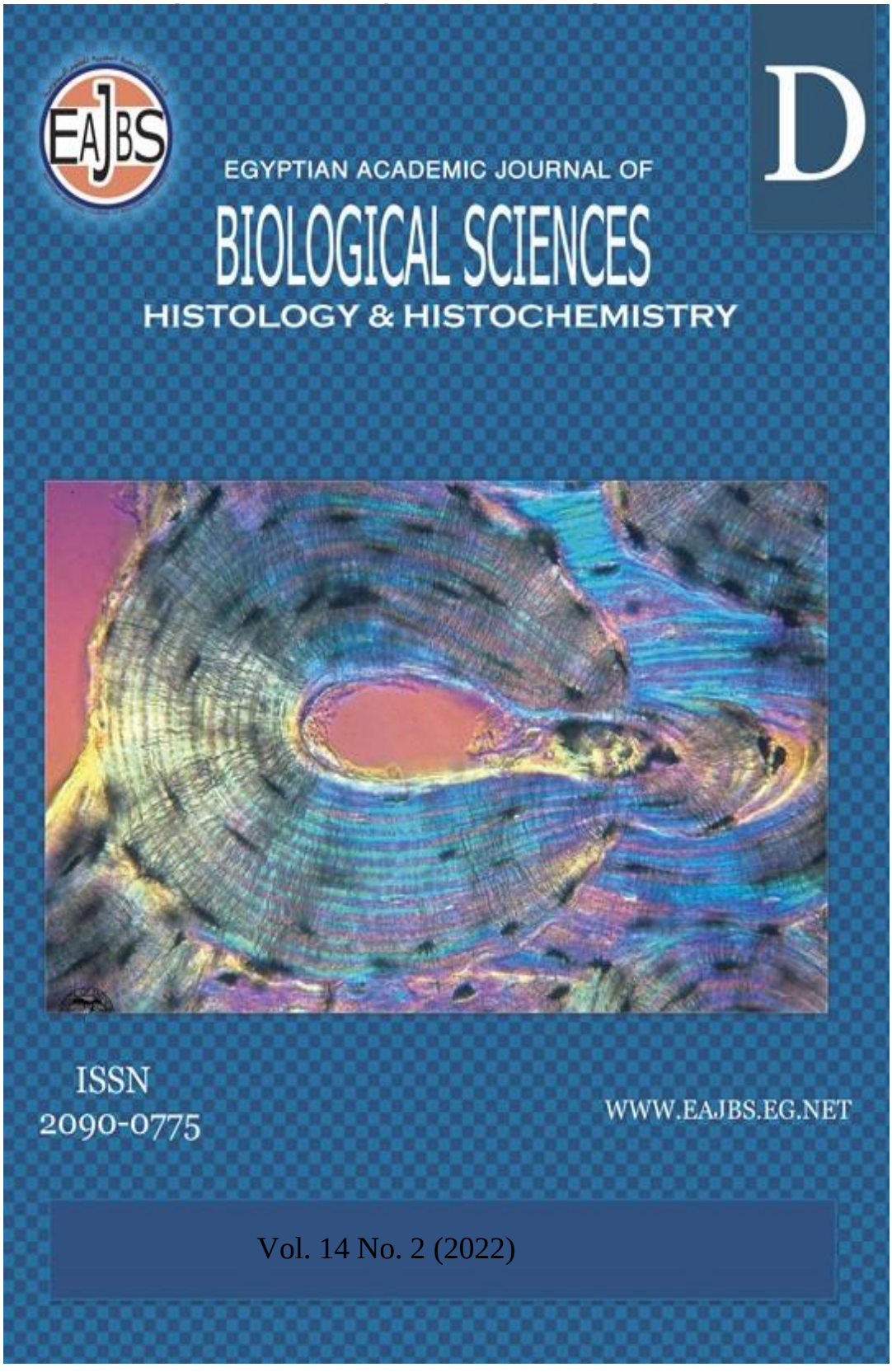

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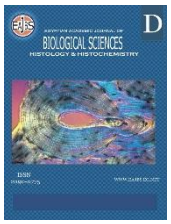

Biological Activities of Black Truffle (TerfeziaClaveryi) Against Dietary Acrylamide-Induced Toxicity in Rats Liver: Biochemical and Histopathological Study

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ABSTRACT

Background: Acrylamide (ACR) is a food toxin capable to produce severe harm to human health.ACR is a hydrosoluble monomer that affects several organs, particularly the liver. Variable antitoxic agents and natural products were suggested to counteract ACR-induced impairs. Black Truffles (BT) are rich in useful compounds and offer a valuable detoxifying agent. **Aim:** This study aims to identify the aptitude of BT extracts in comparison to Vitamin E to attenuate or overcome the induced toxic effect of ACR by investigating the antioxidants and lipid peroxidation in liver tissue homogenates. The coexistence of histopathological, CD4 and COX-2 immune staining was also targeted. **Methods:** The effects of BT at a dose of 400 mg/kg in an active aqueous and methanolic solvent were estimated in 50 healthy male albino Wistar rats intoxicated with ACR (0.11 mg/kg) in 30 % fried rice. The effects of BT extracts are compared to those of vitamin E (100gm/kg). The rats are classified into five groups (10 rats each); control (group I); ACR (group II), vitamin E (group III), BT water extract (group IV), and BT methanol extract group (V). In vitro estimation of vital metabolites in BT extract and their antioxidant activity were identified. In addition, liver cell function, cellular enzymatic and non-enzymatic antioxidant status, and liver histopathological and immunohistochemical changes were identified. **Results:** BT at a dose of 400 mg/kg in aqueous and methanolic extract significantly improved liver function, histology, and antioxidant status compared to vitamin E and ACR-treated rats. The results showed significant improvement in the levels of AST, ALT, bilirubin, albumin, and MDA, 8-Oxo-dG, SOD, CAT, TAC as antioxidant markers, and liver weight to body ratio in BT extract-treated rats. Liver sections of BT-treated groups (IV & V) showed an improved picture with minimal hepatocytes changes. CD4 and COX-2 immunohistochemical staining and area fraction showed decreased reactivity in groups (III, IV & V) with the changes markers significantly improved in BT-treated rats compared to intoxicated ACR ones. The results suggested that BT extracts significantly improved acrylamide liver toxicity via antioxidant and antiapoptotic pathways. **Conclusion:** BT extracts significantly improved ACR-induced liver toxicity on both biochemical, histopathological and immunohistochemical levels.

INTRODUCTION

Acrylamide (ACR) is a water-soluble vinyl monomer that is primarily used for the production of polymers. Polyacrylamides synthesized from ACR are commonly used in much personal care and grooming products like lotions, cosmetics and deodorants (Exon & Environmental Health, 2006). The use of ACR products in industries and molecular laboratories significantly adds to environmental pollution and health risks (Mannaa, AbdelWahhab, Ahmed, & Park, 2006).ACR was detected by chance in certain foods with higher concentrations (D'Agosto, Hughes, Charreyre, Pichot, & Gilbert, 2003; Kork, Yilmaz, & Yagci, 2015). Unfortunately, numerous factors of food processing like temperature, exposure time to high temperatures, and the amino acids and carbohydrates in the food (Blasiak, Gloc, Wozniak, & Czechowska, 2004; Konings *et al.*, 2003), all have an influence on ACR components formation.

Accordingly cooking and processing during the preparation of certain foods like potato and grain-base foods at high temperatures may lead to the formation of ACR monomers(Surdyk, Rosén, Andersson, Åman, & chemistry, 2004). Chemically, the interaction of amino acid asparagine with glucose or other carbohydrates leads to the formation of ACR in food following exposure to higher heat (Surdyk *et al.*, 2004)which explains why overheated diets are considered the main source of ACR(Crowley *et al.*, 2022).

Accumulation of ACR into the human body impairs in particular; the reproductive (Ma *et al.*, 2011) and nervous systems(Seale, Feng, Agarwal, El-Alfy, & Behavior, 2012). In experimental models, ACR demonstrated potent carcinogenic activity in selected organs (Hogervorst *et al.*, 2010), whereas, it has an active and significant binding capacity to cells of the liver, kidney, brain and erythrocyte (Sumner, Selvaraj, Nauhaus, & Fennell, 1997). ACR was classified as B2, a probable carcinogen and as 2B, a possible human carcinogen, respectively by authorities that deal with cancer and related carcinogenic agents (US EPA and IARC)(Friedman, Dulak, Stedham, & Toxicology, 1995).

Although the polymeric form of ACR (polyacrylamides) is nontoxic, occupational intoxication in human or experimental laboratory animals was reported following exposure to ACR monomeric forms(Favor, Shelby, & Mutagenesis, 2005; Hogervorst *et al.*, 2010).Cytotoxicity of ACR showed to induce oxidative stress and cellular apoptosis which increase the probability of cellular damage of proteins, DNA molecules, and initiation of cellular free radicals, activating loss of cell wall permeability, distraction, and lysis of the cells(Sahinturk, Kacar, Vejselova, Kutlu, & Health, 2018).

To overcome the toxic effects of ACR, several trials based upon the use of natural antioxidants, like vitamin E (Al-Serwi, Ghoneim, & ultrastructure, 2015; Rahangadale, Jangir, *et al.*, 2012) considering that vitamin E interacts with free radicals like peroxyl radical, hydroxyl radical, as well as superoxide. Vitamin E also regulated cellular lipid peroxidation and released lipid peroxide radicals like malondialdehyde (MDA). accordingly, mutagen formation as well as repair of membranes and DNA could be ceased or inhibited (Rahangadale, Kurkure, Prajapati, Hedaoo, & Bhandarkar, 2012).

Black Truffles (BT) (TerfeziaClaveryi) is a black diamond of desert fungi species, which grows wildly in desert regions depending on water rainfall. BT has nutritional and health significance worldwide, particularly in Arabian countries (Hussain & Al-Ruqaie, 1999). BT, grows naturally in the northern part of the Arab gulf region like the northern borders of Saudi Arabia, Kuwait, Iraq, and Jordan (Zampieri, Chiapello, Daghino, Bonfante, & Mello, 2016).

Previous studies reported that BT contains a plentiful amount of

vitamin A, vitamin C and β carotene together with antioxidants, carbohydrates, and proteins. It is commonly used as a convalescent and culinary agent (Bokhary, Parvez, & Analysis, 1993; S. Janakat, Al-Fakhiri, Sallal, & Derivatives, 2004; Murcia *et al.,* 2002).Owing to its health-promoting impact, Europeans and other populations worldwide used BT as an edible mushroom and functional food.

Extracts of BT are rich in metabolites with remarkable therapeutic effects such as an anti-inflammatory, anti-carcinogenic, anti-mutagenic, immune suppressor, and anti-microbial (Sorrentino *et al.,* 2018).BT extract has potential antibacterial, anticancer, hepatoprotective and wound-healing effects(Aldebasi, Qureshi, Khan, Aly, & Ahmad, 2015; Gajos, Ryszka, & Geistlinger, 2014; Sorrentino *et al.,* 2018).

The proposed BT extract protection against ACR food toxin-induced hepatic cytotoxicity is the aim of this study. We planned to explore the aptitude of the BT extract as a tissue-protective and antitoxic agent against ACR-induced liver toxicity compared to the already settled vitamin E defensive impact.

MATERIALS AND METHODS

1. Materials:

Vitamin E (sigma chemical Co) was dissolved in 1 ml of corn oil and given orally by gastric tube and applied in a dose of 100 mg/kg body weight (Mehri, Karami, Hassani, & Hosseinzadeh, 2014). In addition, fried rice was applied as a source of dietary acrylamide (1302.36 µg /kg), whereas, 30% fried rice (equivalent to 0.11 mg/ kg/ body weight daily acrylamide) is given (Osman, Romeilah, Elgammal, & Hasan, 2015). In this work, 250 g of fine rice purchased from Othim local market (Almadinah Almounarah, KSA) was fried in one litter of pure sunflower oil at 180 ° C for 10 min (Osman, Romeilah, Elgammal, Ramis, & Hasan, 2016).

BT samples were purchased in Almadinah Almounarah, KSA in March 2020. A typical T. claveryi fruiting body (Variety Zubiedi) was collected. Samples were put in clean polyethylene bags and were labeled carefully with pertinent information i.e., habitats (wild), date of collection, etc. The samples of 2 kg were washed carefully with running water, blotted on kitchen-sucking papers, cut into small pieces, and finely milled with an M20 mill (IKA Werke, Germany) to have a size lower than 600 mm. Then, samples were dried using a hot oven. Finally, samples were ground to powder in order to increase the efficiency of extraction and stored in the dark at 48° C in hermetically vacuum-sealed plastic bags (Tecnotrip, Spain) up to analysis

2. Preparation of BT:

Using the hot maceration method, the samples of BT were dried in an oven at (35–40°C) and ground mechanically. For the preparation of methanol extract, approximately 100 g of the sample was extracted with 500 mL of methanol at 40º C. In the case of water extract, 70% ethanol was added twice to remove small molecular compounds, which can dissolve in ethanol. After being extracted by ethanol, the samples were mixed with deionized water in a ratio of 1:5. The mixture was boiled for 2 h and the extracts were collected and filtered through cheesecloth and then were centrifuged at 4000 rpm for 15 min. To get a dry solid form of the extracts, a rotary evaporator (Heidolph, Germany) was used to concentrate water and methanol extracts. Finally, the extracts were stored at 20º C for further in vivo and in vitro analysis. Yield percentages were calculated as: Yield % = (Weight of extract / Weight of plant ×100)

3. Experimental Design: A total of 60 healthy male albino Wistar rats weighed 180–220 g were included in this study. All rats were kept in normal conditions like 25°C and 40% humidity in polypropylene cages with the 12 h light-dark cycle. The animals were allowed free access to drinking water and provided with normal basal diets containing 21.1% of protein, 5.1% of fat,

60.0% of carbohydrates, 3.9% of fiber, 7.9% of minerals and 2.0% of vitamins. As a preliminary test, only ten rats were applied for measuring the cytotoxicity of BT extract. The rest of the rats (50 rats) were classified into five groups (10 rats each). They are divided as follows:

Group I: is the control group. Rats fed on a normal basal diet.

Group II: treated with fried rice. Rats received 30% fried rice daily which is equivalent to 0.11 mg/ kg b.w. of acrylamide.

Group III: treated with vitamin E and fried rice. Rats received fried rice containing 0.11 mg/ kg b.w. acrylamide and vitamin E (100 mg/kg/ b.w.).

Group IV: treated with fried rice and BT water extract. Rats received fried rice containing 0.11 mg/ kg acrylamide and BT water extract (400 mg/kg/ b.w.)

Group V: treated with fried rice and BT methanolic extract. Rats received fried rice containing 0.11 mg/ kg acrylamide and BT methanolic extract (400 mg/kg/ b.w.)

After the administration period (30 days), all rats were anesthetized by intramuscular injection of ketamine hydrochloride (50 mg/kg, i.p., body weight) in combination with xylazine hydrochloride (10 mg/kg) of body weight. Blood samples were collected, and all rats were sacrificed. Following the excision of the liver, wet weights of the liver were recorded. Liver sections were aliquoted for histopathological examination and remaining tissues and serum samples were kept for biochemical analysis. All biochemical studies were performed in duplicate.

4. Acute Toxicity Test:

The toxicity of BT extracts was evaluated in ten healthy rats administrated the extract (Organization for Economic Cooperation and Development (OECD, 2001)(Osman *et al.*, 2016). In drinking water, the rats received orally a gradual concentration of the BT extracts (50 mg to 1000 mg/rat). After the first 4 h of dosing, rats were observed for toxic symptoms such as behavioral changes and signs of toxicity and/or death and the latency of death. After 24 h, the surviving animals were maintained under daily observation for two weeks. The LD_{50} value was determined (El Allaoui, Filali, Oumokhtar, & Ibijbijen, 2011).

5. Assessments of Phenolic, Flavonoid, And Tannin Contents in BT Extracts:

The major classes of phytoconstituents, such as phenolic, flavonoid, and tannin contents were estimated in the aqueous and the methanolic extracts of the truffles (Dewanto, Wu, Adom, Liu, & chemistry, 2002; Sun, Ricardo-da-Silva, Spranger, & chemistry, 1998). The Folin–Ciocalteu method was applied to identify the phenolic content of BT extracts. For each sample of the extract, the absorbance was determined against a blank at 725 nm by using the spectrophotometer (Lambda 35, Perkin Elmer Co. Ltd., USA). Then, the amounts present were identified from the standard calibration curve for gallic acid monohydrate and expressed as mg gallic acid equivalent (GAE)/g extract. In the case of determining flavonoid content, catechin was used as a standard control for the calibration curve as previously described (Dewanto et al., 2002). In addition, modified vanillin assays were used to estimate tannin content in the aqueous and the methanolic extracts and expressed as mg catechin equivalent (CE)/g extract (Sun et al., 1998).

6. Assessment of Liver Function:

Commercially available kits (bioM4rieux kits, France) were used to estimate the levels of ALT, AST, bilirubin, and albumin as markers of hepato-toxicity in serum samples of BT-treated and non-treated acrylamide intoxicated rats.

6.1. Assessment of antioxidant profile in liver tissues:

-Determination of Antioxidant Enzymes and Lipid Peroxidation:

In this test, the activity of both superoxide dismutase (SOD) and catalase (CAT) in liver tissue samples was identified (Weydert & Cullen, 2010). MDA as a marker of lipid

peroxidation was quantitatively estimated in liver homogenate using high-performance liquid chromatography (Grotto *et al.*, 2007). In addition, 8-Oxo-dG, a marker of DNA damage and total antioxidant activity (TAC) was assayed in liver homogenate using ELISA kit (MyBioSource, USA) for 8-Oxo-dG and colorimetric Assay Kit (BioVision, USA) for TAC respectively. The concentrations equivalent to the antioxidant capacity of the extract were measured at 570 nm and calculated as a function of Trolox concentration (Othman *et al.*, 2020).

-Determination of the Non-Enzymatic Antioxidants:

The contents of both ascorbic acid (Vitamin C) and Vitamin E were identified in the liver tissue homogenates of all rats (Mahmoud, Ashour, Abdel-Moneim, Ahmed, & Complications, 2012).

6.2. Liver Histology and Histopathology:

Liver tissues were fixed in 4% paraformaldehyde for 16 h at 4ºC and paraffin sections of 4µm size were prepared. Then, the sections of liver tissues were stained with Hematoxylin and Eosin (H&E). All slides of liver tissues of control, BT-treated, and non-treated acrylamide-intoxicated rats were examined and photographed by a histopathologist blinded to the experimental protocols.

CD-4 and COX-2 immune staining liver tissue sections were prepared using the standard methodology. Briefly, the immunohistochemical study was performed on 3 µm formalin-fixed, paraffin-embedded sections deparaffinized and rehydrated through alcohol series to water. The endogenous activity was blocked with 3% aqueous hydrogen peroxide for 10 min, antigen retrieval was performed for each antigen in a domestic 750 KW microwave oven with EDTA. Primary antibodies were applied overnight at 4°C according to the manufacturer's protocol. The immunoreaction was developed with 3.3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma Aldrich, St. Louis, MO, USA) as chromogen (Al-Rasheed *et al.*, 2017; Chiaravalli *et al.*, 2013).

Liver tissue-stained samples were photographed and the slide analysis (10 slides/each animal) was achieved using the image analysis software (ImageJ, NIH).

6.3. Statistical Analysis:

All data were expressed as Mean±Standard Deviations (SD). The results obtained were statistically analyzed by GraphPad Prism (version 7). In addition, a one-way ANOVA test followed by Tukey's post hoc analysis was applied to compare and identify the significance between groups. The statistical significance was assigned at p-value <0.05.

RESULTS

1.Acute Toxicity Test:

Oral administration of BT extracts did not induce any lethal effect in the treated rats. None of the animals reported signs of toxicity.

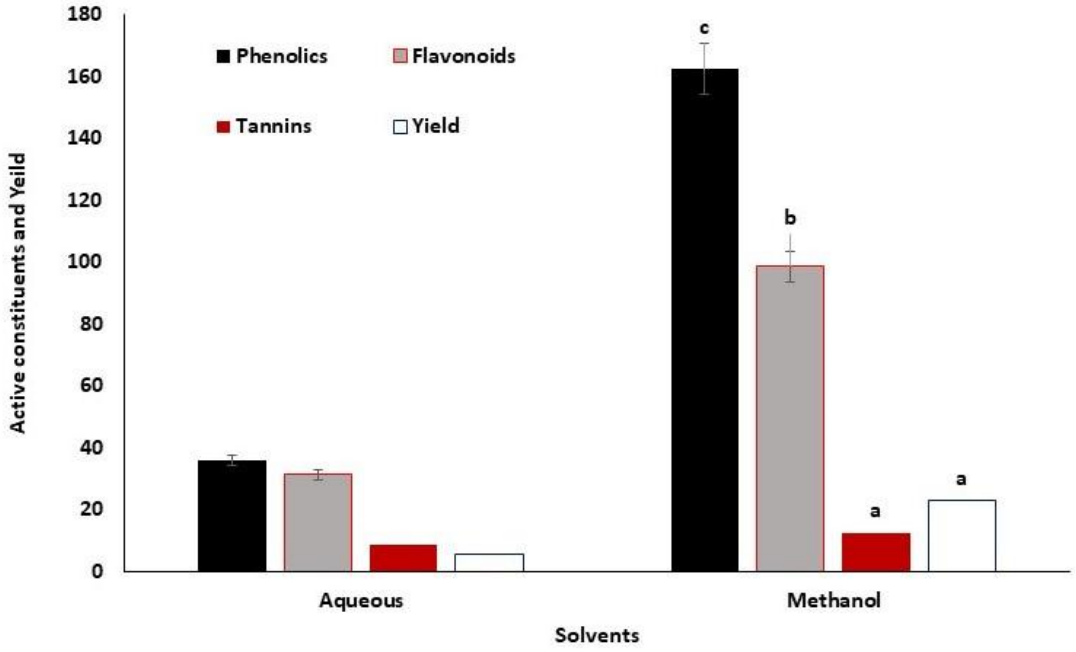
2.Assessments of Active Compounds and BT Extraction Yield:

The results of GC/MS, the Folin–Ciocalteu, and modified vanillin assays were represented in Table (1) and graph (1). The extraction yields varied based on the different solvent polarities. The highest extracted yield was the methanol extraction with 23.1%, while the lowest extraction was the aqueous extraction with 5.3%.

Quantitative analysis showed that phenolic, flavonoids and tannin compounds were identified in higher amounts of methanolic extract of BT compared to aqueous extract. In addition, active compounds such as eugenol, catechin, p-Coumaric acid, chlorogenic acid, gallic acid, and ascorbic acid were quantitively higher in amounts, followed by hesperidin and rutin which were identified in lowest amounts in both aqueous and methanolic extracts.

Table 1: The Yields and main active phytoconstituents present in BT

Phytoconstituents	Ret time (min)	Concentration	
		Aqueous	Methanol
Gallic acid	3.3	1.8	2.1
Catechin	8.7	3.7	4.3
Chlorogenic acid	10.5	2.1	2.8
Rutin	12.6	0.2	0.6
p-Coumaric acid	15.6	2.5	2.7
Ascorbic acid	12.17	1.5	1.9
Hesperedin	18.5	0.89	0.92
Eugenol	23.7	4.85	5.6
Yield (g/100g d.w.)	-	5.3	23.1

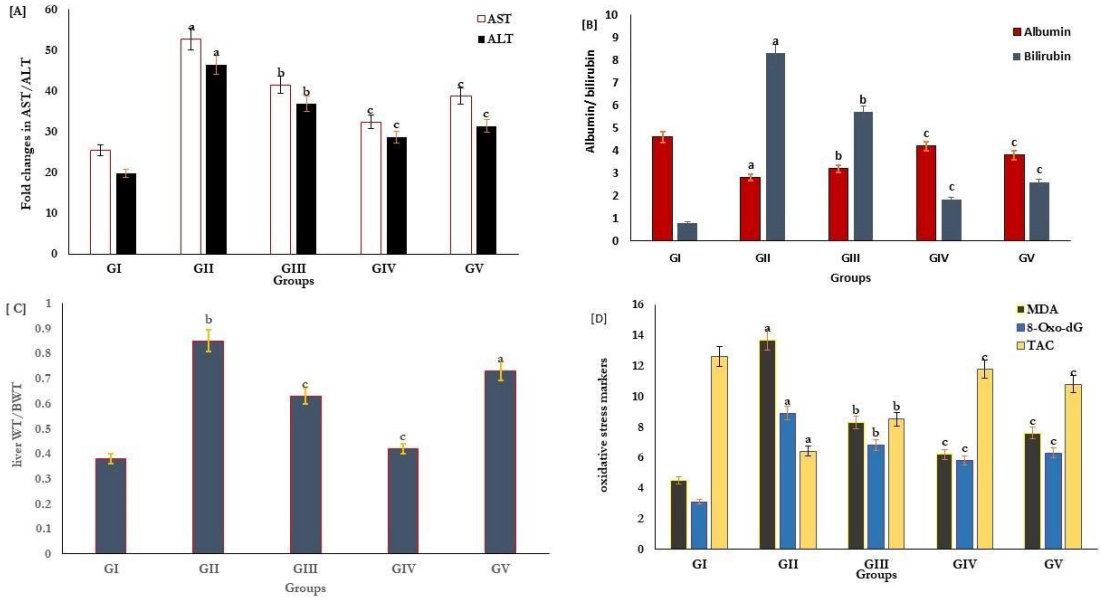


Graph 1: Extraction yields, phenolics, flavonoids, and tannins active contents of BT (n = 3).

3.Effect of BT Extracts on Liver Function and Weight:

Group II rats showed abnormal levels of liver biomarkers. The results showed a significant increase in the levels of AST, ALT, and bilirubin with a decline in the concentrations of albumin compared to normal control rats (Graph 2A&2B). In rats treated with BT extracts treated rats (Groups IV and V) showed an improvement in the levels of liver biomarkers. There was a significant reduction in the levels of AST, ALT, bilirubin, and an increase in the levels of albumin was reported compared to rats treated with acrylamide food toxin (Group II). In addition, liver biomarkers of rats treated with vitamin E (Group III) significantly improved compared to acrylamide-intoxicated rats (Graph 2A&2B).

The results showed also that aqueous BT extract significantly improved the figure of the liver biomarkers compared to methanol extract and vitamin E-treated rats as well. The potential improving activity of the BT extracts was significantly supported by a reduction in liver weight/body weight ratio observed in rats treated with aqueous, methanol, and vitamin-treated rats compared to acrylamide food intoxicated rats (Graph 2C).



Graph 2: Fold change in the liver weight/body weight ratio, liver function, and oxidative stress in the liver tissues of BT treated and fried rice non-treated rats. Data expressed as mean ±SD for ten rats in each group. 30% Fried Rice: equivalent to 0.11 mg/ kg of acrylamide food toxin), MDA: malonaldehyde, TAC: total antioxidant capacity, 8-Oxo-dG: BWT: body weight, AST: Aspartate Aminotransferase, ALT: Alanine Aminotransferase. Significance at $p < 0.05$. a $p < 0.05$ (group II vs group I), b $p < 0.01$ (group III vs group I or II), c $p < 0.001$ (group IV or V vs group II &III).

4.Effect of BT on Enzymatic and Non-Enzymatic Antioxidants:

Acrylamide treatment in the rat (Group II) significantly initiates the production of free radicals increasing the levels of cellular oxidative stress parameters; MDA, 8-Oxo-dG, TAC, SOD, and CAT as shown in Graph (2D) and Table2. The expression of MDA and 8-Oxo-dG is significantly increased, and the activity of SOD, CAT, and TAC is significantly reduced following the administration of acrylamide. Rats treated with vitamin E showed a significant reduction in the expressed MDA and 8-Oxo-dG, along with an increase in the levels of TAC, SOD, and CAT activity compared to rats treated with acrylamide food toxin.

Rats treated with both aqueous and methanolic BT extracts showed a significant improvement in the antioxidant status of the liver tissues. The results showed that TAC, SOD, CAT enzymes as sort of antioxidants significantly increased in the liver of treated rats accompanied by a reduction in the expressed levels of both MDA and 8-Oxo-dG as markers of cellular intoxication with acrylamide. Whenever rats treated with aqueous BT extract showed more enhancements in the levels of TAC, SOD, and CAT enzymes with a reduction in the levels of MDA and 8-Oxo-dG compared to vitamin E and methanolic-treated rats.

In addition, table 2 shows that the concentration of Vit-E and Vit-C as non-enzymatic antioxidants were decreased significantly in the acrylamide-treated rats, whereas, on the administration of vitamin E and BT extracts these cellular antioxidants were increased significantly as compared to group II. Liver tissues of rats treated with aqueous BT extract showed more improvement in the concentration of both Vit-E and Vit-C respectively compared to vitamin E and methanolic-treated rats.

Table 2: Enzymatic and no-enzymatic antioxidant status in liver tissues of the control and experimental rats**.**

Group	Tissue antioxidant status			
	Enzymatic		Non-enzymatic	
	SOD	CAT	Vitamin E	Vitamin C
Group I (control)	10.6±1.4	94.5±4.3	1.8±0.4	2.5±0.5
Group II (30% Fried Rice)	4.5±1.7 [a]	38.3±3.1[a]	0.8±0.3[a]	1.3±0.42[a]
Group III (30% Fried Rice+ Vitamin E; 100 mg/kg)	7.5±2.7 [b]	74.8±4.3[b]	1.1±0.36[c]	2.1±0.65[c]
Group IV(30% Fried Rice+ Aqueous BT; 400 mg/kg)	10.1±1.4 [c]	85.5±2.1[c]	1.5±0.31[c]	2.3±0.72[c]
Group V (30% Fried Rice+ methanolic BT; 400 mg/kg)	8.2±2.3 [c]	73.8±2.8 [c]	1.3±0.28[c]	1.9±0.51[c]

Data expressed as mean ±SD for ten rats in each group. 30% Fried Rice: equivalent to 0.11 mg/ kg of acrylamide food toxin), SOD: superoxide dismutase enzyme (50% NBT reduction/min/mg protein), CAT: catalyze enzyme (µmoles of H_20_2 utilized/min/mg protein). Significance at $p < 0.05$. [a] $p < 0.05$ (group II vs group I), [b] $p < 0.01$ (group III vs group I or II), [c] $p < 0.001$ (group IV or V vs group II &III)

5.Effect of BT on Liver Histology and Histopathology:

Liver samples stained by hematoxylin and eosin showed congestion of the central veins of group II with hydropic degenerative changes in the hepatocytes in the form of vacuolation of the cytoplasm, pyknosis of the nuclei and narrowing of the sinusoids caused by bloated hepatocytes. Vitamin E administration in group III improved the induced tissue changes and decreased the congestion of the central veins and less marked individual hepatocytes' cytoplasmic vacuolation. Few cells show nuclear pyknosis and the sinusoidal spaces appeared narrower than the controls. Group IV had the best histopathological features. The central veins were non-congested, hepatocytes showed minimal cytoplasmic vacuolation, and non-evident nuclear pyknosis and the sinusoidal spaces appear of normal breadth. Group V is more or less close to the previous group (Fig.1).

CD-4 immune expression was minimal in group I and could be only seen in the sinusoidal lining. The intoxicated group (group II) showed extensive dye expression particularly close to the central vein. The immuno-reactivity decreased in group III and tends to be close to the normal level in groups IV and V. These results were confirmed by the image analysis of the percent of CD-4 Immuno-positive cells. In comparison with the control group, the area percent of the immuno-reactivity in group II showed a highly significant increase ($p < 0.001$). The area percent in group III showed a significant increase ($p < 0.01$). Groups IV, and V showed a weak significant increase ($p < 0.05$). In comparison with group II, the decrease in the area percent in groups III, IV and V was significant with the maximum decrease in group V (Fig. 2).

Immuno-stained liver sections with the anti-COX-2 primary antibody in group I showed the absence of immuno-reactivity while group II showed a marked increase of the strongly immuno-stained hepatocytes. Group III showed little immuno-positivity. Groups IV and V had less weak immuno-stained hepatocytes. These results were confirmed by the image analysis of the area percent of COX-2 expression. In comparison with the control group, the area percent expression of COX-2 Immuno-positive cells in group II showed a highly significant increase ($p < 0.001$). The area percent in group III showed a significant increase ($p < 0.01$). Groups IV, and V showed a weak significant increase ($p < 0.05$). In comparison with group II, the decrease in the area percent in groups III, IV and V was significant with the maximum decrease in groups IV and V (Fig.3).

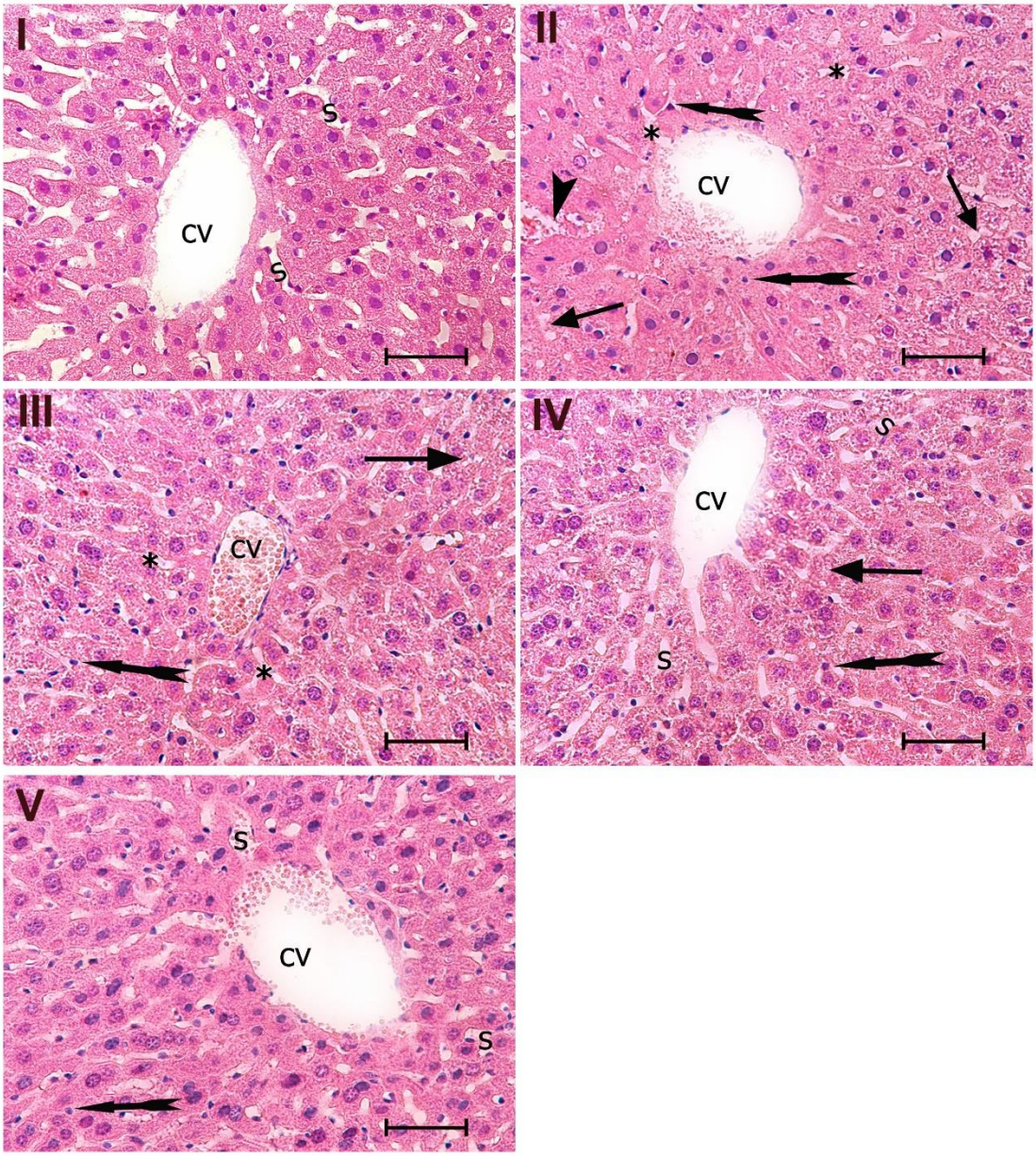


Fig. 1: Liver sections of:
Group I shows the normal liver architecture with plates of hepatocytes separated with sinusoidal spaces (S) radiating from the central vein (CV).
Group II shows congestion of the central vein with hydropic degenerative signs in the hepatocytes in the form of vacuolation of the cytoplasm (arrows), pyknosis of the nuclei (tailed arrows), area of degenerated hepatocytes (arrow heads) and narrowing of the sinusoids (Asterix).
Group III shows congestion of the central veins and less marked individual hepatocytes' cytoplasmic vacuolation (arrow). Few cells show nuclear pyknosis (tailed arrows). The sinusoidal spaces still narrow (Asterix).
Group IV has non-congested central veins and less marked hepatocytes' cytoplasmic vacuolation (arrow). Few cells show nuclear pyknosis (arrow heads). The sinusoidal spaces (S) reappear. Group V similarly has less marked central veins congestion. Most hepatocytes showed normal cytoplasm and nuclei. Scarce pyknosis could be seen (tailed arrow). The sinusoidal spaces (S) are clearly seen.
Scale bar 50µm

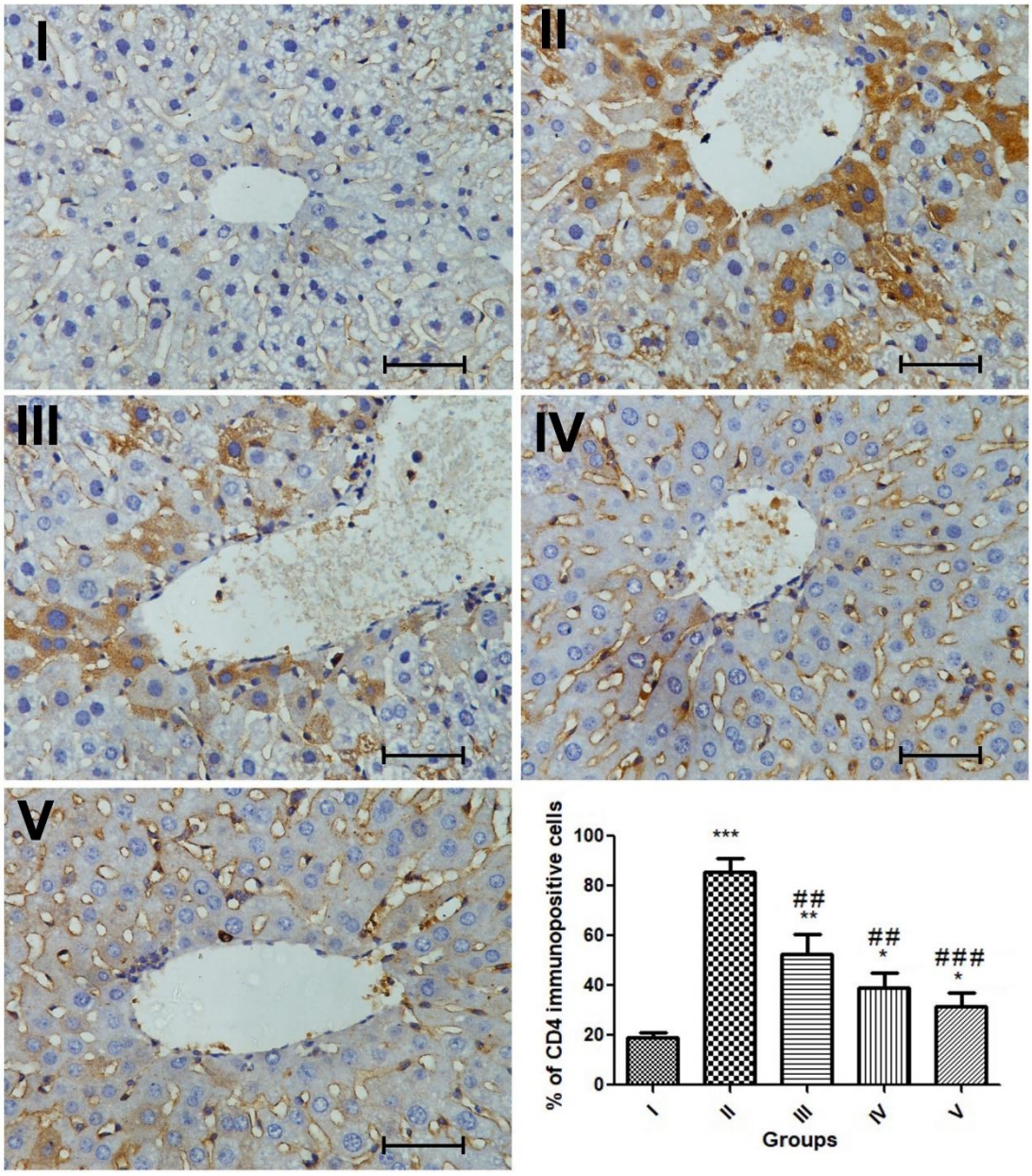


Fig. 2: CD4 immunohistochemical staining liver sections in paraffin-embedded blocks tissue samples. The tissue immuno-reactivity is minimal in the group I and limited to the sinusoidal lining. Group II shows extensive dye expression particularly close to the central vein. The immuno-reactivity decreases in group III and tends to be more or less close to the normal pattern in groups IV and V.

The diagram shows:

1. Comparison between control group and the other groups (referred to as *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$)
2. Comparison between group II and the other treated groups (referred to as ### $p < 0.001$; ## $p < 0.01$, # $p < 0.05$)

Data are the mean ± SE. (n = 8); (one-way ANOVA).

Scale bar 50µm.

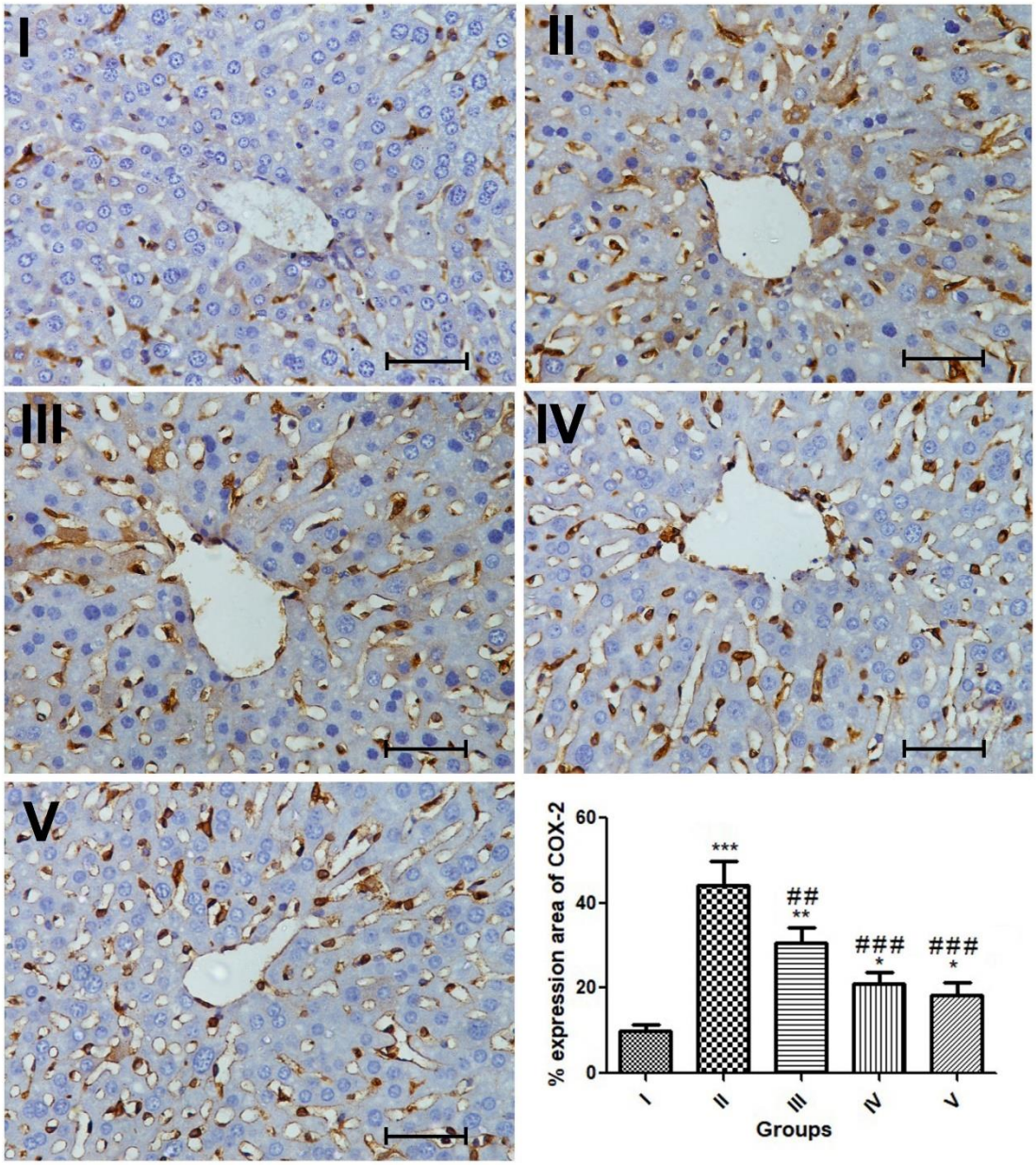


Fig. 3: Light photomicrograph of liver sections Immuno-stained with the anti COX-2 primary antibody. (I) liver section from control rat shows the normal absence of immune reactivity while (II) liver section from rat received ACR shows a marked increase of the strongly immuno-stained hepatocytes between few unstained cells. (III) Liver section shows few immuno-positive hepatocytes between many unstained normal cells. Group IV liver section from rat shows a marked decrease of the density and number of immuno-stained cells. Group V similarly many hepatocytes with immuno-negative reaction while immune positive cells are less with weak positivity.

The diagram shows:

1. Comparison between control group and the other groups (referred to as *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$)
2. Comparison between group II and the other treated groups (referred to as ### $p < 0.001$; ## $p < 0.01$, # $p < 0.05$)

Data are the mean ± SE. (n = 8); (one-way ANOVA).

Scale bar 50µm.

DISCUSSION

Food processing yields high concentrations of hydrocarbons at high temperatures leading to the formation of ACR(Jaegerstad, Skog, & Mutagenesis, 2005). Several trials investigated the protective effects of natural antioxidants; on ACR-induced food toxicity. Black truffles like other natural antioxidants are rich in antioxidants along with carbohydrates and proteins. Its rich content of metabolite of phenolic and flavonoids origin exhibit remarkable anti-inflammatory, anti-carcinogenic, anti-mutagenic, immune suppressor, and anti-microbial properties(Veeraraghavan*et al.*, 2021)thus favoring its proposed antitoxic effect.

Acute toxicity tests proved that different doses of BT extract did not induce any lethal effect in the treated rats indicating the safety of the extract. Matched with other toxicological studies, there are no abnormal changes in body weight and histology in various visceral organs, as well as no negative effects on renal and hepatic functions, were reported after oral administration of BT extracts (El Allaoui *et al.*, 2011).

In this study, the main chemical composition of BT extracts in aqueous and methanolic solvents exhibited high extracted yields of phytosterols, triterpene, vitamin, and aromatic compounds that are responsible for their antioxidant and antiapoptotic properties. Quantitative analysis also reported higher amounts of eugenol, catechin, p-Coumaric acid, chlorogenic acid, gallic acid, and ascorbic acid. These substances besides their higher nutritional values of low fats and higher protein contents can also promote human health(AlAhmed, Khalil, & Journal, 2019).

In vitro analysis showed that aqueous extracts of the BT have significant amounts of radical scavenging activity compared to methanolic solvent extract. The extract reported the strongest DPPH, FRAP, and ABTS radical scavenging activity(Canpolat*et al.*, 2021). However is important to know that the antioxidant activities of BT vary according to the type of Truffles species (Akyüz & nutrition, 2013). Also, the type of solvent has important effects on the yield and antioxidant activity of BT(Al-Rawi, Ibrahim, Majid, Majid, & Ab Kadir, 2013).

Although Vit. E improved the liver functions, and TB extracts induced a remarkable reduction in the concentrations of liver functions compared to ACR-intoxicated rats. This effect can be explained by the high antioxidant contents in TB such as vitamin C and ß-carotene(Teodor, Cuciureanu, Slencu, Zamosteanu, & Cuciureanu, 2011). Both materials help to keep the integrity of the plasma membrane, and the destruction of the plasma membrane which in turn prevents the release of cytosolic enzymes such as ALP, ALT, and bilirubin into the blood stream and suppressed the decrease of albumin concentration (Mehendale *et al.*, 1994). In addition, liver weight ratios were conserved to normal size following the administration of BT extracts. Similarly, this effect might be due to those higher contents of phenolic, flavonoids as well as other antioxidant compounds nullifying the effects of constitutive androstane receptor, which is a central regulator of xenobiotic metabolism which during increasing by ACR toxicity induces hepatomegaly and increased liver size(Huang *et al.*, 2005).

During the cellular metabolism of ACR, an overproduction of ROS was reported, moreover producing deleterious effects on antioxidant enzymes. These deteriorations in the cellular antioxidant status lead to severe intoxication and damage to liver cells (Santhanasabapathy, Vasudevan, Anupriya, Pabitha, & Sudhandiran, 2015). Previous reports attributed this

effect to the presence of mycochemicals like the highly conjugated structure of phenolic derivatives (Guillamón *et al.*, 2010). The ACR-induced free radicals were stabilized by the conjugated active phenolic metabolite present in BT(Choe, Min, & nutrition, 2006).

Although Vitamin E administration improved the induced tissue changes, BT extracts applied the best histopathological features. At the same time, CD-4 and COX-2 immune expression were minimal in the control group. However, the ACR-intoxicated group had extensive dye expression. The inhibited immuno-reactivity in Vitamin E and BT extract was confirmed by the image analysis of the area fraction of both dyes. The evident decreased CD-4 indicates inhibited representation of CD-4 tumor antigen. The degree of expression may be regulated by the presence and absence of inflammatory cytokines (Ramia et al., 2019) with the conclusion that BT exhibited an antitumor effect. Concomitantly, COX-2 inhibited expression by vitamin E and the BT extracts point to their anti-inflammatory effect because COX-2 is known as a generator of prostaglandins (Al-Rasheed *et al.*, 2017).

The superiority of BT water extract over methanolic extract was evident in all biochemical and histopathological studies. This finding can be explained by the fact that water extract usually shows higher content of chemical components (Bae *et al.*, 2012).A similar finding was reported by Janakat et al., where an aqueous extract of BT showed a more powerful effect (S. M. Janakat, Al-Fakhiri, & Sallal, 2005).

Conclusion

The results of the present study suggested that BT extracts in aqueous and methanolic solvents significantly improved liver toxicity induced by ACR. The data showed that BT extract is rich in antioxidants and hepatoprotective components. More studies of molecular bases are recommended.

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