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Olea europaea L. Leaves and *Psidium guajava* L. Leaves Alleviate Cyclophosphamide-induced Oxidative Stress and Immune Disturbance in Wistar Rats

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

a. Nutrition and Food Science Department, Institute of Food Industries & Nutrition Research, National Research Centre, Dokk, Cairo, Egypt, P.O. 12622.
b. Nutrition and Food Sci. Dept., Faculty of Home Economics, Helwan University.,Cairo, Egypt.
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

Many side effects were detected for the use of cyclophosphamide as chemotherapeutic regimen for cancer patients among which are the increased oxidative stress and immune disturbance. The present study was designed to evaluate the effect of leaves of the two antioxidant-rich and commonly used edible plants namely; olive and guava against increased oxidative stress and disturbed immunity induced by cyclophosphamide (CP). Their methanolic and ethanolic extracts were subjected to analysis for the detection of their antioxidant activity by DPPH and their total and differential polyphenolic content by HPLC. A biological experiment was conducted comprising 6 groups of 6 rats in each, three groups of them were intraperitoneally injected with 40 mg/Kg B wt of CP for four successive days, one left as a positive control whereas the other two were given either olive leaves (OLs) or guava leaves (GLs) as 20% of their diet. The remaining three groups were injected by saline for four successive days, one was control negative group fed on basal diet while the other two were fed on either OLs or GLs as 20% of their diet. The experiment lasted for four weeks. Results revealed a relatively high antioxidant potency and polyphenolic content for OLs and GLs. Lymphocytopenia, neutropenia, thrombocytopenia, reduced monocytes and anemia were detected for the CP-injected group in addition to a reduction of reduced glutathione (GSH) and an elevation of the interleukin-6 (IL-6). Also, an alteration of the weight and histopathology of the thymus gland was recorded. All these aforementioned parameters were more or less restored to near their normal values of the control negative group in case of the two CP-injected groups that received either OLs or GLs reflecting their ameliorative impact against increased oxidative stress and disturbed immunity exerted by CP. Thus, it can be concluded that both OLs and GLs can be given as natural antioxidants to cancer patients during the period of cyclophosphamide chemotherapy to combat its concomitant increased oxidative stress and disturbed immunity.

Key words: *Cyclophosphamide; Olive leaves; Guava leaves, Polyphenols; Disturbed immunity, Increased oxidative stress.*

1. Introduction

Increased oxidative stress and disturbed immunity are among the many side effects accompanying the use of some chemotherapeutic drugs for different malignancies. Among these drugs is the cyclophosphamide (CP) which is known for its potency and great efficiency in curing several types of malignancies [1] like breast cancer, prostate cancer, ovarian adenocarcinoma, leukemias and lymphomas [2, 3]. But unfortunately, as it is cytotoxic to the tumor cells, it is also cytotoxic for other rapidly growing cells like mucosal lining of the gastrointestinal tract, hair cells and bone marrow cells. That may explain the side effects of CP that are mainly; nausea, vomiting, diarrhea, stomatitis, hepatotoxicity, hair loss, anemia

*Corresponding author e-mail: mahahanafymmm@yahoo.com (Maha Hanafy Mahmoud).
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and immune disturbance in the form of hematological disorders like myelotoxicity, leukopenia, thrombocytopenia in addition to other several side effects [4, 5].

Increased oxidative stress is nowadays highlighted as it is the causative etiology of many chronic diseases like diabetes mellitus, cardiovascular diseases, different malignancies, some neurodegenerative diseases like Alzheimer's disease, dementia and Parkinson's disease. Presence of free radicals normally occurs in the body cells as a part of their function but the imbalance between the production of these free radicals and the cellular antioxidant defense mechanisms lead to the state of increased oxidative stress [6,7].

Immunity is the ability of each body to defend itself against biological and chemical challenges and the immune system possesses cascade of potent mechanisms for defending and protecting the body against these challenges and maintaining the immune homeostatic balance during normal physiological circumstances [8, 9]. Once there is an external immune-stimulus that is defined by the body, an immune response begins to occur represented by activation of a series of immune cells which may give rise to cellular infiltration, swelling, redness, inflammation and allergy [10]. Various sever clinical and pathophysiological conditions comprising radiation therapy, chemotherapy and organ transplant immunosuppressive therapies in addition to antibiotics and cortisone therapy can aggravate immunosuppression [11]. Cyclophosphamide which is among the most frequently used chemotherapy protocols for many malignancies, is a strong immunosuppressive agent [12].

Nowadays, "Back to nature" is the concern of many specialists and scientists in the era of disease innovative and unconventional therapies. Today, light was thrown on the role of herbal medications as they become of increasing importance in several medical applications since they are of natural sources, relatively lower cost, very low toxicity and high efficiency. They may be used as alternative or even adjuvant therapies for several metabolic disturbances as liver toxicity [13, 14], nephrological disorders [15], diabetes [16] and cardiovascular diseases [17]. Also, it was mentioned previously that the use of antioxidants may reduce the toxicity of cyclophosphamide [4]. Among the natural antioxidants are olive and guava which are plants of dual function serving as both edible and medicinal plants.

Olive tree (Olea europaea L.) which belongs to Oleaceae family is known to be native to the Mediterranean region [18, 19]. It is a tree of medium size that is cultivated in subtropical and tropical regions and predominant mostly in Greece, Spain, Italy, Portugal, France, Cyprus, Palestinian Authority, Jordan, Tunisia, Morocco, Turkey [20] as well as Egypt to some extent. In Mediterranean region, olive tree is considered as the most important and precious fruit trees because of its prominent products which are; the table olives and the olive oil that representing main components of the Mediterranean diet that are also consumed worldwide with large amounts [21]. The leaves from olive tree can be easily reachable from the olive orchard or as by-products of both agricultural and industrial processes [22]. Olive leaves (OLs) include a variety of bioactive components like polyphenols, triterpenoids, tocopherols and polysaccharides [22]. They are considered as an inexpensive raw material in green technologies enabling them to be used as a good source of high-added value bioactive compounds, mainly polyphenols. OLs have been used for many purposes, since their polyphenolic content is greater than that of either whole fruit or even the extra virgin olive oil [23]. OLs are used in food, agronomic, nutraceutical, pharmaceuticals and other biomedical applications like cosmetic [24, 25]. Also, there are other uses of OLs in industry since they are added to the vegetable oils to prevent oxidation and to retain nutritive components of oils leading to elongate the oils' shelf-life [26, 27]. They are mainly used in folk medicine since their possess antidiabetic and cardioprotective properties as well as being used as animal feed [28].

Psidium guajava L which is generally known as guava is a fruitful evergreen shrub that is a member of the Myrtaceae family [29]. Guava is planted in subtropical and tropical regions such as Africa, South Asia and South America [30]. It is known to exert some therapeutic effects like; antioxidant, antibacterial, antivirus, hypoglycemic, anti-inflammatory and antitumor [30]. Guava is generally rich in bioactive compounds, particularly antioxidants and it is worth mentioning that the guava leaves (GLs) have higher antioxidant content than the fruit itself [31]. A wide variety of bioactive compounds are present in GLs like; triterpenoids, tannins,

polyphenols, volatile oils, polysaccharides and quinones [30]. Extracts from leaves and root bark have been used from ancient time for folk remedy for many chronic and acute diseases such as cardiac diseases, diabetes, gastroenteritis, different tumors, dysentery, wounds, diarrhea, ulcers, cough, swollen gums, sore throat and toothache [32].

Based on the previous mentioned knowledge, this study was designed to evaluate the potential role of the leaves of the naturally occurring antioxidant rich edible plants namely, guava and olive against the oxidative stress and immune disturbance exerted by the cyclophosphamide in Wistar rat.

2. Material and methods

Materials

Olive leaves (Olea europaea L.) and Guava leaves (Psidium guajava L.) were collected from herbarium at faculty of agriculture, Cairo University. Cyclophosphamide (CP) with a Cat. No of "PHR1404-1G", that was used for induction of immunosuppression was purchased from Sigma-Aldrich Chemical Co. (St. Louis, Mo, 63103, USA). Ingredients that were used to formulate the semisynthetic balanced diet were as follows; cellulose was obtained from the Laboratory of Rasayan, Fine Chemical Limited, Mumbai, India, while, casein was purchased from Al-Ahram Laboratory Chemicals (Egypt). Ingredients used to constitute the mineral mixture and the vitamin mixture were purchased from BDH Company (England) and Fluka Company (Germany), respectively. The other diet constituents were purchased from the local market. Chemicals and solvents that were used for extraction and estimation of total and differential polyphenolic content by HPLC analysis as well as the antioxidant activity of both olive leaves (OLs) and guava leaves (GLs) were all of analytical grade and were obtained as follows: DPPH or 1, 1-diphenyl-2-picrylhydrazyl, ethanol and methanol were purchased from Sigma-Aldrich Company (St. Louis, Mo, 63103, USA), while Folin-Ciocalteu reagent was obtained from LOBA Chemie Company (UN: 3264), India. ELISA kit used for determination of the concentration of interleukin-6 (IL-6) with a Cat No. of K0331229 was purchased from Koma Biotech., Seoul (South Korea), while the reduced glutathione (GSH) ELISA kit was purchased from Sunlong Biotech Co. (China) with a Cat No. of "SL1410Ra".

Albino Wistar rats of male sex were used in the present study for the biologic evaluation. They were obtained from the Central Animal House, National Research Centre, Dokki, Egypt. An approval for the study protocol was obtained from the Scientific Ethical Committee at the National Research Centre (NRC, Dokki, Egypt) with an approval No. of "18-106". The experiment was conducted in compliance with the guidelines of the Institutional Animal Care and Ethics Committee of the NRC.

Methods

Preparation and extraction of olive leaves and guava leaves

Each of the leaves of both olive and guava were cleaned and allowed to dry in an air ventilated oven at 40°C until complete dryness. Then, the dried OLs and GLs each alone were milled into a fine powder using electric miller (Mienta, Egypt). The obtained powder for each was divided into two portions. The first one was subjected to extraction by either ethanol or methanol for the investigation of the phenolic compounds either total or differential by HPLC and also for the detection of 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. Then, the extraction was carried out as follows: One gm of each powder was put in 10 ml of 70% ethanol followed by sonication for 1 hour at 37°C. Then, the suspension was centrifuged at 3000 rpm for half an 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. The same extraction steps were repeated by replacing ethanol with methanol. The obtained ethanolic extract and methanolic extract for each of OLs and GLs were used for determination of the total polyphenolic content (TPC), the quantitative and differential polyphenolic content by HPLC technique and the antioxidant activity as measured by DPPH test [33].

Determination of total phenolic compounds in OLs and GLs

Folin-Ciocalteu assay was used for the detection of the total phenolic compounds (TPC) in both extracts, ethanolic and methanolic as described by Aboelsoued et al. [34]. Briefly, 250 µl from each extract was diluted with exactly 3.5 ml of distilled water, then 0.25 ml of Folin-Ciocalteu reagent was added. Then, 0.25

ml of 20% sodium carbonate solution was added, mixed well with vortex., and incubated for 20 minutes at 40 °C in water-bath. The absorbance of the formed blue color was read against a blank standard at 765 nm using a spectrophotometer of Shimadzu model, UV-2401 PC (Australia). The TPC were calculated with respect to gallic acid (concentration range: 0-12 µg/ml). The results were expressed as mg of gallic acid per g dry weight 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 antioxidant activity of OLs and GLs by DPPH

The DPPH (1, 1-diphenyl-2-picrylhydrazyl) method was used to determine the radical scavenging activity of each of the two extracts for both two plants; the ethanol extract and the methanol extract according to Hayat et al. [33] with some modifications. A volume of 0.5 ml of each extract was added to 1 ml of DPPH methanolic solution (0.2mM), then incubated in darkness at room temperature for 30 min. The absorbance was then measured at 517 nm using a spectrophotometer of Shimadzu model, UV-2401 PC (Australia). The assay was repeated in the same manner using the solvent only without the extract to serve as control. The scavenging capacity of DPPH for the tested samples was then measured as a decline in the absorbance then, it was calculated according to the following equation:

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

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

Characterization and quantitative estimation of differential polyphenolic content of the methanolic extract for both OLs and GLs by HPLC.

HPLC analysis was conducted with an Agilent 1260 series. The separation was done 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 semisynthetic control diet was prepared as described by Reeves et al. [35]. The powder of either of the dried OLs or GLs was added to the prepared basal diet of the selected groups on the expense of starch as 200 g / Kg diet as described previously [36].

Thirty-six adult albino Wistar rats of a male sex and a body weight of about 200 g were used in this study. They were allocated in separate stainless-steel cages each alone under a temperature of 25 ± 1 °C with a constant light dark cycle of 12 hours for a duration of one week before starting the experiment for acclimatization. They were allowed to have free access to food and water during the adaption period and throughout the whole experiment. Animals were grouped into 6 groups. Three of them were intraperitoneally injected with 40 mg CP / kg body weight for four successive days as described by Zhu et al. [37] and Halaby et al. [14]. The remaining three groups were also injected but using saline instead of the CP solution for the same duration and with the same volume. The 6 experimental groups were:

Group 1: control negative group fed on the basal diet and pre-injected with saline.

Group 2: control positive group pre-injected intraperitoneally with 40 mg CP / kg B. wt. for 4 successive days and fed on control basal diet.

Group 3: fed on control basal diet + 200 g OLs/ kg diet and pre-injected with saline.

Group 4: pre-injected intraperitoneally with 40 mg CP / kg B wt for 4 successive days and fed on control basal diet + 200 g OLs/ kg diet.

Group 5: fed on control basal diet + 200 g GLs/ kg diet and pre-injected with saline.

Group 6: pre-injected intraperitoneally with 40 mg CP / kg B. wt. for 4 successive days and fed on control basal diet + 200 g GLs/ kg diet.

After an experimental period of four weeks, fasting blood samples were obtained from the suborbital vein of each rat under slight xylazine/ ketamine anesthesia. Each blood sample was delivered into two tubes; an

EDTA containing tube to obtain whole blood for complete blood count (CBC) detection and another dry clean tube which was let stand until clotting and centrifuged at 4000 rpm for 15 min to separate the serum. Then, all serum samples were kept in deep freezer at – 80 °C for further biochemical analysis while the whole blood samples were subjected to CBC detection immediately. The thymus gland was separated from each rat, washed carefully with saline, dried on a filter paper and weighed. Then, it was immersed in 10% formalin solution prepared in saline for further histopathological examination.

Complete blood count (CBC) of whole blood samples was done using a coulter or an automated haematology analyser (Sysmex, XT-1800i, Hamburg, Germany) which uses fluorescence flow cytometry (FFC) technology. The analyzed hematological parameters include hemoglobin concentration, RBCs (red blood corpuscles) count, PCV (packed cell volume), platelet count. Then, differential leukocyte count was microscopically examined to obtain the percent of each of lymphocytes, neutrophils and monocytes for each blood sample.

Serum reduced glutathione (GSH) and interleukin-6 (IL-6) were assessed by ELISA technique according to the manufacturer's instructions by using ELISA reader with a model of Sunrise, Tecan Austria GmbH 5082 Grödig, (Austria).

Histopathological examination

Thymus gland specimens for each group of rats were 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 [38]. Finally, thymus was examined using an Olympus (U.TV0.5C-3) light microscope with Olympus digital camera for photographing of slides.

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 "6.0" (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.

3. Results and discussion

In the past few decades, there was a considerable interest in magnifying the value of using the natural antioxidants from plant sources rather than the synthetic sources. Guava (Psidium guajava) is a very popular fruit used as dessert and has been found to contain many nutrients as minerals and vitamins. It is a good example for the potent antioxidant containing edible and medicinal plants. Other parts of the guava tree have been traditionally used like leaves, root and bark for treating some diseases, since extracts from these parts are well known for their powerful antioxidant potency [39]. Guava has been used in traditional medicine for many purposes, like respiratory and gastrointestinal disturbances for its antioxidant and anti-inflammatory effects. Guava leaves (GLs) contain several bioactive components like essential oils, saponins, phenols and polysaccharides [40]. Leaves, bark and roots are used in treating dysentery, diarrhea, gastroenteritis, while leaves are used for rheumatic pain, ulcers, applying on wounds and to relieve toothache in addition to the most popular and common use of the leaves for treating cough [41]. Another example for edible and medicinal plants is the olive tree (Olea europaea) which was chosen amongst natural antioxidant sources to be one of the highest antioxidant activity owners with its various parts; fruit, oil and leaves [42]. Antioxidant molecules in olive tree in general are well recognized. It contains tyrosol, hydroxytyrosol, caffeic acid, ligstrosides and oleuropein to which the prevention of many diseases can be attributed [42, 43]. These compounds have many health-promoting effects, like anti-inflammatory, cardioprotective, antioxidant, hypotensive, spasmolytic and antithrombotic [44, 45]. From our point of view, compounds having antioxidant activity are expected to exert immune stimulatory impacts since one of the etiologic causes of immune disturbances in the body is the increased oxidative stress. Also, those which possess anti-inflammatory effects are expected to be immunomodulators. Both OLs and GLs have antioxidant and anti-inflammatory activities.

Table (1): Total polyphenolic concentration and antioxidant activity by DPPH of ethanolic and methanolic extracts of both guava leaves and olive leaves.

	Polyphenol (mg/g dry wt.)		DPPH (%)	
	Ethanol	**Methanol**	**Ethanol**	**Methanol**
GLs	978.99 ± 18.05[a]	1202.10± 30.01[a]	76.597 ± 1.70[a]	82.34 ± 1.72[a]
Olive leaves	282.55 ± 7.32[b]	468.71± 30.44[b]	46.27± 0.84[b]	49.72 ± 1.90[b]

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 column are non-significant while, values that sharing different letters at the same column are significant.

Polyphenolic compounds are substances synthesized in plants to exert many functions like protection against microbial infections and they were found to possess potential therapeutic benefits when used for humans. They were said to be potent antioxidants [46] and anti-inflammatory agents [47], so they can be of therapeutic importance in a wide range of diseases. In the present study, the total polyphenolic content of either OLs or GLs in both ethanolic and methanolic extracts as illustrated in table (1), shows relatively high concentration for the two plants with both extracts of the GLs having about three folds higher than their corresponding extracts of olive leaves. Gutiérrez et al. [41] mentioned similar results for guava leaves stating that GLs have high concentration of total polyphenolic compounds. Also, Rocchetti et al. [23] mentioned that the polyphenolic content of OLs is higher than that in whole fruit or olive oil rendering the OLs of higher antioxidant activity and can be used as a rich source for high-added value bioactive compounds. As illustrated from the data, the methanolic extract for both plants gave better values for polyphenolic content. Also, the same can be noticed for the antioxidant activity as determined by the DPPH method, since the antioxidant activity for both GLs and OLs are relatively high with the methanolic extract rather than the ethanolic one is of better yield for both of the two plants.

Methanolic extract rather than ethanolic extract was selected as the extract of choice for polyphenols' differential and quantitative HPLC analysis based on the obtained data in this study as it gave a better result for the total polyphenolic compounds when determined by Folin-Cicaltue reagent. As detected by HPLC, the OLs methanolic extract contains 14 polyphenolic compounds with five compounds having a high concentration as follows: gallic acid 653, quercetin 596.25, chlorogenic acid 498, naringenin 263.75 and hesperetin 103.75 µg/g dry weight in addition to other nine compounds with varying concentrations (table 2). However, the GLs extract comprises 11 polyphenolic compounds, among which five compounds with high concentration which were; gallic acid 3909.25, naringenin 853, catechin 387.75, coumaric acid 201, caffeic acid 152, ferulic acid 151.75 and quercetin 134.25 µg/g dry weight and other four compounds with varying concentrations. Gutierrez et al. (2008) [41] mentioned that guava leaves extract contains quercetin, and kaempferol in addition to other polyphenols that render it with potent antioxidant activity. Gallic acid was reported to possess many benefits among which antimicrobial, antioxidant, anticarcinogenic and anti-inflammatory properties in addition to its therapeutic activities in many diseases such as metabolic, cardiovascular, gastrointestinal, neuropsychological as mentioned by Kahkeshani et al. [48]. Also, Cai et al. [49] added that gallic acid modulate immunity in weaning piglets. As obvious from the obtained data in table (2), gallic acid constitutes the polyphenolic compound of the highest concentration in both olive leave and guava leaves extracts which renders both of them as potent antioxidant, anti-inflammatory and immune-modulatory supplements. Quercetin was also reported to improve the cytokine levels in vitro using virus O'nyng-nyong infected synovial human fibroblasts [50]. Based on the present results, olive leaves are rich in quercetin and so are guava leaves with lower extent, thus both of them are expected to exert immunomodulatory effects. Moreover, naringenin was evidenced to have anti-inflammatory and antioxidant effects against a wide range of oxidative stress and inflammation-related disorders like nephrotoxicity, skin damage, diabetic neuropathy [51].

Lymphocytopenia is the state of reduced total lymphocytic count. Among the many causes for lymphocytopenia, the medications and chemotherapy are involved [52]. Also, neutropenia which is the state of reduced neutrophils may be due to many causes like toxins, immune dysfunction, some medications

Table (2): Differential polyphenolic profile by HPLC of methanolic extracts of each of olive leaves and guava leaves.

Polyphenolic compounds	Concentration	
	OLs (µg/g d.w)	GLs (µg/g d.w)
Gallic acid	653	3909.25
Chlorogenic acid	498	272
Catechin	ND	387.75
Methyl gallate	ND	55.25
Caffeic acid	31.75	152
Syringic acid	15.25	ND
Rutin	29.25	71.5
Ellagic acid	77.5	ND
Coumaric acid	12	201
Vanillin	40.25	ND
Ferulic acid	11	151.75
Naringenin	263.75	853
Querectin	596.25	134.25
Cinnamic acid	1.5	2
Kaempferol	55.25	ND
Hesperetin	103.75	ND

and chemotherapy [53]. Also, neutrophils are among the components of the immune system that helping in killing microorganisms and sever neutropenia magnifies the chances of bacterial and fungal infections [54, 55]. In the present study, a significant reduction in the mean percentage of each of lymphocytes, neutrophils and monocytes count was observed in the group that was injected with CP compared to the control negative group as shown in table (3). This marked reduction was moderately restored in case of the lymphocyte percent for the two groups that were injected by CP and given either OLs or GLs to the extent that it became significantly higher than the group that was injected with CP alone or the control positive group but still significantly lower than the control negative group. However, in case of neutrophilic percent the two injected groups with CP that were given either OLs or GLs with their diet showed a marked but non-significant improvement compared to the control positive group but still significantly lower than the value of the control negative group, while in case of the monocytes, no improvement was recorded for the same previously mentioned two groups. Kumar and Venkatesh [11] mentioned similar results for lymphocytopenia in CP injected rats. They attributed this reduction to the alkylating effect of CP on the cellular protein functional groups and also that CP causes restraining of the function of the medulla hematopoietic. Also, Madondo et al. [56] reported that the use of CP leads to lymphodepletion. Moreover, Shabbir et al. (2016) [57] reported that CP injection in mice causes a reduction in all differential leukocytic count elements leading to a reduction in neutrophils, lymphocyte and monocytes. Dhabhar [58] mentioned that some risky stimuli such as tobacco abuse, alcohol, chemotherapy, antibiotics, and other drug therapies can cause immunosuppression, in particular affecting the activity of the natural killer cells negatively which is one of the most important lymphocyte elements and that seems to be in the same line with our findings in the present study of reduced lymphocyte count due to the immunosuppressing effect of cyclophosphamide. Shabbir et al. [59] reported similar results that GLs extract was able to counteract the immunosuppressive action of CP on all the leukocytic elements including the lymphocytes, the neutrophils and the monocytes. Both of OLs and GLs, with their potent antioxidant activity as previously mentioned [59] and according to the data obtained in the present study of the antioxidant activity as measured by DPPH test in table (1), were capable of neutralizing the toxic effect of CP to some extent and hence hindering its alkylating action. Also, it is worth mentioning that the predominant polyphenolic compound in both of OLs and GLs as obvious from the obtained data of HPLC analysis in the present study in table (2), which is gallic acid was said to have immunomodulatory effect in weanling piglets [60], hence it was capable of restoring the reduced count of lymphocytes, neutrophils and monocytes.

Also, the other hematological parameters for the CP-injected group which were hemoglobin concentration (Hb), red blood cells' count (RBCs), packed cell volume (PCV) and platelets' count (PLT) recorded a marked reduction compared to the control negative group. This reduction was significant in case of hemoglobin and RBCs, while insignificant in case of PCV and PLT. Anyway, the reduction either significant or non-significant was more or less restored for all parameters in the group that was injected with CP and given OLs compared to the CP-injected group, although this improvement was still insignificant with the control positive group, but it became also insignificant with the control negative group meaning

Table (3): The mean percentages of lymphocytes, neutrophils and monocytes of all studied groups.

Groups	Parameters		
	Lymphocytes %	Neutrophils %	Monocytes %
Control negative	80.23 ± 3.75^{c}	24.50 ± 0.90^{b}	2.50 ± 0.18^{b}
Control positive	65.95 ± 1.88^{a}	11.63 ± 1.87^{a}	1.75 ± 0.16^{a}
Control + OLs	80.50 ± 0.13^{c}	23.75 ± 0.30^{b}	2.68 ± 0.15^{b}
OLs + CP	72.00 ± 0.77^{b}	15.00 ± 0.26^{a}	1.40 ± 0.20^{a}
Control + GLs	84.15 ± 1.49^{c}	24.25 ± 1.60^{b}	2.50 ± 0.32^{b}
GLs + CP	74.20 ± 1.35^{b}	14.50 ± 1.70^{a}	1.80 ± 0.30^{a}

OLs: olive leaves, CP: cyclophosphamide, GLs: guava leaves. 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 column are non-significant while, values sharing different letters at the same column are significant.

that the CP-injected group that received OLs began to be normal However, no improvement was noticed for any of the mentioned four parameters in the group that was injected with CP and given GLs except for a slight increase in the same group for PLT which although it was still insignificant with the CP-injected group, also insignificant with the control negative group. Shabbir et al. [57] reported similar results for the reduction in Hb content RBCs count and PLT count in mice injected by CP. Again, this data can be explained on the basis of the toxic effect of CP that having strong alkylating potency and also affecting negatively the function of medulla hematopoietic [11]. It is worth mentioning that OLs that were given to one of the CP-injected groups showed more potency than GLs, since OLs were able to minimize the hazardous effect of CP on each of hemoglobin, RBCs, PCV and PLT, while GLs improved the PLT count only.

Table (4): Hemoglobin concentration, red blood corpuscles' count, packed cell volume percent and platelets' count for all studied groups.

Groups	parameters			
	Hb (g/dl)	RBCs (10^{6} × µl)	PCV %	PLT (10^{3} × µl)
Control negative	14.30 ± 0.25^{b}	8.62 ± 0.12^{b}	48.25 ± 0.67^{b}	712.00 ± 21.84^{ab}
Control positive	12.58 ± 0.67^{a}	7.79 ± 0.33^{a}	46.82 ± 0.64^{ab}	678.23 ± 52.58^{a}
Control + OLs	13.97 ± 0.23^{b}	8.51 ± 0.08^{ab}	47.32 ± 0.396^{ab}	767.00 ± 20.64^{ab}
OLs + CP	13.28 ± 0.18^{ab}	8.34 ± 0.18^{ab}	47.83 ± 0.38^{ab}	731.17 ± 45.79^{ab}
Control + GLs	14.00 ± 0.37^{b}	8.38 ± 0.15^{ab}	48.00 ± 0.61^{ab}	798.32 ± 25.75^{b}
GLs + CP	12.68 ± 0.42^{a}	7.78 ± 0.40^{a}	46.62 ± 0.59^{a}	695.50 ± 20.87^{ab}

OLs: olive leaves, CP: cyclophosphamide, GLs: guava leaves, Hb: hemoglobin, RBCs: red blood corpuscles, PCV: packed cell volume, PLT platelets. 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 column are non-significant while, values sharing different letters at the same column are significant.

A marked non-significant decrease in the concentration of reduced glutathione was noticed in the CP-injected group compared to the control negative group. While a marked increase was recorded for the control group that received GLs (Fig. 1-A). Anyhow, the recorded reduction in the CP-injected group was restored to some extent in case of the other two CP-injected groups which received either OLs or GLs with their diet indicating an ameliorative and antioxidant impact for both OLs and GLs against the strong oxidizing effect of the CP. It was reported that CP upon administration, it undergoes hydrolysis in hepatocytes by the action of cytochrome enzyme P450 to the pharmacologically activated form which is 4-hydroxycyclophosphamide and its isomer aldophosphamide. Then, in the cytosol of the target cell, the latter is converted via spontaneous β-elimination into acrolein or phosphoramide mustard which is the active product that is responsible for facilitating tumor cell death [61]. This product, which is acrolein, is responsible for the depletion of GSH in the cell, since it forms a stable conjugate with GSH and hence reducing the amount of free GSH in cytosol [56]. This explanation seems to satisfy the obtained results in the present study of the reduced GSH in the CP-injected group.

However, OLs and GLs were able to some extent to neutralize the oxidizing effect of CP. OLs and GLs were known to be potent antioxidants since they contain many bioactive components among which the polyphenols which are known to exert potent antioxidant effects as mentioned above in the obtained data in table (1). Rocchetti et al. [23] mentioned that olive leaves contain the highest polyphenols' concentration among other parts of the olive tree either the whole fruits or even the extra virgin olive oil rendering the olive leaves to be used in the traditional medicine in the Mediterranean basin. Also, other studies reported that olive polyphenols exert protective impacts against many diseases through a putative anti-inflammatory and antioxidant activities [25, 62] which is obvious here from the obtained data of the present study of protecting and restoring the levels of GSH in the CP-injected group that received OLs. Moreover, although all parts of guava were reported to have potent antioxidant activity yet, the guava leaves' extract was said to possess the highest polyphenolic content and the highest antioxidant potency among other guava parts including the fruit itself and the stem bark [59] and that may explain the ability of GLs here in the present study to counteract the effect of CP on GSH.

A significant increase was recorded for interleukin-6 (IL-6) of the CP-injected group compared to the control negative group. This increase was restored significantly in the two CP-injected groups that received either OLs or GLs with their diet indicating that both OLs and GLs possess potent anti-inflammatory effects (Fig. 1-B). CP was said to be converted into acrolein which in turn induce the elevation of reactive oxygen species in macrophages through a comprehensive mechanism leading finally to the activation of the pro-inflammatory cytokines [56] and this appears to explain the increase of the pro-inflammatory cytokine; IL-6 for the CP-injected group in the present st**ud**y. Barbalho et al. [63] mentioned that GLs extract possesses anti-inflammatory, analgesic, hepato-protective, antimicrobial and antioxidant activity which is probably attributed to its high content of polyphenolic compounds and that is why GLs was able to maintain the IL-6 near the normal value of the control negative group in the CP-injected group that was given GLs since it possesses anti-inflammatory effect. Also, it was mentioned previously that olive can protect against many diseases through its potent antioxidant and anti-inflammatory properties [25, 62]. Moreover, Rocchetti et al. [23] have found from their *in vitro* study that OLs extract was found to downregulate some of the pro-inflammatory cytokines and these results seem to reinforce the obtained results in the present study of the ability of OLs to prevent the elevation of IL-6 in the CP-injected group that receive OLs rendering the value of IL-6 insignificantly similar to its corresponding value of the control negative group.

It is worth mentioning that the two control groups that received either OLs or GLs with their diet didn't show any considerable changes for all the previously mentioned biochemical parameters reflecting that the use of these two supplements as 200 g/ Kg diet is safe with no adverse effect on the screened parameters.

Weight of thymus gland

Regarding the thymic weight there was no significant change among rats that were fed on olive leaves or guava leaves in their basic diet when compared to the control negative group (Fig. 2). On the other hand, the thymic weight for rats of the CP-injected group shows a reduction by 32% compared to its corresponding value of the control negative group (Fig 2). Huang et al. [64] reported similar results of reducing the thymus weight percent of the CP-injected mice. However, the weight of thymus from rats of the CP-injected group and fed on OLs in their basic diet showed a significant improvement comparing to the weight of CP-injected rats. Also, an improvement of the thymic weight for the CP-injected group that received GLs was recorded but this improvement is still insignificant with the CP-injected group and it became more or less similar to the value of the control negative group but still significantly lower. Luo et al. [65] and Chen et al. [9] mentioned that the thymus gland is one of the most vital immune organs in the body that is responsible for lymphocyte maturation, differentiation and immune response. They added that CP reduced the thymic index due to its immunosuppressive property [9]. OLs and GLs were able to protect the body's immune organs by stimulating the development of the thymus gland to improve the host's immune function. This effect of the OLs and GLs may be attributed to their antioxidant and anti-inflammatory activities [25, 62].

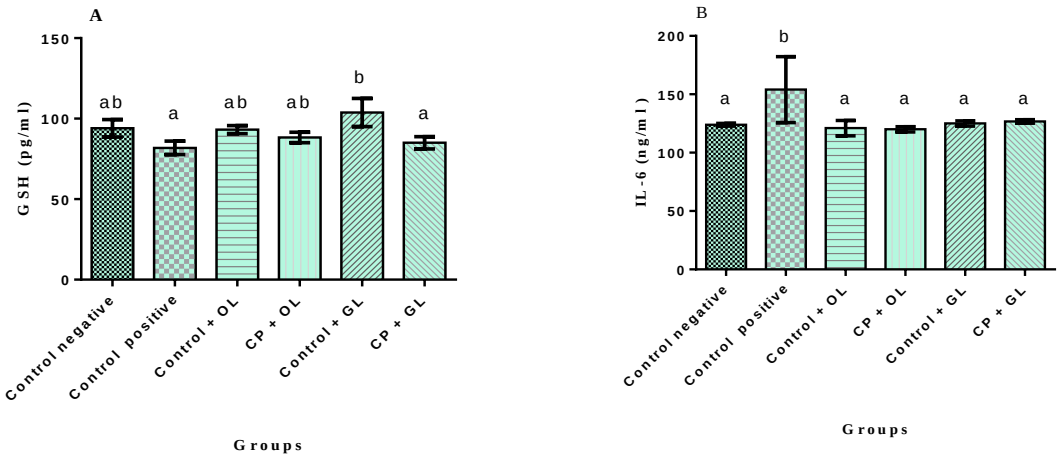

Fig. (1): Serum concentration of: A-Reduced glutathione (GSH) B- Interleukin-6 (IL-6) for all studied groups. OLs: olive leaves, CP: cyclophosphamide, GLs: guava leaves. Columns that share the same letters reflect non-significant values, while columns that share different letters have significant values.

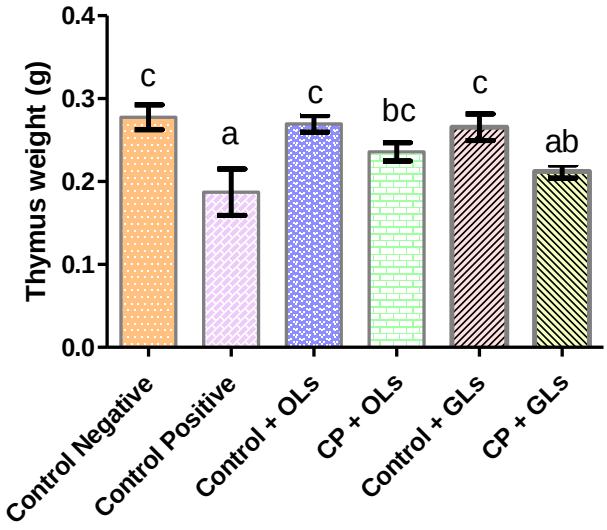

Fig. (2): Thymus weight of all studied groups. OLs: olive leaves, CP: cyclophosphamide, GLs: guava leaves. Columns that share the same letters reflect non-significant values, while columns that share different letters have significant values.

Histopathology of thymus gland

Macroscopic examination of the thymus revealed that the thymus was unchanged for rats that were fed on OLs or guava leaves in their basic diet compared to those rats of the control negative group. On the other hand, the thymus from rats that were injected by CP appeared quite different from thymus of rats from the control negative group. However, the thymus of both the two groups that were injected with CP and fed on either OLs or GLs showed improvement compared to the thymus of the group of rats that were injected with CP only to the extent connective tissues (Fig. 3-A). Also, Fig (3-B & 3-C) show no histopathological changes for the control groups that received GLs and OLs, respectively.

On the other hand, the examined thymic section from rats of the CP-injected group shows that the cortex was relatively thin with lower density of deeply stained lymphocytes and the thymic medulla appeared with some masses of lightly stained lymphocytes were observed (Fig. 3-D). Pearse [66] reported similar changes in the examined sections of thymus from male F344 rats that were injected with four daily doses of CP which seems to be in accordance with the

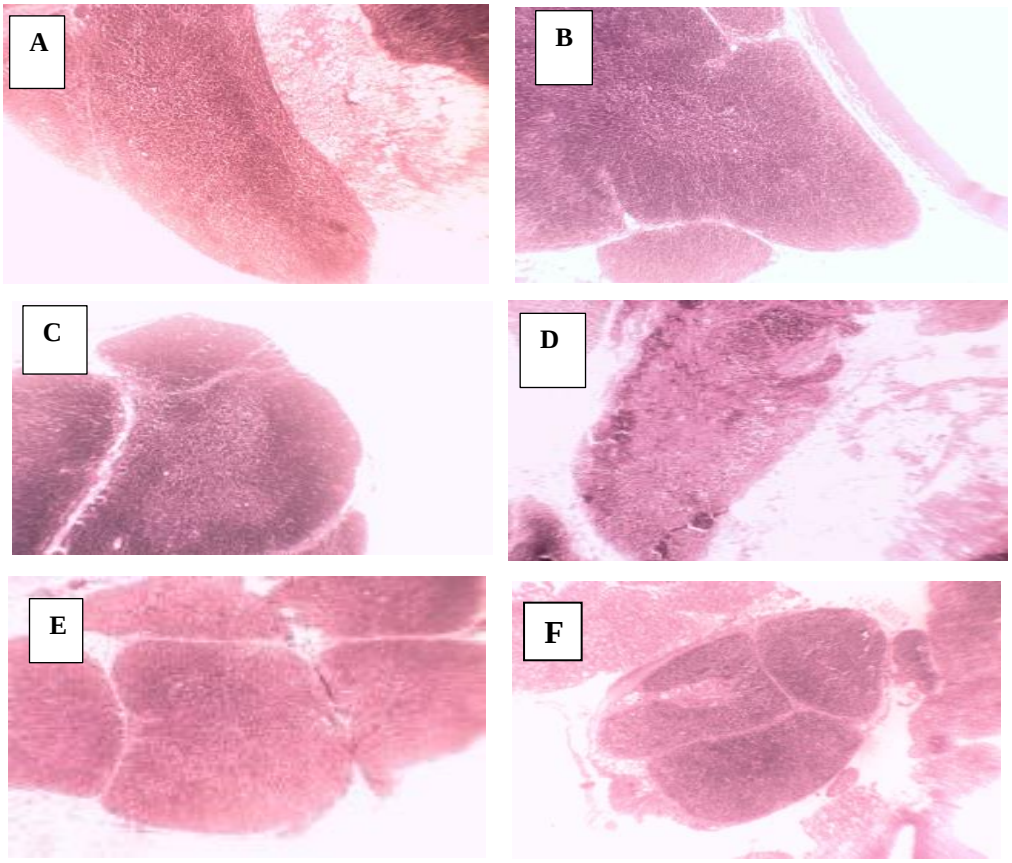

Fig. 3: A- Section of thymus from the control negative group shows normal architecture of thymic tissue with the cortex extremely rich with numerous densely packed lymphocytes with epithelial cells and the cortex was enclosing faint staining medulla with epithelial cells and groups of lymphocytes and the lobuli appeared separated by thin band of connective tissues (H & E 100X). B- Thymus section from the control group that received GLs showing no histopathological changes. C- Thymus section from the control group that received OLs showing no histopathological changes. D- Section of thymic tissue from the CP-injected group showing thin cortex with lower density of deeply stained lymphocytes and the thymic medulla appeared with some masses of lightly stained lymphocytes. E- Thymic section from the CP + GLs showing that the cortex was lightly stained with low density of lymphocytes enclosing faint medulla and no obvious changes from the CP-injected group. F- Thymic section of the CP + OLs group showing a complete recovery process with normal cortex and numerous densely packed lymphocytes enclosing normal medulla with lightly staining.

observed changes in the present study. However, the thymic section from the CP + GLs group shows that the cortex was lightly stained with low density of lymphocytes enclosing faint medulla and no obvious changes from the CP-injected group meaning that a very slightly improvement was observed for this group compared to the CP-injected group. On contrast to the CP + GLs group, the thymic section from the group of CP + OLs shows a complete recovery process with normal cortex and numerous densely packed lymphocytes enclosing normal medulla with lightly staining suggesting the potent efficiency of the OLs for alleviating the hazardous effect of CP on the thymic tissue. Anyway, these obtained histopathological results confirm the obtained biochemical findings.

4. Conclusion

Based on the obtained data from the present study, it can be concluded that fortification of the diet of the immunocompromised animals (CP-injected rats) by either OLs or GLs has led to improving the increases oxidative stress as represented by restored GSH concentration as well as improving their

immune state as evidenced by reduction of the elevated IL-6, restoring the normal weight and histopathology of the immune organ which is the thymus and more or less normalizing all altered hematological parameters as an indication of an immunomodulation effect of both OLs and GLs with varying degrees for improvement. Thus, it can be recommended to keep cancer patients that receive cyclophosphamide as chemotherapy on a continuous supplement from both OLs and GLs in the form of a suitable dietary supplement to minimize the strong oxidative stress and the disturbed immune conditions that are concomitant to cyclophosphamide therapy.

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6. Conflict of interest

The authors declare that there is no conflict of interest.

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