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Research Article
Analysis of Cytochrome c Expression on Liver Histology of Hepatitis Rats after Administration of Tin and Olive Leaf Ethanol Extract

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Abstract

Background and Objective: Hepatitis is a liver illness caused by a viral infection, autoimmune conditions or the use of certain medicines. In molecular hepatitis treatment, cytochrome c can be used as a potential predictor of the severity of liver impairment. In Asia, particularly in Indonesia, antioxidant-rich plants include *Ficus carica* and *Olea europaea.* This study aimed to see what impact cytochrome c in hepatitis after these two botanicals were administered. **Materials and Methods:** Rats were grouped as follows: Normal rats with no additions or herbs (G_0), the physiological solution group (G_1), the intravenous administration of the quercetin-copper (II) (G_2), Olive leaf extract or OLE (300 mg kg^{-1} b.wt.) (G_3) and Tin leaf extract or TLE (100 mg kg^{-1} b.wt.) (G_4). For an animal model of hepatitis, the rats were given thioacetamide 280 mg kg^{-1} b.wt., 8 days later. The rats were dissected and blood and liver samples were collected for enzyme and immunohistochemistry examination. **Results:** Malondialdehyde (MDA), superoxide dismutase (SOD) and cytochrome c expression levels differed significantly ($p<0.05$) across treatment groups in rat's models of hepatitis. Hepatocytes first displayed symptoms of lipid degradation, inflammatory and necrosis cells. When administered quercetin and the two herbs, necrosis and inflammatory cells were reduced, demonstrating that OLE and TLE can enhance liver histology and lower cytochrome c expression in a mouse model of hepatitis. **Conclusion:** Administration of Olive leaf extract (OLE) and Tin leaf extract (TLE) can improve liver histology in hepatitis model rats while decreasing cytochrome c expression, which is a mechanism for hepatocyte cell death.

Key words: *Ficus carica*, hepatocytes, hepatitis, immunohistochemistry, liver, cytochrome c, *Olea europaea*

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Hepatitis is a liver illness caused by a viral infection, drinking alcohol, toxic substances, autoimmune illnesses or specific medicines are common causes of this condition[1]. Hepatitis can be transmitted faecal-oral and then spread through the digestive system through food or drinks infected with hepatitis faeces[2]. Furthermore, getting tainted blood or blood products as well as invasive medical treatments employing contaminated equipment[1,2]. Liver function tests are required to monitor the amounts of proteins or enzymes in the bloodstream, which can suggest liver disease[3]. A liver biopsy is also required to evaluate the aetiology of liver tissue destruction[4].

Apoptosis, the process of cellular self-destruction characterised by nucleus fragmentation and organised cells, is known to cause a variety of disorders, including liver ailments such as hepatitis[5]. Cytochrome c is produced from mitochondria during stress-induced apoptosis and binds to Apoptotic Protease-Activating Factor-1 (Apaf-1), triggering caspase-9[5,6]. The death process is then accelerated by cytochrome c activating downstream caspases[7]. This role of mitochondria in cytochrome c release causes the mitochondrial membrane to become more permeable, a process known as the mitochondrial permeability transition (MPT), which opens the high conductance membrane in the channel, known as the permeability transition pore (PTP)[8]. Serum cytochrome c may be a potential predictor of the severity of the liver injury[9]. Molecular hepatitis treatment must be improved further, both chemically and herbally.

Ficus carica (FC) or Tin plant is a traditional medicine that grows in tropical or subtropical areas, particularly in the Middle East and Asia[9]. The FC leaves have a low toxicity and anti-inflammatory, antioxidant, renoprotective and hepatoprotective properties[10]. The FC leaves contain 126 phytoconstituents, mostly polyphenol compounds, making them more antioxidant-rich than red wine and tea. Scientific studies on the active ingredients have revealed significant hypoglycemic, antioxidant and anti-inflammatory effects, supporting the anti-diabetic benefit of FC leaves[9-11].

Olive (*Olea europaea*) is a plant that grows to a maximum height of 15 m and has a single silvery green leaf. Furthermore, olives are well-known for their health benefits. When the Olive leaf is extracted, it provides additional benefits[12]. Flavonoids, secoiridoids, iridoids, flavanones, biophenols, triterpenes, benzoic acid derivatives, isochromes and other secondary metabolites are found in the leaves of this plant[13]. This plant's leaves have antidiabetic, anticonvulsant, antioxidant, anti-inflammatory, immunomodulatory, analgesic, antibacterial, antiviral, antihypertensive, anticancer and antihyperglycemic properties as well as antinociceptive, gastroprotective and wound healing properties[12-14]. The findings of phytochemical and pharmacological investigations broaden this herb's medicinal potential and give compelling evidence for future clinical usage in modern medicine.

This study aimed to look at the expression of cytochrome c in the liver histology of hepatitis rats after administration of extract (OLE) and Tin leaf extract (TLE) extract. This research can help us establish whether cytochrome c is a new possible marker for hepatitis. This study is likely to lead to the development of a medication candidate for future hepatitis.

MATERIALS AND METHODS

Study area: The research project was conducted from July to December, 2021. The research was carried out in the Animal House in the Department of Biology, Faculty of Mathematics and Natural Sciences, University of Sumatera Utara, Medan, Indonesia.

Preparation of Tin ethanol extract (TLE) and Olive leaf ethanol extract (OLE): A thick extract of Tin or Olive leaf Simplicia, 200 g of simplicia leaf powder and 2 L of 96% ethanol were combined in a macerator. Soaked for 6 hrs, stirring occasionally, before letting it stand for 18 hrs. The macerate was separated by filtration and the extraction process was performed again with the same solvent type and amount. All of the macerates were collected and evaporated with a vacuum or low-pressure vaporizer until a thick extract was obtained. The resulting yields were weighed and recorded[15].

Phytochemical test:

- **Tannins:** About 0.5 g of sample was placed in a beaker, followed by 20 mL of distilled water, which was then heated and filtered. Following that, 0.5 mL of the filtrate was treated with 0.1% ferric chloride and the colour change was noticed
- **Saponins:** A total of 2 g of the sample are placed in a beaker, followed by 20 mL of distilled water, which is then heated to a boil and filtered. Shake briskly 10 mL of the filtrate with 5 mL of distilled water until foam forms. Then, add 3 drops of Olive oil to the foam and shake it again to see whether an emulsion forms

- **Flavonoids:** A total of 2 g of the sample was placed in a beaker and mixed with 20 mL of distilled water before being heated and filtered. 0.5 mL of the filtrate was then mixed with 5 mL of dilute ammonia and 5 mL of concentrated sulfuric acid and observed
- **Steroids and triterpenoids:** About 2 mL of the test solution was evaporated, the residue was diluted in 0.5 mL of chloroform and 0.5 mL of anhydrous acetic acid was added. Add 2 mL of sulfuric acid through the tube wall. The creation of a blue-green ring suggests the presence of steroids and rings[16]

Animal handling: The white rat was employed in the experiment (*Rattus norvegicus*). The animals were housed four in a cage and kept at a regulated room temperature and humidity, with a 12:12 hrs chronobiological schedule for the light cycle:dark cycle. All animals were fed rats and water as needed. The study was carried out after obtaining an ethical clearance letter for experimental animal research (Number: 0050/KEPH-FMIPA/2022).

Research design: Twenty-five healthy male rats (8-11 weeks) weighing 200-250 g were used. Five treatment groups were assigned at random. Thioacetamide (280 mg kg^{-1} b.wt.) was given intravenously to hepatitis model rats. Rats were grouped as follows:

G_0 : Healthy rats with no added additives or herbs
G_1 : Group that received physiological solutions
G_2 : Group that received an intravenous injection of a quercetin-copper (II) complex compound at a dose of 0.0125 mmol kg^{-1} (7 mg kg^{-1} b.wt.)[17]
G_3 : Olive leaf extract (300 mg kg^{-1} b.wt.)[18]
G_4 : Tin leaf extract (100 mg kg^{-1} b.wt.)[19]

At the designated time points, all rats were terminated with a 280 mg kg^{-1} thioacetamide pentobarbital injection 8 days later[20]. After 24 hrs, blood was taken from the heart with a 1 mL syringe after being anticoagulated with heparin and centrifuged for 20 min at 8000 g at 4°C. Plasma samples were kept at -20°C until they were tested for liver enzymes in serum. Within 2-4 min, the liver is removed, cleaned with cold saline, dissected and weighed. For biochemical examination, liver samples were immediately frozen in liquid nitrogen and stored at -80°C until use[15].

Serum enzyme tests: Serum levels of Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) are indicators of liver function problems. Measured in units per litre (IU L^{-1})[21].

Extract extraction and antioxidant enzyme tests on liver cells: Using an Ultra-Turrax homogenizer, frozen liver samples were homogenized in ice-cold saline (1:10, w/v). The total activity of superoxide dismutase (SOD) was determined indirectly in the liver of rats using a spectrophotometric technique.

Hematoxylin-eosin (H&E) staining: The formalin-fixed liver was taken from the storage room and put in xylol for 15 min. Following that, the tissues were alternately soaked in 96 and 70% pure alcohol for 5 min before being washed in distilled water. After 5 min of haematoxylin dye application, the tissues were washed in distilled water for 3 min. For 1 min, eosin dye was applied. The slides were dehydrated in 70, 96 and 100% alcohol before being immersed in xylol and placed on a cover glass. A light microscope with a 5x field of view was used to examine the sample[15].

Immunohistochemistry: Before immunohistochemistry, 5 m thick paraffin-embedded placental slices were deparaffinized and treated with 1% H_2O_2 in methanol for 30 min to inhibit endogenous peroxidase activity. The slides were then washed in 0.01 M tris-buffered saline (TBS pH 7.4). After blocking non-specific binding to 1% skimmed milk in PBS, tissue slices were treated for 2 hrs with primary cytochrome c antibody (1:100 dilution in PBS containing 1% BSA). The vectastain elite ABC kit (Vector Laboratories, Burlingame, CA, USA) was used to identify immunoreactivity, which was counterstained with Mayer's haematoxylin[22].

Data analysis: Data is gathered and processed to reinforce the conclusions drawn from the research findings. The univariate analysis includes normality tests, data homogeneity and data descriptions such as mean and standard deviation. Then there's bivariate analysis, such as the test of differences between treatment groups with One-way ANOVA for numerical data with normal and homogeneous distribution or the Kruskal-Wallis test for categorical data (ordinal) or numerical data but not regularly distributed[22].

RESULTS

Phytochemical screening of Olive leaf extract (OLE) and Tin leaf extract (TLE): According to the findings of research on both extracts (Table 1) given to rats triggered by hepatitis, TLE does not include terpenoids and OLE does not contain Tannis. However, because these two plants contain phenol and flavonoids, they are antioxidants.

AST and ALT values after administration of Olive leaf extract (OLE) and Tin leaf extract (TLE)) in hepatitis model rats: There were significant differences between the treatment groups based on the measurement of AST values in the hepatitis rat model ($p<0.05$). The G1 group had the highest AST levels, followed by the G_0 group. G_2 had lower AST levels than all other medications. There was a significant difference between the therapy groups ($p<0.05$) in the ALT group (Table 2). It is known that both the G_0 and G_1 groups had normal ALT levels. The G_2 had lower AST levels than all other medications. The ALT increased when OLE and TLE were administered.

Value of superoxide dismutase (SOD) and malondialdehyde (MDA) after administration of Olive leaf extract (OLE) and Tin leaf extract (TLE)) in hepatitis model rats: The SOD data were acquired on day 8 after the rats were slaughtered in a trial in which TLE and OLE were administered to rats given hepatitis-causing chemicals that can damage the liver (Table 3). In the rat model of hepatitis, there was a significant difference between the treatment groups ($p<0.05$) in the SOD group. The G_0 group was found to have the lowest SOD levels, followed by the G_1 group. The SOD levels in group G_2 were higher than in all other treatments. The SOD levels began to fall after being given OLE and TLE.

The MDA results were acquired on day 8 after the rats were dissected in a trial in which TLE and OLE were administered to rats treated with hepatitis-causing drugs that can damage the liver (Table 4). In the rat model of hepatitis, there was a significant difference between the treatment groups ($p<0.05$) in the MDA group. The G_0 group is known to have lower MDA levels than the G_1 group. The MDA increased when quercetin (G_2) was administered. The highest MDA level was discovered in TLE extract made from the Tin plant. Based on these findings, it is known that TLE and OLE treatment can raise MDA levels.

Histological description of the liver after administration of Olive leaf extract (OLE) and Tin leaf extract (TLE)): Data on liver histology were acquired based on a study conducted by administering TLE and OLE to rats treated with hepatitis-causing drugs that can harm the liver (Fig. 1). The liver histology of the hepatitis rat model showed a significant difference between treatment groups ($p<0.05$). The G_0 group is known to have a higher level of cell inflammation and necrosis than the G_1 group. The G_2 group as well as the G_3 and G_4 groups, saw a decrease in inflammatory and necrotic cells. Based on these findings, it is known that TLE and OLE treatment can lower the number of inflammatory cells and necrotic liver cells.

The microscopic description of liver histology revealed that group G_0 (Fig. 2a) had hydropic degenerated hepatocytes with vacuolated cytoplasm, inflammatory areas marked with

Table 1: Phytochemical profile of Tin and Olive leaves

Secondary metabolites	Tin leaves	Olive leaf
Alkaloids	+	+
Terpenoids	-	+
Phenol	+	+
Flavonoids	+	+
Lipophilic	+	+
Tannins	+	-

Table 2: AST and ALT values after administration of TLE (Tin leaf extract) and OLE (Olive leaf ethanol extract) for 8 days

Groups	AST (IU L^{-1})	ALT (IU L^{-1})
G_0	224.3 ± 6.5^{c}	329.9 ± 17.2^{c}
G_1	233.7 ± 10.9^{d}	336.0 ± 21.4^{c}
G_2	27.3 ± 3.8^{a}	32.1 ± 2.2^{a}
G_3	58.8 ± 3.4^{b}	69.3 ± 2.7^{b}
G_4	54.7 ± 2.7^{b}	64.7 ± 4.5^{b}

G_0: Without material administration, G_1: Physiological solution, G_2: Quercetin, G_3: Olive leaf extract (300 mg kg^{-1} b.wt.) G_4: Tin leaf extract (100 mg kg^{-1} b.wt.) and different small superscripted alphabets indicate statistically significant differences ($p<0.05$) using the One-way test ANOVA

Table 3: SOD values of rats after administration of TLE and OLE for 8 days

Groups	SOD (ng mL^{-1})
G_0	1.2544 ± 0.1113^{a}
G_1	1.3849 ± 0.1185^{a}
G_2	1.8975 ± 0.0613^{c}
G_3	1.5731 ± 0.0574^{b}
G_4	1.6089 ± 0.1847^{b}

G_0: Without material administration, G_1: Physiological solution, G_2: Quercetin, G_3: Olive leaf extract (300 mg kg^{-1} b.wt.) G_4: Tin leaf extract (100 mg kg^{-1} b.wt.) and different small superscripted alphabets indicate statistically significant differences ($p<0.05$) using the One-way test ANOVA

Table 4: MDA value after administration of TLE and OLE for 8 days

Groups	MDA (μg mL^{-1})
G_0	0.8233 ± 0.0722^{a}
G_1	0.9203 ± 0.0332^{a}
G_2	0.1549 ± 0.0450^{b}
G_3	0.2372 ± 0.1411^{c}
G_4	0.1847 ± 0.0302^{c}

G_0: Without material administration, G_1: Physiological solution, G_2: Quercetin, G_3: Olive leaf extract (300 mg kg^{-1} b.wt.), G_4: Tin leaf extract (100 mg kg^{-1} b.wt.) and different small superscripted alphabets indicate statistically significant differences ($p<0.05$) using the One-way test ANOVA

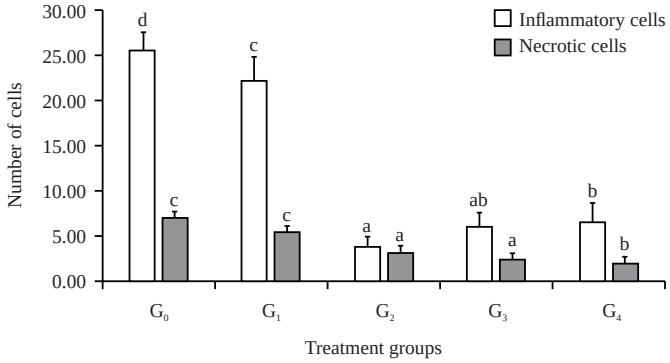

Fig. 1: Number of inflammatory cells and necrosis after administration of TLE and OLE

[a,b,c,d]Different small alphabets indicate statistically significant differences $p<0.05$, G_0: Without material administration, G_1: Physiological solution, G_2: Quercetin, G_3: Olive leaf extract (300 mg kg^{-1} b.wt.) and G_4: Tin leaf extract (100 mg kg^{-1} b.wt.)

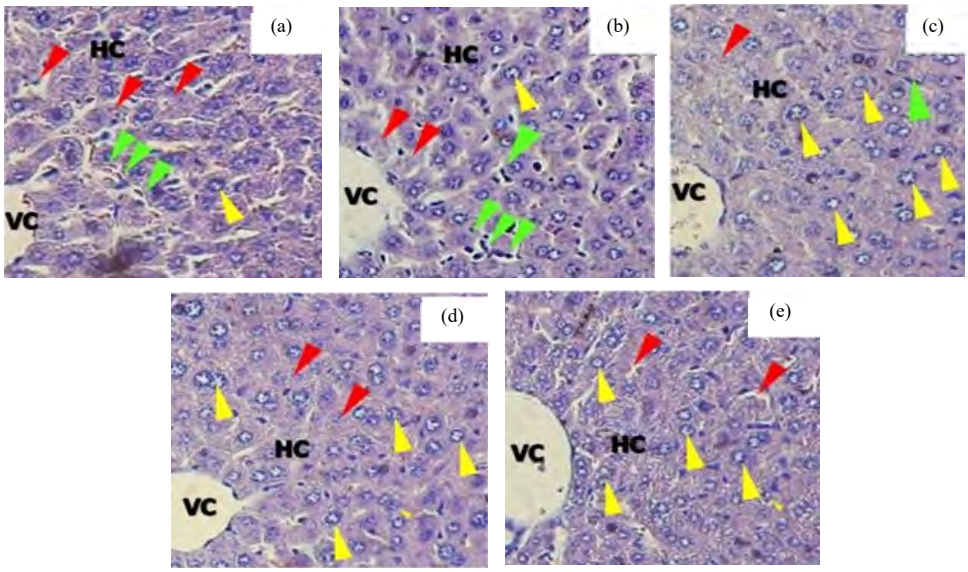

Fig. 2(a-e): Histological features of rats after administration of TLE and OLE, (a) Without material administration (G_0), (b) Physiological solution (G_1), (c) Quercetin (G_2), (d) Olive leaf extract (300 mg kg^{-1} b.wt.) (G_3) and (e) Tin leaf extract (100 mg kg^{-1} b.wt.) (G_4)

Red arrows: Necrosis cells, Yellow arrows: Normal hepatocyte cells, Green arrows: Inflammatory cells, VC: Central vein, HC: Hepatocyte cells, magnification 10×40 and staining of HE

green arrows and a large number of necrotic cells, this also occurred in group G_1 (Fig. 2b) but the hepatocytes in this group began to show signs of lipid degeneration. When group G_2 was given quercetin (Fig. 2c), necrosis and inflammatory cells diminished and the histological image was nearly identical to when TLE (Fig. 2d) and OLE (Fig. 2e) were administered. According to the histology picture, TLE and OLE can improve liver histology in hepatitis model rats.

Cytochrome c analysis after administration of Olive leaf extract (OLE) and Tin leaf extract (TLE)) in hepatitis model rats: TLE and OLE treatment showed a significant difference ($p<0.05$) in rats treated with hepatitis-causing chemicals that can harm the liver in a non-invasive test, The Kruskal-Wallis parametric test and the Mann-Whitney follow-up test (Table 5). The mean rank value calculated with SPSS software demonstrates that G_0 has the highest cytochrome c expression, whereas, G_3 (TLE) and G_4 have the lowest (OLE). The nucleus shown with red arrows was an expression of cytochrome c and the yellow colour indicated that the hepatocytes were normal, according to histology of liver tissue stained with cytochrome c antibody. Statistical analysis and histology were used. The nucleus in the G_0 group showed the highest cytochrome c expression (Fig. 3a), which thereafter

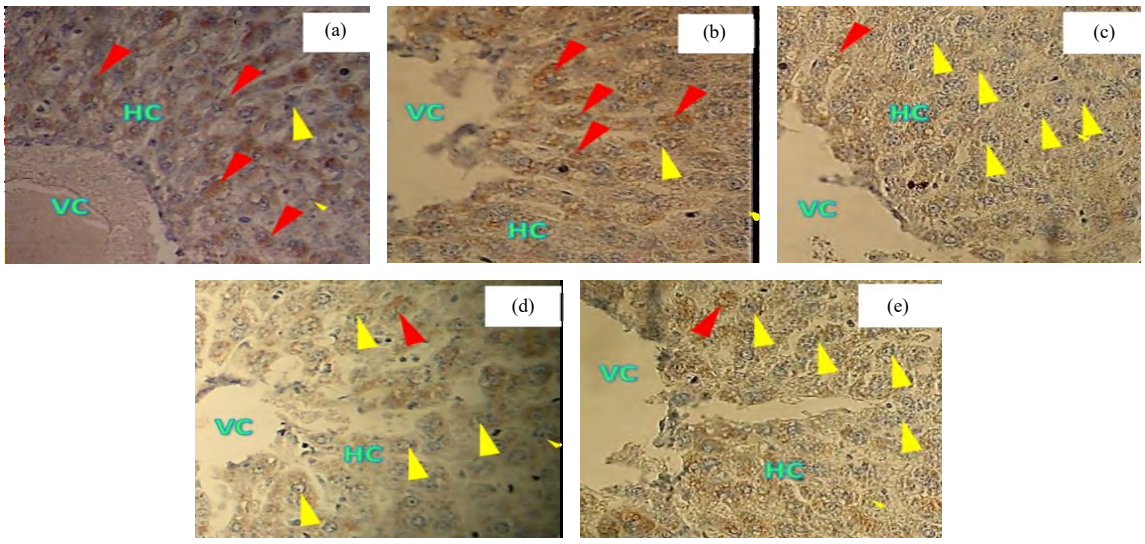

Fig. 3(a-e): Cytochrome c expression of rat's liver after administration of TLE and OLE, (a) Without material administration (G_0), (b) Physiological solution (G_1), (c) Quercetin (G_2) , (d) Olive leaf extract (300 mg kg^{-1} b. wt.) (G_3) and (e) Tin leaf extract (100 mg kg^{-1} b.wt.) (G_4)
Red arrow: Cytochrome expression c, yellow arrows: Normal cells, VC: Central vein, HC: Hepatocyte cells and magnification 10×40

Table 5: Kruskal Wallis and Mann-Whitney analysis for cytochrome c hepatocyte cells

			Mann-Whitney				
Groups	Mean rank	Kruskal-Wallis	G_0	G_1	G_2	G_3	G_4
G_0	21.80	0.003		0.151	0.013*	0.013*	0.008*
G_1	17.60				0.104	0.020*	0.011*
G_2	11.20					0.448	0.154
G_3	8.10						0.575
G_4	6.30						

G_0: Without material administration, G_1: Physiological solution, G_2: Quercetin, G_3: Olive leaf extract (300 mg kg^{-1} b.wt.), G_4: Tin leaf extract (100 mg kg^{-1} b.wt.) and *$p<0.05$ using Kruskal-Wallis test

reduced in the G_1 group (Fig. 3b). The cell damage, however, is not extensive and is still considered normal. When given quercetin as an antioxidant, cytochrome c expression dropped and normal hepatocytes were more visible (Fig. 3c). TLE (Fig. 3d) and OLE (Fig. 3e) administration resulted in nearly identical histologic findings as well as decreased cytochrome c expression.

DISCUSSION

Phytochemical experiments used to detect flavonoids in Tin (*Ficus carica*) leaves revealed that Tin (*Ficus carica*) leaves contained anthocyanins, flavones and bioflavonoids[9,10]. The flavonoid luteolin was found in an ethanol extract of Tin leaves in silico[11]. 1-Acetoxypinoresinol, Cis-2-Hexen-1-ol, Cis-3-Hexenyl acetate, Methyl nominate and Oleuroside is all chemicals found in Olives[9]. This was consistent with the findings of this study (Table 1), which found that the two leaves of this plant contain antioxidant-rich compounds such as positive containing alkaloids, phenols, flavonoids, lipophilic and tannins.

The liver is a vital and complicated organ that performs numerous activities, including fat metabolism, glycogen storage, body defence, red blood cell rebuilding and detoxification of human wastes, hormones, medications and other foreign chemicals[1,2]. Alanine transaminase (ALT) is an enzyme that transforms protein into energy for liver cells to use. Aspartate transaminase (AST) is an enzyme involved in amino acid metabolism[3]. The G_0 and G_1 showed elevated ALT levels. G_2 had lower AST and ALT values than all other treatments. When fig and Olive leaf extracts were administered, the two enzymes increased (Table 2). If the liver is not functioning properly, ALT and AST are released into the blood, causing ALT levels to rise.

The presence of lipid peroxidation and decreased levels of endogenous antioxidants as a result of elevated

malondialdehyde (MDA) levels can be utilized as indications of oxidative stress. MDA levels normally decrease when SOD levels rise[23]. The body has enzymatic and nonenzymatic systems to inactivate free radicals in reaction to free radicals, including superoxide dismutase (SOD), glutathione (GSH), catalase and other endogenous antioxidants[24]. The SOD found in OLE and TLE can lower levels of free radical scavenging enzymes like MDA (Table 3 and 4). Tissue damage is hypothesized to be caused by its action as an endogenous antioxidant that responds promptly when free radicals are present and can also stabilize membrane structures by removing acyl peroxides produced by lipid peroxidation[23,24]. Because of their strong antioxidant content, OLE and TLE can be turned into herbal medications.

When hepatocyte cells are damaged or injured due to a variety of reasons, they undergo a series of morphological changes that can be sublethal, namely degenerative or lethal in the form of necrotic (Fig. 1). The degeneration process is the starting point for hepatocyte destruction. The predominantly hydropic degeneration is assumed to be caused by an increase in free radicals in the liver tissue. Alterations in the characteristics of the cell membrane and cytoplasmic membrane are among the changes caused by free radicals (Fig. 2a-e). The lipid peroxidation damages biological components such as mitochondria and lysosomes toxic effects can reach the nucleus after disrupting the cell membrane, resulting in aberrant cell structure and, eventually, necrosis and apoptosis[5].

Cytochrome c causes apoptosis, which is characterized by an inflammatory process and is characterized by the presence of inflammatory cells on histology (Fig. 3a-e). Increased cytochrome c circulating levels (Table 5) in people with various liver illnesses as well as levels that correlate with the apoptotic index in the liver, imply that serum cytochrome c is derived from apoptotic cells[25]. However, further investigations to determine the mechanism of cytochrome c leakage from apoptotic cells are needed. Although cytochrome c is a new candidate, it is significant not only for liver damage but also for brain injury and prognosis[26]. The cytochrome c protein could be used to predict the severity of liver damage[25]. According to the findings of this investigation, cytochrome c expression is highly sensitive to liver injury and can be utilized to detect it[25]. Phytochemicals discovered in OLE include flavonoids, secoiridoids, iridoids, flavanones, biophenols, triterpenes, benzoic acid derivatives, isochromes and other forms of secondary metabolites. Because TLE includes phytoconstituents, including polyphenol components, it has more antioxidant activity than red wine and tea[9]. Scientific studies on the active ingredients have revealed significant hypoglycemic, antioxidant and anti-inflammatory properties[12]. Based on this data, OLE and TLE can be tested in the creation of medications rich in antioxidants, particularly in the liver.

CONCLUSION

Olive leaf extract (OLE) and Tin leaf extract (TLE) administration can enhance liver histology in hepatitis model rats and lower cytochrome c expression, which is a mechanism for hepatocyte cell apoptosis. The presence of OLE and TLE can also have a substantial effect on AST and ALT values as well as boost superoxide dismutase (SOD) in fighting free radicals by lowering MDA levels.

SIGNIFICANCE STATEMENT

This study discovers that Tin and Olive leaf ethanol extract can as herbal for liver damage problem therapy molecularly. This study will help the researcher to uncover the role of *Ficus carica* and *Olea europaea* in the molecular signalling of other target proteins for drug development in hepatitis. Thus, a new theory on the role of Tin and Olive leaf ethanol extract in the hepatitis animal model may be arrived at.

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