoxification property of the BOP is attributed to its potent antioxidant activity which in turn is due to the high content of polyphenolic compounds in BOP as mentioned above in fig. (1-A & 1-B). Also, Suryawanshi [43] reported that bitter orange in general helps in digestion and in addition, it relieves flatulence which might help in improving the absorption and thus improves FER.

A state of hepatotoxicity was recorded in the present study as a result of cyclophosphamide injection to rats. Cp was reported previously to cause hepatotoxicity when used for chemotherapy regimens [11]. This hepatotoxicity was evidenced by a remarkable elevation in serum transaminases' activity AST and ALT for the control positive group that was injected by cyclophosphamide compared to the control negative group, but this rise was significant only in case of ALT. Also, a significant reduction in serum total protein and albumin concentrations as well as the in the ratio of albumin to globulin (A/G) were detected in the control positive group that was injected by cyclophosphamide compared to the control negative group as illustrated in table (3). Joshi et al. [11] reported that serum aminotransferases' elevation represents a frequent side effect during or post cytotoxic chemotherapy. These altered liver function parameters reflect the toxic impact of cyclophosphamide on hepatocytes that affects their function negatively. The increased activity of liver transaminases AST and ALT may be attributed to the state of increased oxidative stress that was induced by cyclophosphamide injection and was evidenced by the decrease in the concentration of liver tissue reduced glutathione or GSH (Fig. 2-B). This increased oxidative stress in turn has led to increased lipid peroxidation of the fatty acids constructing the hepatocytes' membrane as evidenced by elevated lipid peroxidation product which was represented by the malondialdehyde or MDA (Fig. 2-A) causing impaired membrane permeability. Hence, leakage of hepatic enzymes in the circulation occurred which explained the increased serum transaminases' activity as previously reported by Nooman et al. [45] & Mahmoud et al., [34]. Also, the reduced concentration of total protein and albumin may be explained on a similar basis that the increased oxidative stress affects negatively the hepatocytes' function rendering them malfunctioning, which are responsible for the production of total protein and albumin in addition to other proteins from amino acids resulting from protein metabolism that also takes place in liver. Also, another explanation for the reduced concentration of serum total protein and albumin is the inflammation state that occurred to hepatocytes which was caused by cyclophosphamide injection and was documented by increased concentration of the inflammatory cytokine; tumor necrosis factor-α or TNF-α (Fig. 2-C).

Table 3: Activity of serum ALT and AST, concentration of serum total protein and albumin and the A/G ratio for all groups.

parameters	Groups			
	Control negative	Control positive	Control + BOP	CP + BOP
ALT (UL^{-1})	21.77 ± 1.56^{a}	41.17 ± 1.74^{b}	20.28 ± 0.24^{a}	20.00 ± 0.65^{a}
AST (UL^{-1})	59.32 ± 1.27^{a}	67.60 ± 5.39^{a}	59.00 ± 8.02^{a}	53.68 ± 1.42^{a}
TP (g/dL)	11.23 ± 0.63^{b}	9.57 ± 0.20^{a}	10.74 ± 0.37^{ab}	10.15 ± 0.63^{ab}
Alb. (g/dL)	3.21 ± 0.05^{b}	2.49 ± 0.06^{a}	3.40 ± 0.09^{b}	2.57 ± 0.13^{a}
A/G	0.44 ± 0.01^{b}	0.36 ± 0.01^{a}	0.47 ± 0.03^{b}	0.38 ± 0.02^{a}

CP: cyclophosphamide, BOP: bitter orange peel, TP: total protein, Alb: albumin, A/G: albumin to globulin ratio. Values are represented as mean ± SE and $P < 0.05$ was considered as the level of significance. Values sharing the same letters at the same raw are non-significant while, values sharing different letters at the same raw are significant.

This inflammation rendered the hepatocytes unable to perform their functions normally, thus serum total protein and albumin formation was diminished. However, the CP-injected group that was fed on a diet fortified with the bitter orange peel shows a significant improvement of ALT activity and a remarkable non-significant improvement in AST activity as well as restoring the concentration of total protein so as it became non-significantly changed from the control negative group, while a slight non detectable improvement was noticed for both the albumin concentration and the A/G ratio of the same group. Lim et al. [46] reported similar results for the effect of BOP extract to reduce increased liver transaminases' activity in cholestatic liver fibrosis-induced mice. The obtained improvement in liver function may be attributed to the high polyphenolic content of the BOP that makes it of potent antioxidant activity which in turn enables the body to overcome the increased oxidative stress, consequently, ameliorates the altered liver function by stabilizing the hepatocyte membrane, thus preventing leakage of the enzymes into the circulation and hence normalizes the activity of hepatic transaminases [45]. Also, ceasing the increased oxidative stress by the potent antioxidant activity of the BOP renders the hepatocyte to retain its normal function, thus improving the concentration of the serum total protein.

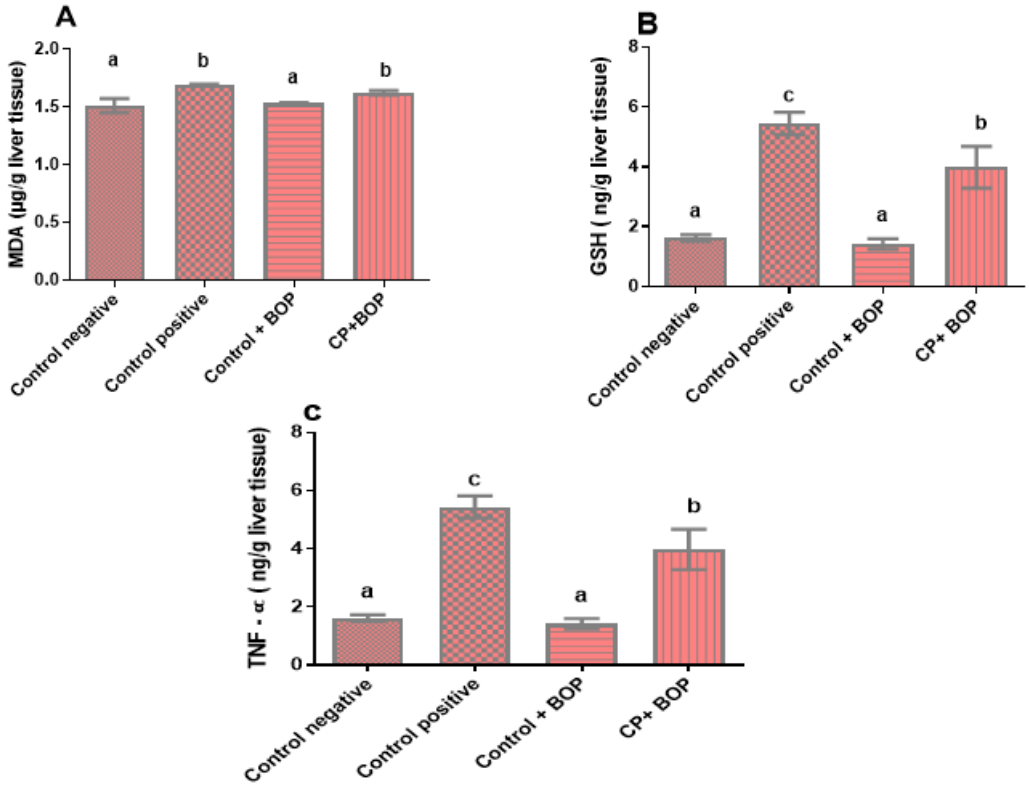

Fig. 2: Concentration in the liver tissue of: A- Malondialdehyde (MDA). B- Reduced glutathione (GSH). C- Tumor necrosis factor alpha (TNF-α). BOP: bitter orange peel. Columns that share the same letters reflect non-significant values, while columns that share different letters have significant values.

Liver tissue concentration of each of lipid peroxidation product as detected by malondialdehyde (MDA), reduced glutathione (GSH) and tumor necrosis factor alpha (TNF-α) for all groups are illustrated in fig. (2- A, B & C), respectively. A significant elevation in the concentration of both liver MDA and TNF-α was recorded for the group of the control positive compared to the control negative group which was very high in case of TNF-α. On the other hand, a significant reduction in the concentration of reduced glutathione was noticed for the control positive group compared to the control negative one. This obtained results of the altered oxidative stress parameters; MDA and GSH and the altered inflammatory parameter; TNF-α may be attributed to the state of strong oxidative stress that resulted from cyclophosphamide injection which was reported previously by Wang et al. [44] that the oxidative stress was found to be the main factor of cyclophosphamide-induced side effects. The increase was highly and significantly reduced in case of the TNF-α but slightly and unsignificant in case of MDA for the CP-injected group that received bitter orange peel compared to the control positive group, but still significantly elevated than the normal control. However, the reduction in GSH was more or less restored back to near the value of the control negative group in case of the CP-injected group that was fed on BOP with its diet to the extent that it became significantly different from the control positive group and unsignificant changed from the control negative group. Bitter orange was reported before to exert antioxidant and anti-inflammatory effect in the hepatotoxicity-induced rat model due to carbon tetra chloride treatment [40], it seems that these findings are more or less similar to those obtained in the present study. It was reported previously that CLA reduce lipid peroxidation by increasing oxidative stability in serum of rats [47]. Orange bitter peel was found to be a new potent natural antioxidant [41, 48]. The antioxidant and the anti-inflammatory effect of the BOP is due to its high content of the polyphenols (fig, 1-A), especially, the naringenin, hesperidin, quercetin and rutin (table 1) which were reported previously to have high antioxidant activity. Kim et al. [49] reported previously that the anti-inflammatory effect of the Korean *Citrus aurantium* was found to be due to the proinflammatory mediators' inhibition by blocking the mitogen-activated protein kinase (MAPK) and the nuclear factor-kappa B (NF-ƘB) pathways.

It is worth mentioning that none of the detected biochemical parameters (ALT, AST, total protein, albumin, A/G, GSH, MDA, TNF-α) showed any alterations for the control negative group that were fed on BOP along the whole experimental period indicating the safety of the BOP on most body functions.

Histopathology of liver

Histopathological examination of a hepatic section of rat from the control negative group shows normal hepatocellular architecture of liver tissue (Fig. 3-A). Also, the group that received the BOP with the control diet shows normal hepatocellular architecture of liver tissue (Fig 3-E). On the other hand, liver section of a rat from the control positive group that was injected with CP shows histopathological changes in liver parenchyma represented by several vacuoles inside the cell cytoplasm, congested hepatic blood vessels and amylodosis in liver parenchyma (Fig. 3-B, C, D). In fact, histopathological observations confirmed the obtained biochemical results of this study and reflected the hepatotoxicity of the CP which was reported previously by Grigorian & O'Brien [12]. However, the liver section of a rat from the group that was injected with CP and fed on BOP shows more or less the normal hepatic histopathological architecture except for a slight hepatic blood vessels congestion (Fig. 3-F & G). According to Lim et al. [46], the histopathological examination of hepatic tissue revealed that the administration of BOP extract significantly reduced liver fibrosis in mice induced for cholestatic liver injury. This ameliorative effect of BOP was attributed to its high content of polyphenolic components as explained above in this study.

4. Conclusion

In conclusion, BOP methanolic extract was found to contain seventeen polyphenolic compounds which are mainly responsible for its relatively high antioxidant activity. Moreover, biological evaluation of the BOP revealed its high ameliorative impact against the hepatotoxicity induced by CP injection in rats. Consequently, it can be recommended to use bitter orange peel for those patients who develop hepatotoxicity due to drugs, especially those cancer patients who receive CP among their chemotherapy regimens for cancer treatment.

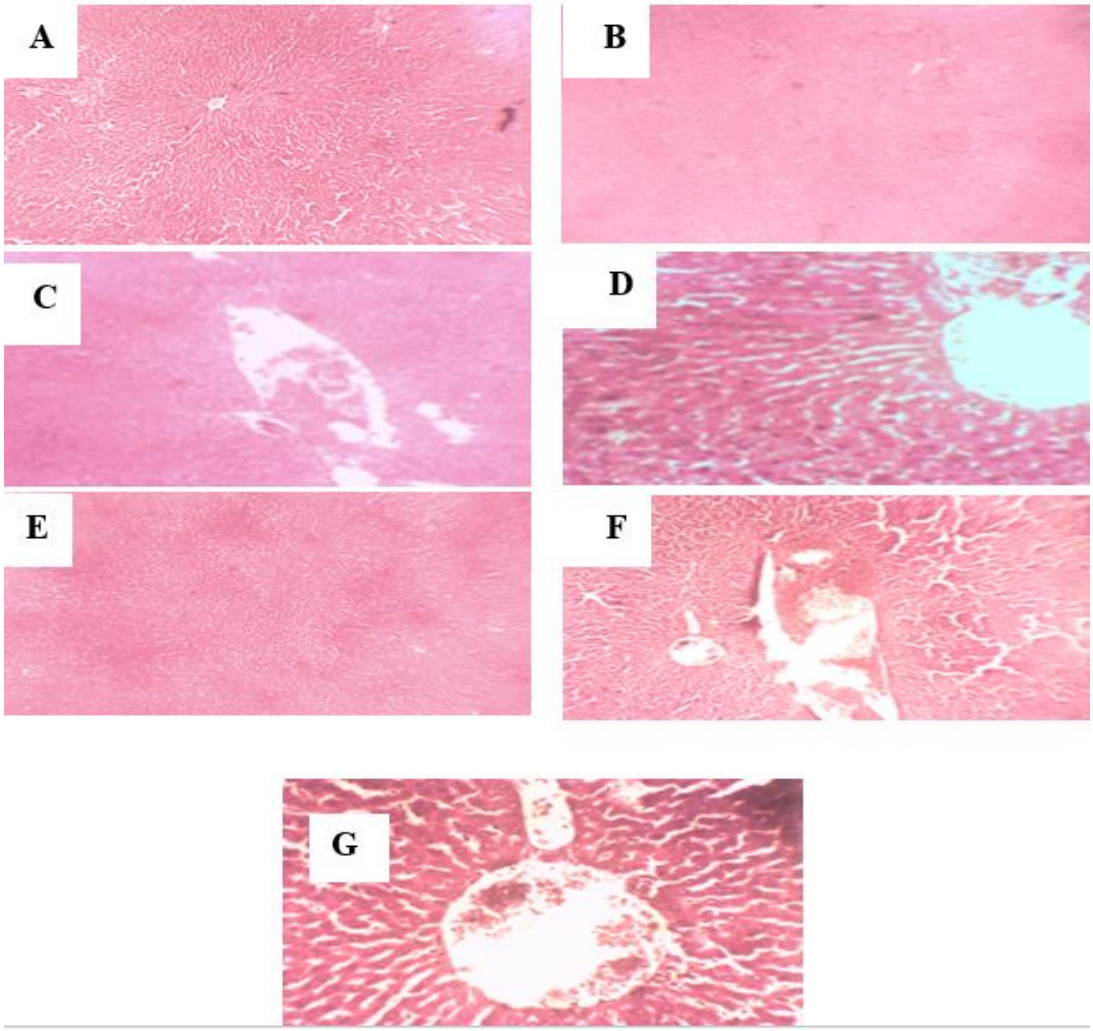

Fig. 3: A- Section of liver from the control negative group shows normal hepatocellular architecture of liver tissue (H & E 100X). B, C, D- Liver section from the control positive group shows histopathological changes in liver parenchyma represented by several vacuoles inside the cell cytoplasm, congested hepatic blood vessels and amylodosis in liver parenchyma provided that B = X100, C= X100 & D=X400 H & E. E- Liver section from the group of control + BOP shows no histopathological changes (H & E X100). F & G- Liver section from the group that was injected with CP and fed on BOP shows more or less the normal hepatic histopathological architecture except for a slight hepatic blood vessels congestion (F = 100X & G = 400X H & E).

5. Acknowledgment

The authors would like to express their deepest appreciation and gratitude for the financial support by the National Research Centre, Egypt that was given for this study.

6. Conflict of interest

The authors declare that there is no conflict of interest.

7. References

1. Ali, A.H.A., Al-Ghamdi, S., Alanazi, G.G., et al., Protective effects of ginger extract against the toxicity of cyclophosphamide on testes: an experimental laboratory-based study. Int. J. Med. Sci. Publ. Health., 9 (1), 27–33 (2020). https://doi.org/10.24911/IJMDC.51- 1558600986.
2. Harahap, Y., Vianney, A. M., & Suryadi, H.. Method Development and Validation for Measuring O6-Methylguanine in Dried Blood Spot Using Ultra High-Performance Liquid Chromatography Tandem Mass Spectrometry. Drug Design, Development and Therapy, 15, 963–971 (2021).
3. Sung H., Ferlay J., Siegel R. L., Laversanne M., Soerjomataram I., Jemal A., Bray F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36

Cancers in 185 Countries. CA CACER J CLIN, 71, 209-249 (2021).
4. Ou Z., Zhu L., Huang C., Ma C., et al., Betulinic acid attenuates cyclophosphamide-induced intestinal mucosa injury by inhibiting the NF-κB/MAPK signalling pathways and activating the Nrf2 signalling pathway. Ecotoxicology and Environmental Safety, 225, 112746. (2021)
5. Reddy H., Duffy A., Holtzman N.G., Emadi A. The role of β- elimination for the clinical activity of hypomethylating agents and cyclophosphamide analogues. Am J Cancer Ther Pharm., 3, 1–8 (2016).
6. Van de Perre D., Li D., Yao K-S., Lei H-J., Van den Brink P.J., Ying G-G., The effects of the chemotherapy drug cyclophosphamide on the structure and functioning of freshwater communities under sub-tropical conditions: A mesocosm study. Science of the Total Environment, 806, 150678 (2022).
7. Lien, E.J., Ou, X., Carcinogenicity of some anticancer drugs: a survey. J. Clin. Pharm. Ther., 10, 223–242 (1985). https://doi.org/10.1111/j.1365-2710.1985.tb00921.x.
8. Peterson, A.R., DNA synthesis, mutagenesis, DNA damage, and cytotoxicity in cultured mammalian cells treated with alkylating agent. Cancer Res., 40, 682–688 (1980).
9. Boisseaux P., Noury P., Thomas H., Garric J., Immune responses in the aquatic gastropod Lymnaea stagnalis under short-term exposure to pharmaceuticals of concern for immune systems: diclofenac, cyclophosphamide and cyclosporine A. Ecotoxicol. Environ. Saf., 139, 358–366 (2017). https://doi.org/10.1016/j.ecoenv.2017.02.003.
10. Grzesiuk M., Mielecki D., Pilżys T., Garbicz D., Marcinkowski M., Grzesiuk, E., How cyclophosphamide at environmentally relevant concentration influences Daphnia magna life history and its proteome. PLoS One, 13, e0195366 (2018). https://doi.org/10.1371/journal.pone.0195366.
11. Joshi M., Sodhi K.S., Pandey R., et al., Cancer Chemotherapy and Hepatotoxicity: An update. Indo American Journal of Pharm Research., 4(06), 2976-2984 (2014).
12. Grigorian A., O'Brien C. B., Hepatotoxicity Secondary to Chemotherapy. Journal of Clinical and Translational Hepatology, 2, 95–102 (2014). DOI: 10.14218/JCTH.2014.00011.
13. Pandit A., Sachdeva T., Bafna P., Drug-induced hepatotoxicity: a review. J.Appl.Pharm.Sci., 2, 233–243 (2012). doi:10.7324/japs.2012.2541
14. Shu Y., He D., Li W., et al., Hepatoprotective Effect of Citrus aurantium L. Against APAP-induced Liver Injury by Regulating Liver Lipid Metabolism and Apoptosis. Int. J. Biol. Sci., 16(5), 752-765 (2020). doi: 10.7150/ijbs.40612
15. Maor Y., Malnick S., Liver Injury Induced by Anticancer Chemotherapy and Radiation Therapy. International Journal of Hepatology, 815105, 1- 8. (2013).
16. Zhao, S. Y., Liu, Z. L., Shu, Y. S., et al., Chemotaxonomic Classification Applied to the Identification of two closely-related citrus TCMs using UPLC-Q-TOF-MS-based metabolomics. Molecules, 22(10), 1721٢٠١٧)).
17. El-Shobaki, F.A., Mahmoud M. H., Badawy, I.H., Marwa H.M., A dietary supplement to Ameliorate Hyperglycemia and associated complications in Streptozotocin injected rats. International Journal of ChemTech Research, 8(10), 399-410 (2015).
18. El-Desouky, M.A., Mahmoud, M.H., Riad, B.Y., Taha, Y.M., Nephroprotective effect of green tea, rosmarinic acid and rosemary on N-diethylnitrosamine initiated and ferric nitrilotriacetate promoted acute renal toxicity in Wistar rats. Interdisciplinary toxicology, 12(2), 98-110 (2019).
19. Mahmoud M.H., Wahba H.M.A., Mahmoud M.H., Badawy I. H., Newly formulated antioxidant rich dietary supplement in jelly form for alleviation of liver diseases in rats. Journal of Biological Sciences, 17 (7), 334-346 (2017).
20. El-Shobaki, F.A., Wahba, H.M.A., Mahmoud M.H., Kazem, Y.I., Badawy, I.H., Zahran, A.S., Formulation and evaluation of an adaptogenic formula to improve performance of athletes practicing anaerobic exercises. J Biol Sci, 18(5), 231-242 (2018).
21. Rafiq S., Kaul R., Sofi S.A., Bashir N., Nazir F., Nayik G. A. Citrus peel as a source of functional ingredient: A review. Journal of the Saudi Society of Agricultural Sciences, 17, 351–358 (2018).
22. Mahmoud M.H., Wahba H.M. A., Mahmoud M.H., Abu-Salem F.M., Antagonizing the Hazardous Impact of Increased Oxidative Stress in Wistar Rats by Biscuits with Dried Orange Peel. Journal of Biological Sciences, 18(1): 21-31 (2018).
23. Karabıyıkl Ş., Değirmenci H., Karapınar M., Inhibitory effect of sour orange (Citrus aurantium) juice on Salmonella Typhimurium and Listeria monocytogenes. LWT-Food Science and Technology, 55(2), 421-425 (2014).
24. Firenzuoli F., Gori L., Galapai C., Adverse reaction to an adrenergic herbal extract (Citrus aurantium). Phytomedicine, 12, 247–248 (2005).
25. Anagnostopoulou M.A., Kefalas P., Kokkalou E., Assimopoulou A.N., Papageorgiou V.P., Analysis of antioxidant compounds in sweet orange peel by HPLC–diode array detection–electrospray ionization mass spectrometry.

Biomedical Chromatography, 19(2), 138-148 (2005).
26. Aschoff J.K., Kaufmann S., Kalkan O., Neidhart S., Carle R., Schweiggert R. M., In vitro bioaccessibility of carotenoids, flavonoids, and vitamin C from differently processed oranges and orange juices [Citrus sinensis (L.) Osbeck]. Journal of agricultural and food chemistry, 63(2), 578-587 (2015).
27. Liu N., Li X., Zhao P., Zhang X., Qiao O., Huang L., Guo L., Gao, W., A review of chemical constituents and health-promoting effects of citrus peels. Food Chemistry, 130585 (2021).
28. Sawalha S.M.S., Arráez-Román D., Segura-Carretero A., Fernández-Gutiérrez A. Quantification of main phenolic compounds in sweet and bitter orange peel using CE–MS/MS. Food Chemistry 116 567–574 (2009). doi:10.1016/j.foodchem.2009.03.003
29. Hayat, K., Zhang, X., Farooq, U., Abbas, S., Xia, S., Jia, C., & Zhang, J. (2010). Effect of microwave treatment on phenolic content and antioxidant activity of citrus mandarin pomace. Food chemistry, 123 (2), 423-429.
30. Aboelsoued D., Abo-Aziza F.A.M., Mahmoud M.H., Megeed K.A., El Ezz, N. A., Abu-Salem F.M. Anticryptosporidial effect of pomegranate peels water extract in experimentally infected mice with special reference to some biochemical parameters and antioxidant activity. Journal of Parasitic Diseases, 43(2), 215-228 (2019).
31. Reeves P.G., Nielsen F.H., Fahey Jr.G.C. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J. Nutr. 123, 1939–1951 (1993).
32. Mahmoud M. H., Taha M. M., Shahy E. M. Germination of glycine max seeds potentiates its antidiabetic effect in streptozotocin induced diabetic rats. International Journal of Pharmaceutical and Clinical Research, 8 (10): 1429-1437 (2016).
33. Zhu G., Luo J., Du H., Jiang, Y., Tu Y., Yao Y., Xu, M., Ovotransferrin enhances intestinal immune response in cyclophosphamide-induced immunosuppressed mice. International journal of biological macromolecules, 120, 1-9.٢٠١٨)).
34. Mahmoud M.H., Badawy I.H., Mohammed F.E.S. The relationship between high consumption of fresh whole milk or yogurt and the risk for both cardiovascular diseases and liver disorders in hyperlipidemic Wistar rats. Journal of Microbiology, Biotechnology and Food Sciences, 10 (6), e3485 (2021).
35. Young D.S. Effects of drugs on clinical laboratory tests. Third edition, 3, 6-12 (1990).
36. Koller, A., Total serum protein. In: Clin Chem, Kaplan A. et al., (Eds.). The C.V. Mosby Co., St. Louis, Toronto, Princeton, , pp. 1316-1324 and 418 (1984).
37. Doumas, B.T., Watson W.A., Biggs H.G., Albumin standards and the measurement of serum albumin with bromcresol green. Clin Chim Acta., 31(1), 87-96 (1971). DOI https://doi.org/10.1016/0009-8981(71)90365-2
38. Li J., Wang T., Liu P., Yang F., Wang X., Zheng W., Sun, W., Hesperetin ameliorates hepatic oxidative stress and inflammation via the PI3K/AKT-Nrf2-ARE pathway in oleic acid-induced HepG2 cells and a rat model of high-fat diet-induced NAFLD. Food & Function, 12 (9), 3898-3918 (2021).
39. Carleton, H. Histolgical Technique. 4 Th Ed. London, Oxford University Press, New York, Toronto. Chemists' Society, Chicago, v.37, p.447-451(1976).
40. Hsouna A.B., Gargouri M., Dhifi W., Saibi W., Antioxidant and hepato-preventive effect of Citrus aurantium extract against carbon tetrachloride-induced hepatotoxicity in rats and characterization of its bioactive compounds by HPLC-MS. Archives of Physiology and Biochemistry, 125 (4), 332-343 (2018). https://doi.org/10.1080/13813455.2018.1461233
41. karoui I. J. & Marzouk B. Characterization of Bioactive Compounds in Tunisian Bitter Orange (Citrus aurantium L.) Peel and Juice and Determination of Their Antioxidant Activities. BioMed Research International, 2013 (2), 345415 (2013).
http://dx.doi.org/10.1155/2013/345415
42. Suntar I. Haroon Khan ,2 Seema Patel ,3 Rita Celano ,4 and Luca Rastrelli. An Overview on Citrus aurantium L.: Its Functions as Food Ingredient and Therapeutic. Agent Oxidative Medicine and Cellular Longevity, 2018, 7864269, (2018) https://doi.org/10.1155/2018/7864269
43. Suryawanshi, J.A.S. An overview of Citrus aurantium used in treatment of various diseases. Afr. J. Plant Sci, 5, 390–395 (2011).
44. Wang X.H., Yuan Z.H., Zhu L.J., et al., Protective effects of betulinic acid on intestinal mucosal injury induced by cyclophosphamide in mice. Pharmacol. Rep. 71, 929–939 (2019). https://doi.org/10.1016/j.pharep.2019.05.004.
45. Nooman M.U., Mahmoud M.H., Al-kashef A.S., Rashad M.M., Hypocholesterolemic impact of newly isolated sophorolipids produced by microbial conversion of safflower oil cake in rats fed on high fat and cholesterol diet. Grasas y Aceites, 68 (3) e212 (2017). http://dx.doi.org/10.3989/gya.0219171
46. Lim S.W., Lee D.R., Choi B.K. et al., Protective effects of a polymethoxy flavonoids-rich Citrus aurantium peel extract on liver fibrosis induced by bile duct ligation in mice. Asian Pacific

Journal of Tropical Medicine, 9 (12), 1158–1164 (2016).
47. Halaby M.S., Farag M.H., Mahmoud M.H., Badawy I.B., Abd Rabou N.S., Mohammed F.S., Effect of consumption of Milk and Yogurt on Lipid metabolism and Atherosclerosis in adult male rats. International Journal of ChemTech Research, 8 (10), 388 - 398 (2015).
48. Tundis R., M. R. Loizzo, M. Bonesi et al., Comparative study on the antioxidant capacity and cholinesterase inhibitory activity of Citrus aurantifolia Swingle, C. aurantium L., and C. bergamia Risso and Poit. peel essential oils. Journal of Food Science, 77 (1), H40–H46 (2012).
49. Kim J.-A., Park H.-S., Kang S.-R., et al., Suppressive effect of flavonoids from Korean Citrus aurantium L. on the expression of inflammatory mediators in L6 skeletal muscle cells. Phytotherapy Research, 26 (12), 1904–1912 (2012).