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A Study on the Therapeutic Use of Different Olive Leaves Extracts against Cholestasis and Cholangitis

Hanadi M. Baeissa [1], Aminah A. Barqawi [2], Manal A. Tashkandi [1], Reem S. Alazragi[1], Israa J. Hakeem[1], Hanan S. Alnahdi[1], Najlaa O. Ayaz[1], Rasha H. Hussein[1, 3]and Ammar AL-Farga[1]

[1]Department of Biological Sciences, College of Science, University of Jeddah, Jeddah, Saudi Arabia.
[2]Department of Chemistry, College of Science, Umm Al Qura University, Makkah al Mukarramah, Saudi Arabia.
[3]Department of Biochemistry and Nutrition, Faculty of Women for Arts, Science and Education, Ain Shams University, Cairo, Egypt.

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ABSTRACT

Cholangitis and cholestasis are significant hepatobiliary disorders characterized by impaired bile flow and inflammation of the bile ducts, leading to severe complications such as liver damage, infections, and increased morbidity. Primary biliary cholangitis (PBC), a progressive autoimmune disease characterized by bile duct damage and autoantigen generation, disproportionately affects females, with a median female-to-male ratio of 10:1. This study evaluated the therapeutic potential of olive leaf extracts, prepared using two different extraction methods, against α-naphthyl-isothiocyanate (ANIT)-induced cholangitis and cholestasis in rats. Forty rats were divided into four groups: G1 (control), administered saline solution (0.9% NaCl); G2 (PBC), induced with ANIT (1 g/kg powdered rat chow); G3 (PBC + OL-Etha), treated with olive leaf extract obtained via simple extraction (400 mg/kg/day b.w.); and G4 (PBC + OL-Sox), treated with olive leaf extract obtained via Soxhlet extraction (400 mg/kg/day b.w.). ANIT-treated rats exhibited significant liver damage, as evidenced by elevated biomarkers of liver dysfunction, cholangitis, and cholestasis, along with increased levels of TNF-α, IL-1β, and antimitochondrial antibodies (AMA-M2). Histological analysis confirmed these findings. Treatment with olive leaf extracts significantly reduced liver function test markers, indices of cholangitis, inflammatory cytokines, AMA-M2 levels, and improved anthropometric measurements, gene expression of TGF-β, and histological alterations. The study concluded that olive leaves contain bioactive compounds with antioxidant and anti-inflammatory properties that mitigate ANIT-induced cholangitis and cholestasis by regulating oxidative stress and inflammatory pathways. Primarily, the Soxhlet extraction method was more effective in isolating active components compared to the methanolic maceration method.

Keywords: Antimitochondrial antibodies (AMA-M2); Cholangitis; Cholestasis; Hepatoprotection; α-Naphthyl-Isothiocyanate (ANIT); Olive leaves; Primary Biliary Cholangitis (PBC).

INTRODUCTION

Cholangitis and cholestasis are significant hepato-biliary disorders characterized by impaired bile flow and inflammation of the bile ducts, leading to severe complications such as liver damage, infections, and increased morbidity. Primary biliary cholangitis (PBC) is defined by progressive bile duct damage and autoantigen generation, which can result in liver failure. This condition is more prevalent in women, with an estimated one in every 1,000 women over the age of forty affected (Lu *et al.*, 2018). Meanwhile, cholestasis is considered as a clinical condition marked by disturbances in bile acid flow and is used to screen for various illnesses, including primary sclerosing cholangitis (PSC), primary biliary cirrhosis (PBC), pregnancy-related intrahepatic cholestasis, and progressive familial intrahepatic cholestasis. The condition has various structural causes, involving both inherited and acquired pathologies (Wu et al., 2020). Bile, produced in liver cells, is essential for the absorption of fatty acids. Bile acids stimulate the production of sterol regulatory element-binding protein-1c, which regulates cholesterol biosynthesis. Bile acids (BAs) are key endogenous compounds that serve multiple biological functions. In addition to managing nutritional absorption, bile acids (BAs) play critical roles in various biological functions, including the modulation of glucose and lipid metabolism, maintenance of immunological equilibrium, and preservation of gut microbiota (Zou et al., 2023). Their physiological importance extends to the regulation of metabolic processes, where BAs act as signaling molecules that influence systemic inflammation and energy homeostasis through receptors such as the farnesoid X receptor (FXR) and Takeda G protein-coupled receptor 5 (TGR5) 34.

The diagnosis of primary biliary cholangitis (PBC) relies heavily on the sensitivity and specificity of serum antimitochondrial antibodies (AMA), where measurable AMA levels and a continuous rise in cholestatic enzyme levels are sufficient for diagnosis (Ashby et al., 2018). Patients diagnosed with PBC are typically treated with ursodeoxycholic acid, which remains the first-line therapeutic option for this condition (Assis, 2024). Ursodeoxycholic acid is a

* *Corresponding author e-mail: dr.rasha.asu@gmail.com*

hydrophilic bile acid whose mechanism of action is associated with its choleretic effects, promoting bile flow while also facilitating the displacement of more hydrophobic bile acids from the enterohepatic circulation (Manne and Kowdley, 2019). This therapeutic approach underscores the significance of BAs in both disease management and metabolic regulation.

Old medicine showed profound value on liver illness. Herbal plants have achieved popularity as possible therapeutic agents for the prevention and treatment of diverse liver illnesses due to their high efficiency and little side effects. Olive leaves, a common byproduct of tree pruning, are waste and useless generation. Indeed, it has been calculated that each olive tree produces 25 kg of garbage every year, comprising leaves and branches (Ronca *et al*.,2024).

Olive tree (*Olea europaea* L.), one of the world's most ancient cultivated plants, is referenced in the Holy Qur'an and Ahadith, highlighting its long-standing therapeutic and nutritional significance (El-Seedi et al., 2019). This tree is primarily found in the Mediterranean region, which accounts for approximately 98% of global olive production, although it is also cultivated in the Arabian Peninsula, India, and parts of Asia (Medfai et al., 2020). Olive leaves are recognized as a cost-effective raw material and a treasured source of phenolic compounds. The total concentration of polyphenols in *Olea europaea* L. decreases during summer, progressively increases in autumn, and reaches its peak at the beginning of winter. Numerous in vitro and in vivo studies have demonstrated that olive leaf extract (OLE) reduces oxidative stress and inflammation, while also providing beneficial effects against cardiovascular diseases, metabolic disorders, and bacterial infections (Silvestrini et al., 2023). The polyphenolic compounds present in olive leaves can be categorized into five groups: a) oleuropein; b) flavones; c) flavonols; d) flavan-3-ols; and e) substituted phenols. Additionally, olive leaves contain triterpenes and chalcones, which have been traditionally used as treatments for fever and malaria (El-darier *et al*., 2018; de Oliveira *et al*., 2024) Several studies have highlighted the importance of these compounds in reducing cardiovascular risk factors and cancer (Kermanshah et al., 2020).

Previous research has highlighted the hepatoprotective properties of various natural compounds, including those found in olive leaves, which are rich in polyphenolic compounds known for their antioxidant and anti-inflammatory effects. These properties suggest that olive leaf extracts may mitigate the oxidative stress and inflammation associated with ANIT-induced liver injury, a well-known cholestasis-inducing agent that damages the small bile ducts in the liver, leading to intrahepatic cholestasis and subsequent liver cell injury. The injury characteristics induced by ANIT closely resemble those observed in clinical primary biliary cholangitis (PBC) (Mariotti *et al.,* 2018). Therefore, this study aims to investigate the protective mechanisms of two types of olive leaf extracts against cholangitis and cholestasis caused by ANIT. Taking advantage of the traditional medicinal uses of olive leaves, the study seek to elucidate their potential benefits in alleviating liver damage related to cholangitis and cholestasis conditions brought by α-naphthylisothiocyanate (ANIT).

MATERIALS AND METHODS

Chemical used

α-naphthyl-isothiocyanate (ANIT) acetonitrile anhydrous (99.8%, molecular weight 41.05) and Ethyl alcohol pure (molecular weight 46.07) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Plant materials and nutritional composition

Olive leaves were obtained from the local market, rinsed thoroughly with clean water, and air-dried for two weeks. The dried leaves were then ground into a fine powder, which was used for extract preparation. Ultimately, 100 g of the powdered olive leaves contained 4.7 g of water, 11.7 g of protein, 10.2 g of lipids, 68.4 g of carbohydrates, and 4.2 g of oleuropein (Omagari *et al.,* 2021).

Extraction technique and preparations

Maceration technique using ethanol

The fine powder of air-dried, ground olive leaves (20 g), was macerated in 150 ml of 80% ethanol. The extract was filtered and concentrated to dryness under reduced pressure using a rotary evaporator.

Soxhlet Extraction Method (Sox)

Twenty grams of dried olive leaves were placed in a Soxhlet apparatus thimble and extracted with 150 ml of 20% acetonitrile at 60 °C for 4 hrs. After extraction, the mixture was cooled to room temperature and then filtered. The extract was evaporated using a rotary evaporator at room temperature under vacuum conditions. The collected extract was stored at 4^{o}C until use (Yateem *et al*., 2014).

Induction of cholangitis and cholestasis

Chronic cholangitis and cholestasis were induced in rats by administering a diet containing alpha-naphthyl-isothiocyanate (ANIT) at a dosage of 1 g/kg of powdered rat chow, provided *ad libitum* for periods of 4, 7, and 14 days, as described by Tjandra *et al.* (2000). ANIT is known to act as a toxin that specifically targets bile ducts, leading to inflammation and damage in the biliary system. This model allows for the investigation of the pathophysiological changes associated with cholestasis.

Experimental animals

The experiment was performed on forty female adult Albino rats weighing (120-150 g). Rats were kept in poly propyl cages in a cycle of 12:12 light/dark in a temperature-controlled room (25 ± 2^{o}C). Rats had access to food and water *ad libitum* and were provided with a standard diet. Rats were habituated for one week before starting the experiment. The Ethics Committee of King Abdulaziz University's Faculty of Medicine authorised this research (Reference Number 210-22) Animal Study).

Experimental Design

After the acclimatization period (one week), animals were divided into 4 groups: G1: served as control, administered saline solution (0.9% NaCl), four times per week for 28 days. G2: Primary Biliary Cholangitis (PBC) induced: Chronic cholangitis was induced by feeding rats on a diet supplemented by a-naphthyl-isothiocyanate (1 g/kg powdered rat chow) for 28 days. G3: PBC + OL (Etha): cholangitis rat group, supplemented orally olive leaves extracted by simple extraction method, using 80% ethyl alcohol as a solvent by gavage (400 mg/Kg/day bw) for 28 days. G4: PBC+OL(Sox): cholangitis rat group, supplemented orally with olive leaves extracted by Soxhlet apparatus using 20% acetonitrile as a solvent by gavage (400 mg-/Kg/day bw) for 28 days according to Al-Attar and Abu Zeid (2013).

Sample collection

At the end of 28 days, rats were sacrificed after overnight fasting under ether anaesthesia. Blood samples were collected from hepatic portal vein in a dry clean centrifuge tube (Khalil *et al*., 2015) Serum was separated by centrifugation at 3000 r.p.m. for 10 min at 4°C, and kept for biochemical analysis. The liver was separated, washed up with saline, dried, and weighed to detect its relative weight part of hepatic tissue was, and kept in 10% formalin for histopathological assessment and another part kept in - 20C for hepatic TGF-β gene expression measurement.

Anthropometric measurements

Animals were weighed twice a week by electric balance, the final body weight (bw) of animals were determ-ined according to Chapman *et al*. (1959). Gain in body weight was calculated by the formula:

Gain in bw (g) = Final bw (g) - Initial bw (g).

Under deep anaesthesia using diethyl ether, to detect body mass index, body length (from nose to anus) was measured using non-stretchable tape to calculate body mass index (BMI):

BMI = bw (g) / square of nasal-anal length (cm), normal BMI: female [0.4504–0.5044] g/cm^2.

Daily feed intake (FI) /group was estimated during the investigational period, while feed efficiency ratio (FER) was calculated by the formula:

$$FER = \frac{Body\ weight\ gained\ (g)}{Consumed\ Food\ (g).}$$

Antioxidant content in Olive Leaves: Comparison of ethanolic maceration and Soxhlet extraction techniques

The total phenolic content (TPC) was determined using Folin-Ciocalteu reagent, expressed as milligrams of gallic acid equivalents per gram of sample (mg GAE/g). Additionally, the total flavonoid content was assessed.

Indices of hepatic cell damage, biliary cell damage and cholestasis

Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were measured using commercially available clinical test kits purchased from Biovision Kit, CA. USA. according to manufacturer instructions.While, Serum levels of alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), direct bilirubin (DBIL), serum cholesterol level, total bilirubin (TBIL) and total bile acid concentrations (TBA) and were detected (Kamiya Biomedical Co. CA. USA), according to manufacturer instructions.

Cytokines assays

Cytokine levels, comprising Tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and interleukin-10 (IL-10), were detected in samples by enzyme-linked immunosorbent assay (ELISA) using kits (Biocompare Kits, USA) according to manufacturer instructions.

Detection of serum antimitochondrial antibodies (AMA-M2)

Serum Antimitochondrial Antibodies (AMA) were detected by ELISA quantitative determination of IgG antibodies to the mitochondrial antigen M2 according to manufacturer instructions (Eagle-Bio, Amherst, NH 03031, USA). Reference values: Positive > 15 U/L, Negative ≤ 15 U/L.

Gene expression of Transforming growth factor beta TGF-β

Extraction of RNA

Total RNA was extracted from liver tissues with RNase Mini Kit instructions (Catalogue no.74104) (Qiagen Company, USA) according to manufacturer instructions.

Real-Time RT-PCR (RT-PCR)

Primers of Genes primers were constructed depending on the gene sequences of *Rattus norvegicus* on the NCBI homepage. Extremely refined salt-free primers used to amplify TGF-β. β-actin cDNA was amplified as previously described and considered as control. Sequences of primers were shown in Table (1). The strata gene MX3005P software determined amplification curves and threshold cycle (Ct) values that used for detecting gene expression difference on different samples of RNA and comparing it with control group (Artika *et al*.,2022).

Table (1): Primer sequences employed in SYBR Green real-time PCR.

Gene to be detect	Primer sequence (5'-3')	Accession No.
ß-actin	F 5'-CTCTAGACTTCGAGCAGGAGATG-3' R 5'-CACTGTGTTGGCATAGAGGTCTT-3'	V01217
TGF-β	F 5'-AACCCCCATTGCTGTCCCGT-3' R 5'-CCTTGGTTCAGCCACTGCCG-3'	U24174

Histopathological examination

Specimens from the liver tissues were collected and fixed in 10% neutral buffered formalin, then dehydrated using ascending grades of ethyl alcohol (50-100%), then cleared using xyelene, then embedded in melted paraffin wax, blocked, cutted and stained using the routine staining method (H&E) according to Perry *et al* (2016).

Statistical Analysis

Differences among means were tested by ANOVA using SPSS package version 19.0. Results were represented as mean ± Standard deviation (SD) of the mean. Statistical significance was considered at $p \leq 0.05$ level.

RESULTS

Total phenols and flavonoids of the two different types of olive leaves extract was shown in Table (2). The chemical analysis revealed that the Soxhlet extraction method yielded higher amounts of phenols and flavonoids compared to the simple ethanolic maceration method.

Anthropometric measurements

Results obtained in Table (3) showed different treatments on anthropometric measurements. The control group shows the highest significant weight gain (8.21 g/day), indicating normal metabolic function. However, ANIT administration induced a significant *(p* ≤ 0.05) reduction in body weight, likely due to hepatic dysfunction and metabolic disturbances. Meanwhile, treatment with OL(Etha) partially restores weight gain (6.74 g/day), indicating a potential protective effect of the ethanolic extract compared to the OL(Sox) group which showed a lower weight gain (4.86 g/day). This suggests that, under these experimental conditions, the Soxhlet extract may be less effective than the ethanol extract in supporting weight gain recovery. In parallel the measured BMI, wet liver weight, food consumption and feed efficiency ratio, recorded significant differences among all groups (Table 3). PBC significantly increases liver weight (11.65 g) compared to the control (8.37 g). Meanwhile, the treatment with olive leaves extracted either by ethanol or Soxhlet method induced a significant ($p \leq 0.05$) improvement in these parameters. Regarding food consumption, no significant difference was detected between PBC and PBC + OL(Etha) rats. In general, the obtained data indicate that PBC negatively impacts growth and metabolic efficiency, while the addition of olive oil ethanolic form, in particullary) appears to offer some improvement and protective effects. However, further investigation is in need to understand the mechanisms behind these changes and the implications for dietary interventions as protective drug.

Cholangitis and Cholestasis indices

As shown in figure (1 A-I), ANIT caused significant ($p \leq 0.05$) elevation in hepatic transaminases (ALT,. AST, ALP) of cholangitis and cholestasis that caused including serum levels of hepatic transaminases, LDH, GGT and total and direct bilirubin, bile acids concentration and cholesterol levels. On the other hand, treatment with either olive leaves extract markedly recovered serum levels of these parameters.

Table (2): Total phenols and total flavonoids in the two types of olive leave extracts.

Extraction method	Total phenols (GAE mg/100g)	Total flavonoids (CE mg/100g)
Simple ethanolic maceration	220	18
Soxhlet extraction	300	25

Inflammatory cytokine indices

As shown in the figure. (2 A-D), A significant ($p \leq 0.05$) increase of inflammatory indices (TNF-α, IL-1β and IL-6) accompanied by a significant ($p \leq 0.05$) reduction in IL-10 level as compared to the normal rats. While, the change was significantly ($p \leq 0.05$) attenuated by administering both types of olive oil extract.

Antimitochondrial Antibodies M2 of Serum (AMA-M2)

The AMAs present in these samples predominantly directed against the E2 subunit of alpha ketoacid dehydrogenase complex (PDC-E2), the major AMA autoantigen. Results revealed that AMA level in ANIT treated rats was positive compared to normal control rats, which was later counteracted after olive leaves extract treatments.

Histological results

Figure (4) represented the effects of olive leaf extracts on histology in rats with induced primary biliary cholangitis (PBC). Liver of the control group (G1) showed normal liver architecture with a clear central vein and well-organized hepatic cords and nuclei are well defined. Meanwhile, ANIT induced PBC that changed the texture of liver tissue with heaptocytes degeneration and leukocyte infiltration. Hepatocyte arrangement appears slightly disrupted. On the other hand, treatment by any method of extraction (G3 and G4) showed some improvement, with hepatocytes appearing more intact, although there are still signs of mild structural disruption, mild leucocyte infiltration accompanied by mild focal inflammatory reaction.

Gene expression of transforming growth factor

PCR examinations were achieved by exact primers

Table (3): Effect of different treatments on Anthropometric measurements

Treatment Groups	Measured parameters				
	Gain in body weight (g /day rat)	BMI (g/cm2)	Wet Liver weight (g)	Food consumed (g /day/rat)	Feed efficiency ratio
Control	8.21 ± 1.25^{a}	0.473 ± 0.03^{a}	8.37 ± 1.86^{a}	23.54 ± 2.65^{a}	0.348 ± 0.04^{a}
PBC	3.76 ± 1.43^{b}	0.109 ± 0.06^{b}	11.65 ± 1.77^{b}	16.54 ± 1.88^{b}	0.227 ± 0.05^{b}
PBC + OL(Etha)	6.74 ± 0.95^{c}	0.375 ± 0.08^{c}	10.00 ± 1.83^{c}	16.88 ± 1.84^{b}	0.399 ± 0.01^{c}
PBC+ OL(Sox)	4.86 ± 1.12^{d}	0.287 ± 0.08^{d}	9.62 ± 1.23^{d}	24.73 ± 2.94^{c}	0.195 ± 0.06^{d}

*Means with different superscript letters, per column, are significant different at *(p* ≤0.05*)*, n=10.

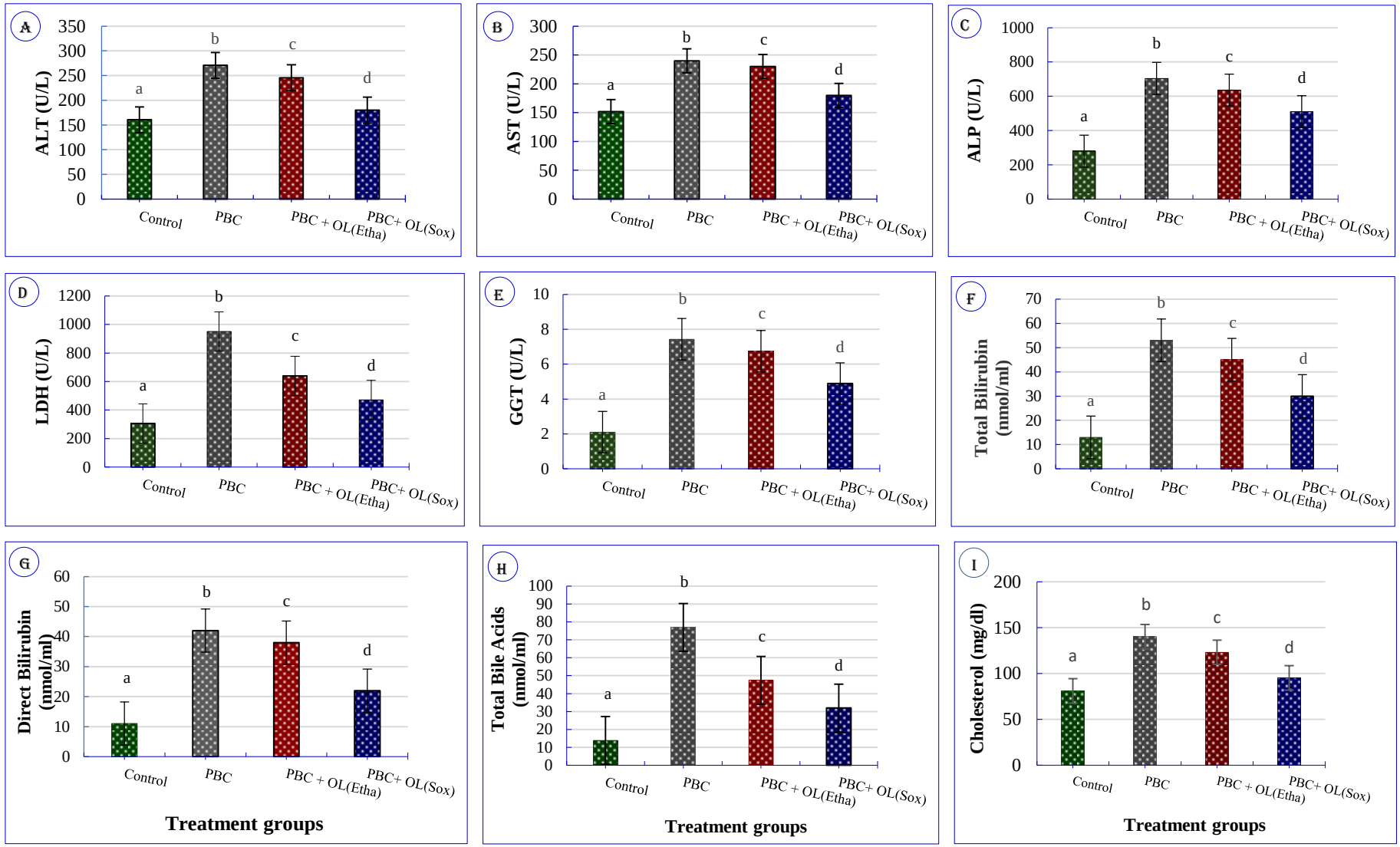

Figure (1A-I): Effects of olive l leaf extracts on liver enzymes and serum parameters in rats with induced primary biliary cholangitis (PBC). Liver enzymes assessed include alanine transaminase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH), and gamma-glutamyltransferase (GGT). Serum parameters evaluated are total bilirubin (TBIL), direct bilirubin (DBIL), total bile acids (TBA), and cholesterol (CHOL). Treatment groups: control, PBC, PBC treated with olive leaf extract using ethanol extraction (PBC + OL(Etha)), and PBC treated with olive leaf extract using Soxhlet extraction (PBC + OL(Sox)). Columns with different superscript letters indicate significant differences ($p \leq 0.05$, based on Duncan's Multiple Range Test, n = 10 per group).

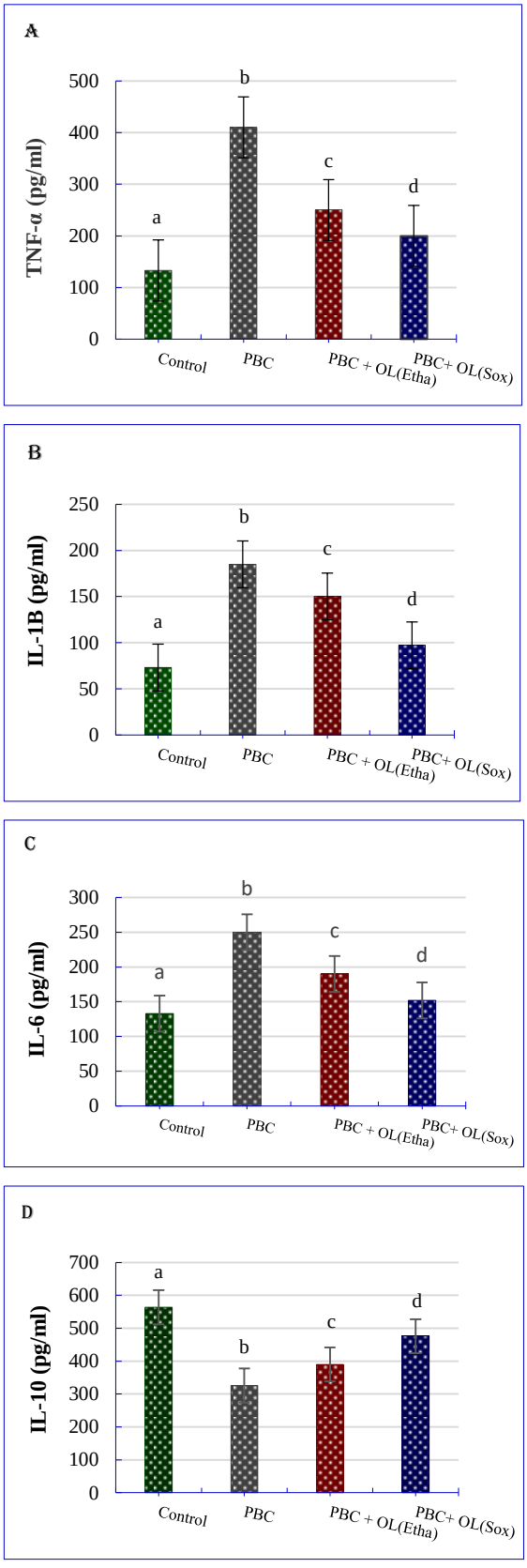

Figure (2A-D): Effects of olive l leaf extracts on serum-level in rats with induced primary biliary cholangitis (PBC). The parameters include TNF-α, IL-1, IL-6 and IL-10. Treatment groups: control, PBC, PBC treated with olive leaf extract using ethanol extraction (PBC + OL(Etha)), and PBC treated with olive leaf extract using Soxhlet extraction (PBC + OL(Sox)). Columns with different superscript letters indicate significant differences ($p \leq 0.05$, based on Duncan's Multiple Range Test, n = 10 per group).

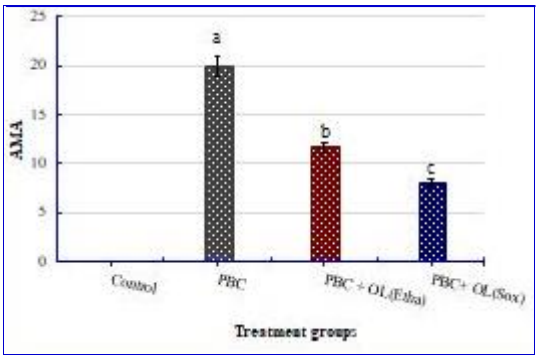

Figure (3): Effects of olive l leaf extracts on serum level of antimitochondrial antibodies M2 (AMA-M2) in rats with induced primary biliary cholangitis (PBC). Treatment groups: control, PBC, PBC treated with olive leaf extract using ethanol extraction (PBC + OL(Etha)), and PBC treated with olive leaf extract using Soxhlet extraction (PBC + OL(Sox)). Columns with different superscript letters indicate significant differences ($p \leq 0.05$, based on Duncan's Multiple Range Test, n = 10 per group).

for TGF-β and β-actin Table (1). Gene expression analysis of TGF-β by PCR was done to estimate the effect of different treatments on TGF-β, ANIT treatment increased TGF-β mRNA (Fig. 5), the effect was avoided by administering olive leave extract either extracted by ethanol or simple green method of extraction, on the other hand, comparing these results emphasized the efficacy of simple green method of extraction on improving TGF-β gene expression. In addition, the use of different olive leaf extracts against cholestasis and cholangitis showed TGF-β expression (lane 1, control group) was reflect by a faint or nearly absent TGF-β band, indicating baseline expression. However, PBC group (lane 2) presented by a strong TGF-β band, indicating upregulation of this profibrotic marker in primary biliary cholangitis induced in rats. Meanwhile, Lane 3 (PBC + Ethanolic extract) was represented by a reduction in TGF-β expression compared to Lane 2, showing a potential beneficial effect of the ethanolic extract. In the same pattern but with less effect, PBC + Soxhlet Extract (lane 4) showed reduced TGF-β expression.

β-Actin bands (as loading control, Fig. 5) showed a similar intensity across all lanes, confirming equal protein loading and normalization reliability. These results prove the therapeutic effect of both olive leaf extracts in downregulate TGF-β expression with proposing that ethanolic extract appearing more effective than the Soxhlet extract in reducing fibrotic marker levels.

DISCUSSION

The chronic liver disease represents a general health problem worldwide. Female Albino rats were chosen for this study, as PBC is more in female sex. Despite recent treatment advancements, many liver illnesses continue to worsen because specialised medications to address the underlying aetiologies of the liver disorders are lacking. As a result, until more effective therapeutic and anti-fibrotic medicines are produced, the need for liver transplantation is projected to rise (Rajapaksha *et al.*, 2019).

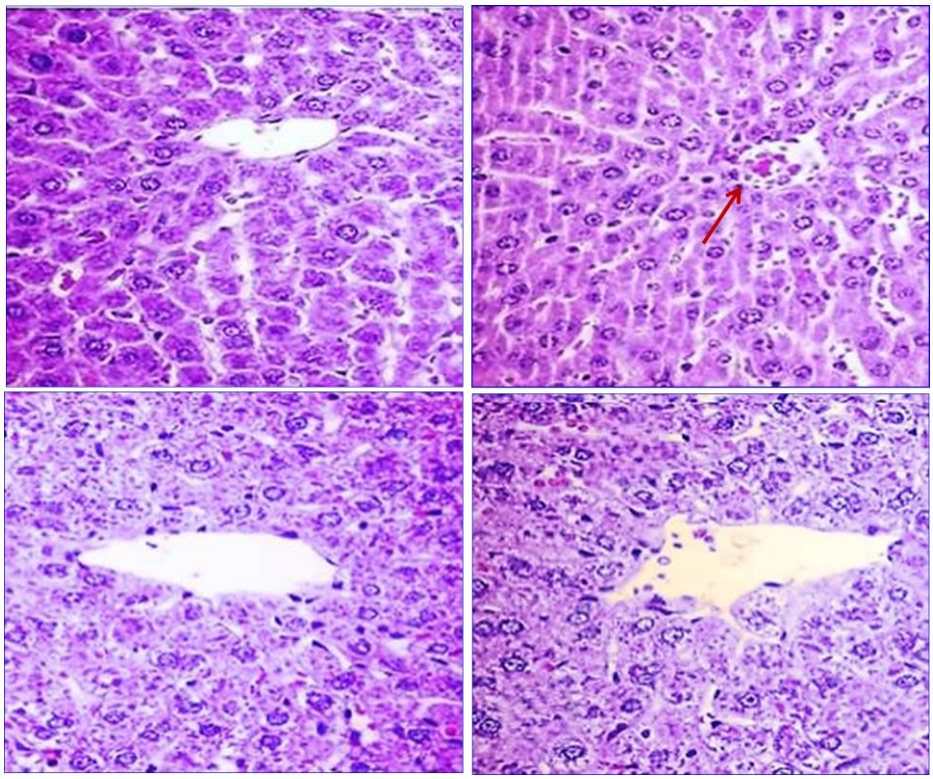

Figure (4): Photomicrographs of liver tissues (A) Normal control group: shows normal architecture of the liver sections, no infiltration of bleeding; (B) Primary biliary cholangitis (PBC): shows focal hepatic haemorrhage (arrow), hepatocytes degeneration, leukocyte infiltration appeared a vacuolated hepatocytes appears in focal zone, unequal lipid precipitations and alteration in the shape of nucleus (C) PBC + OL(Etha): Shows mild leucocyte infiltration, and reduced amounts of focal inflammatory cell structure appear slightly normal (D) PBC+ OL(Sox): Shows reduced inflammatory cell counts reduced hemorrhage, cell structure appear slightly normal.

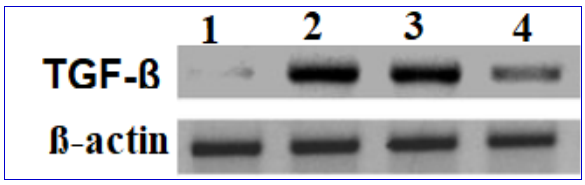

Figure (5): Gene expression analysis of TGF-β in a cholestasis and cholangitis induced in rats. Treatment groups: lane 1, control; lane 2, PBC; lane 3, PBC treated with olive leaf extract using ethanol extraction (PBC + OL(Etha)), and lane 4, PBC treated with olive leaf extract using Soxhlet extraction (PBC + OL(Sox)).

In this study two types of extraction methods have been chosen: the simple extraction (maceration) method with 80% Ethanol (Eth) and the Soxhlet extraction method using 20% acetonitrile as a solvent. A previous study by Yateem *et al* (2014) found that using pure solvents (100% ethanol and 100% acetonitrile) was inaccurate in obtaining the maximum phenolic compounds of olive leaves. While using them as (80% ethanol and 20% acetonitrile) will give high oleuropein content. Meanwhile, Soxhlet extraction produced greater oleuropein content than maceration method. The Soxhlet extraction is an automatic continuous extraction method with high extraction efficiency that require less time solvent consumption than maceration or percolation. The high temperature and long extraction time in the Soxhlet extraction will increase the possibilities of thermal degradation. However, maceration procedure has certain limitations such as low extraction yield, lower efficiency and use of large amount of solvents which have some health hazards. Furthermore, the selection of appropriate solvent is important along the methodology for the extraction of particular plant extract (Mutlag *et al.*, 2020; Ramazan *et al*., 2024).

Alpha-naphthyl-isothiocyanate ANIT was used to stimulate cholangitis and cholestasis as it is a frequently applied chemical toxin to stimulate hepatic cholestasis by damaging biliary epithelial cells (Hua *et al.*, 2020).The present study revealed that ANIT administration-induced cholangitis and cholestasis are characterized by disturbed biomarkers of liver injury, including serum levels of ALT, AST and biomarkers showed the grade of bile cell injuries and cholestasis, including serum levels of GGT, DBIL, TBIL, bile acids concentrations as well as serum Antimitochondrial Antibodies AMA, accompanied by disturbing markers of liver inflammation (TNF-α, IL-1β, IL-6 and IL-10), indicating that ANIT stimulated intrahepatic chol-

angitis and cholestasis in rats . Similar to this study's results, previous study by Palmer *et al* (2019) proved that PBC is correlated with a cholestatic pattern of elevated serum activities of ALP, GGT, or both and the presence of circulating antimitochondrial antibodies. ANIT-induced biliary epithelial cells due to bile acid transporters shift as it binds to glutathione (GSH) in hepatocytes. ANIT is subsequently dissociated from glutathione in bile, and the liberated ANIT preferentially destroys biliary epithelial cells, producing cholangitis and, as the condition progresses, intrahepatic cholestasis (Yao *et al*., 2018).Inflammatory cytokines (IL-6, TNF-α , IL-1β and IL-10) were evaluated in different experimental rat groups, and their highest levels were reached following ANIT administration, whereas IL-10 decreased, indicating inflammation and fibrosis in the liver of ANIT animals. ANIT-evoked hepatotoxins, which cause severe neutrophilic inflammation around portal tracts and bile ducts, thus induced significant inflammatory reactions that lead to cholestatic hepatitis (Tresserra-Rimbau, 2020). Similar results were obtained by Onofrio and Hirschfield (2020).

Anti-mitochondrial autoantibodies M2 AMA-M2 results were considered negative in the control rat group, while it was positive in the other groups. The M2 antigen was found to be highly specific to PBC also it is the characteristic PBC antibody due to its presence in 90-95% of diagnosed patients (Sun *et al*., 2019). TGF-β is the main cytokine implied in liver fibrogenesis. Moreover, it is directly involved in different stages of liver disease, from initial liver injury to fibrosis, cirrhosis and cancer (Tang *et al*., 2021).

Study results showed that supplementing rats with olive leaves extract restored body weight in ANIT supplemented group. Meanwhile, one study by (Lodyga and Hinz, 2020) demonstrated the effect of olive leaves extract on managing body weight gain of obese rats. Furthermore, it has been observed that these polyphenols can suppress the synthesis of eicosanoid compounds and lipoxygenases, as well as decrease platelet aggregation and low-density lipoprotein oxidation (Kashaninejad *et al*., 2020).

The olive leaves's main constituent is oleuropein, which is responsible for pharmacological effects. Da Silva *et al* (2021) stated that polyphenols content in the olive leaves is of higher concentration than in olive fruit or olive oil. Another work by (Elnahas *et al*., 2021) showed that treatment with olive leaves extract significantly improved the activities of hepatic markers. Similarly, a study by (Acar-Tek and Ağagündüz, 2020).showed that treatment with olive leaves extracts reduced elevated liver enzymes levels. In addition, using olive leaves extract in treatment to regulate bile inflammation and liver injury in obstructive jaundiced rats. The olive leaves total extract (OLE) and its derived compounds, mainly oleuropein and hydroxytyrosol, have been tested in several in vitro and in vivo systems showing. They can reduce inflammation and oxidative species production in experimental models of gastric and intestinal diseases, they also exert beneficial effects in metabolic syndrome, atherosclerosis and cardiovascular diseases by suppressing the inflammatory response, reducing lipid peroxidation and attenuating hypertension (Silvestrini et al., 2023).

The study showed that olive leaves extract reduced serum elevated cholesterol levels and controlled cholestasis. Concerning the effects of polyphenols of the olive leaves on hypocholesterolemia, Vogel *et al* (2015) evaluated the effects of administering oleuropein and hydroxytyrosol in diabetic mice at doses 16 and 8 mg/kg of bw. It presented a reduction in cholesterol levels in diabetic rats that received oleuropein and hydroxytyrosol compared with the untreated rat group.

Additionally, the study found that administration of olive leaves extract restores serum levels of inflamematory indices (TNF-α, IL-6, IL-10 and IL-1β). Furthermore, in the oleuropein-supplemented group, both IL-6 and IL-1 were reduced. IL-6 is a pleiotropic cytokine that regulates cellular development, proliferation, and apoptosis. Recent research has discovered various roles for cytokines in chronic inflammation, including interleukin-6 (IL-6), which has been extensively studied. In recent decades, IL-6 has been related to a variety of diseases, including rheumatoid arthritis (RA), diabetes, cancer, and multiple sclerosis (MS), and its overall concentration is higher in acute and chronic inflammation, making it a prominent driver of autoimmune disorders (Gupta *et al*., 2023). Transforming growth factor TGF-β is a key cytokine involved in the pathogenesis of fibrosis in many organs, whereas interleukin IL-6 plays an important role in the regulation of inflammation. Recent reports demonstrate interaction between the two cytokines in disease states (Zhang *et al*., 2005). Olive polyphenols have been found a preventative activity concerning inflammation by inhibiting the Toll-like receptor signalling pathway. In line with previous research in various experimental designs, Badr and Fouad (2016) showed the ability of olive leaves extract to suppress the TNF-α pathway demonstrating its anti-inflammatory activity in rats' livers intoxicated by cisplatin. By increasing antioxidant activity and reducing inflammation and apoptosis, olive leaves extract significantly protects rats against cisplatin-induced nephrotoxicity, according to a different study by ALHaithloul et *al* (2019). Similarly, Kaneko *et al* (2019) reported that olive leaves extract therapy suppressed inflammatory cytokine release in human placental tissue culture. Furthermore, Omagari *et al* (2021) proposed that olive leaves extract may increase serum adiponectin concentration, decreasing liver inflammation and fibrosisdue to its content of luteolin. It is considered that olive leaves possess the highest antioxidant and anti-inflammatory activity amongst the different parts of the olive tree due to their high content in bioactive phenolic compounds. These relevant properties may be due to their ability to chelate metal ions that catalyze free radical generation reactions, as well as their ability to inhibit many inflammatory

enzymes, such as lipoxygenases. Moreover, it has been observed that olive-leaves extracts are able to attenuate inflammation, and this positive effect has been associated with a modulation of the altered immune response due to its antioxidant capacity (Silvan *et al*., 2021).

In this investigation, olive oil extract decreased TGF-β mRNA levels and inhibited its expression, avoiding liver fibrosis and cholangitis caused by ANIT (Soliman *et al*.,2019). Meanwhile, Alteration in hepatic gene expression in response to histological intensity may reveal a strong link involving liver damage and bile acid metabolism regulation (Lockyer *et al.*,2017). Results obtained in the study showed the degenerative effect of ANIT represented as hepatocytes degeneration, leukocyte infiltration as compared to control group.

On the other hand, treatment with olive leaf extracts obtained using two different extraction methods showed significant improvement in tissue histology. This effect is attributed to their polyphenol content, which suppresses inflammation through antioxidant pathways and the regulation of pro-inflammatory processes (Silvestrini et al., 2023). PBC pathology is primarily localized to the intrahepatic small and medium-sized bile ducts. Over time, the intrahepatic small bile ducts progressively deteriorate, leading to the disappearance of intrahepatic bile channels and the development of chronic cholestatic features (Beretta-Piccoli et al., 2017).

Damage of interlobular and septal bile ducts is the hallmark histological lesion of primary biliary cirrhosis (PBC). The damaged ducts are approximately inflamed and engulfed by a mononuclear cell infiltrate that includes lymphocytes and plasma cells and may have granulomatous characteristics. Lymphocytes and varied quantities of plasma cells accompany the granulomatous inflammation, which is localised to the bile duct and can be well-formed or poorly formed. Even when all stages are considered, florid duct lesions are present in less than 40% of PBC biopsy samples due to their localised nature (Martini *et al*.,2023).

CONCLUSION

The findings of this investigation showed that olive leaves extract, either extracted by simple extraction (maceration) method with ethanol or Soxhlet extraction method, had a possible beneficial influence on ANIT - induced cholangitis in rats, that established as a decrease of biomarkers of cholangitis and cholestasis and improvement of liver histological morphology via antioxidant pathways and regulating the pro-inflammatory processes. The study also showed that using the Soxhlet method would be more potent in extracting active components of olive leaves. Additional future studies should explore the molecular mechanisms in greater detail, test different extracts dosages and combinations between different extract types, also long-term studies should be conducted to assess chronic effects and safety.

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