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Hepato and neuro-protective influences of biopropolis on thioacetamide-induced acute hepatic encephalopathy in rats

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Draft

Abstract

Hepatic encephalopathy (HE) is a neuropsychiatric syndrome ultimately occurs as a complication of acute or chronic liver failure; accompanied by hyperammonemia. This study aimed to evaluate the potential of biopropolis as a hepato and neuroprotective agent using thioacetamide (TAA)-induced acute HE in rats as a model. Sixty Wistar rats were divided into five groups: Group 1 (normal control) received only saline and paraffin oil. Group 2 (hepatotoxic control) received TAA (300 mg/kg, once). Groups 3, 4 and 5 received TAA followed by vitamin E (100 mg/kg) and biopropolis (100 and 200 mg/kg), respectively, daily for 30 days. Evidences of hepatic encephalopathy were clearly detected in TAA-hepatotoxic group including significant elevation in the serum level of ammonia, liver functions, increased oxidative stress in liver and brain, apoptotic DNA fragmentation and overexpression of iNOS gene in brain tissue. The findings for groups administered biopropolis, highlighted its efficacy as a hepato and neuro-protectant through improving the liver functions, oxidative status and DNA fragmentation as well as suppressing the brain expression of iNOS gene. In conclusion, biopropolis, at a dose of 200 mg/kg/day protected against TAA-induced HE through its antioxidant and antiapoptotic influence; therefore, it can be used as a protective natural product.

Keywords: Biopropolis; hepatic encephalopathy; thioacetamide; iNOS; vitamin E.

1. Introduction

Hepatic encephalopathy (HE) is a neuropsychiatric syndrome resulting from acute or chronic liver failure (Lizardi-Cervera et al. 2003). HE affects a huge number of patients worldwide leading to a mortality rate of 50 to 90% (Raghavan and Marik 2006). HE causes a wide range of clinical manifestations, including psychomotor dysfunctions, sensory abnormalities, poor concentration, increased reaction time and impaired memory. Patients commonly develop stupor, coma and death in severe cases. The mechanism of HE has not been fully explained. However, hyper-ammonemia has been suggested to play the main role in HE pathogenesis and to be responsible for the direct and indirect alterations in cerebral metabolism and hence encephalopathy (Frederick 2011; Mustafa et al. 2013).

Thioacetamide (TAA) is used to induce an experimental rat model mimicking acute hepatic failure and HE. This model has been validated to be a satisfactory model of HE (Avraham et al. 2006). TAA undergoes extensive metabolism to highly reactive metabolites that binds to liver macromolecules resulting in hepatic necrosis, hyper-ammonemia (Swapna et al. 2006) and extensive oxidative stress (Sathyasaikumar et al. 2007). Studies demonstrated that centrilobular necrosis is apparent after single dose of TAA, while cirrhosis, liver cell adenomas and liver cancer usually develop after chronic administration (Waters et al. 2005).

Oxidative stress has long been involved in the pathogenesis of acute and chronic liver damage in many conditions such as toxin exposures, bile duct obstruction, excess alcoholism, liver ischemia, and viral infection (Stehbens 2003). Overproduction of reactive oxygen species (ROS) and nitrogen species, along with decreased antioxidant capacity, disturbs various cellular functions through lipid peroxidation (Fang et al. 2002; Saad et al. 2014). Therefore, great attention has been paid to antioxidants or free radicals scavengers for the prevention and treatment of acute and chronic liver damages.

Biopropolis (PP; bee glue) is a sticky resinous hive substance that is collected from plants by honeybees. It has been used as a folk medicine in Europe for its many biological activities such as antimicrobial, anti-allergic, dermatoprotective, laxative, antidiabetic,

immunomodulatory, antitumor and antibiotic activities (Campos et al. 2015). Recently, PP has also been used as antiulcerous and antitumor agent (Kakehashi et al. 2016). PP is characterized by its unique chemical constituents that target numerous biochemical and genetic pathways. The anti-inflammatory and oxyradical scavenging properties of PP attribute to its wide range of biological activities (Nakamura et al. 2013).

Oral administration of PP and its main constituent was shown to be protective against acute liver damage induced by various hepatotoxicants such as carbon tetrachloride, alcohol, D-galactosamine and acetaminophen (Ramadan et al. 2015; Seo et al. 2003). This hepato-protection may be attributed to its several pharmacological actions due to its ability to inhibit lipid peroxidation (Nakamura et al. 2013). However, it is still unclear whether PP exerts protective effects against hepatic encephalopathy in rats.

The current study was designed to evaluate the possible hepato- and neuro- protective effect of PP against TAA-induced HE and its associated liver and brain oxidative damage and comparing its effect to vitamin E as a well-established antioxidant.

2. Materials and methods

2.1. Animals

Adult male Wistar albino rats, weighing 130-150 g body weight were used. The animals were obtained from the Animal House Colony of National Research Centre (Dokki, Giza, Egypt) and were housed under conventional laboratory conditions. Animals were provided with standard laboratory food pellets and tap water *ad libitum*. The study was conducted in accordance with the National Research Centre- Medical Research Ethics Committee (NRC-MREC) for the use of animal subjects and following the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.2. Chemicals

TAA was obtained from Sigma (St. Louis, MO, USA). TAA (2.5%) was prepared freshly by dissolving in sterile saline and stirred well until all crystals were dissolved.

2.3. Preparation of drugs

Vitamin E (Vitamin E®, 200 mg soft gelatin capsules; Pharco Pharmaceuticals, Egypt) and biopropolis (Biopropolis®, 400 mg soft gelatin capsules; Sigma Pharmaceutical Industries SAE Company, Egypt) were used after dissolution in paraffin oil. All other chemicals used throughout the experiment were of the highest analytical grade available.

2.4. Experimental protocols

Sixty rats were divided into five groups, 12 animals each. Group 1 received saline (2 mL/kg, intraperitoneally (i.p), once) followed by paraffin oil (2 ml/kg, p.o, daily for 30 days). Groups 2-5 were injected (i.p) with TAA (300 mg/Kg) once to induce HE. Group 2 were received paraffin oil (2 ml/kg, p.o, daily for 30 days) and kept as hepatotoxic control group; while groups 3, 4 and 5 were administered daily oral doses of vitamin E (100 mg/kg), and PP (100 mg/kg) and (200 mg/kg), respectively, for 30 days after 24 h of TAA injection.

Along the experimental period, animals received dextrose water and ringer lactate solutions (10 mg/kg/day, i.p.) to prevent renal failure, hypoglycemia and electrolyte imbalance (Mustafa et al. 2013). After 24 h from the last treatment and overnight fasting, all rats were anesthetized for blood collection from the retro-orbital venous plexus, and then rats were sacrificed. Livers and brain were washed in ice-cold saline, blotted on filter paper, weighed, and finally frozen immediately at -80 °C for further investigation. Part of samples was fixed in 10% neutral buffered paraformaldehyde for histopathological examinations.

2.5. Preparing serum and tissue homogenates

Collected blood samples were allowed to stand for 10 min at room temperature then centrifuged at 4 °C using cooling centrifuge (Laborezentrifugen, 2k15, Sigma, Germany) at 3000 rpm for 10 min and sera were separated. Tissue homogenates (10%) were prepared in ice-cold saline.

2.6. Hepatic biochemical parameters in serum

Serum ammonia concentration was measured immediately according to Da Fonseca-Wollheim (1973). ALT and AST activities in serum were measured according to the method of Reitman and Frankel (1957). Colorimetric determination of ALP activity was done according to the method of Belfield and Goldberg (1971). All these parameters were assessed using commercially available kits (Quimica Clinica Aplicada, Spain).

2.7. Oxidative stress markers activities in tissue homogenates

Lipid peroxidation product, malondialdehyde (MDA), was measured using the thiobarbituric acid reactive substances (TBARS) assay, as described by Ruiz-Larea et al. (1994) and expressed as nmol/g wet tissues. Reduced glutathione (GSH) content was determined in liver and brain homogenates according to the method of Ellman (1959) and modified by Bulaj et al. (1998) and expressed as mg/g wet tissue.

2.8. DNA damage evaluation

Apoptotic DNA damage in the brain tissue was assessed by estimation of DNA fragmentation percentage and Comet assay.

2.8. 1. DNA fragmentation assay

DNA fragmentation assay is a method used for quantitative grading of DNA damage (Perandones et al. 1993). Brain samples were lysed in 0.5 ml of hypotonic lysis buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA and 0.2% triton X-100), and centrifuged at 14,000 xg for 20 min at 4 °C. The pellets were resuspended in hypotonic lysis buffer. To the supernatants and the resuspended pellets, 0.5 ml of 10% trichloroacetic acid (TCA) was added. The samples were centrifuged for 20 min at 10,000 xg at 4 °C, and the pellets were suspended in 500 µl of 5% TCA. Subsequently, each sample was treated with a double volume of diphenylamine (DPA) working solution and incubated at 4 °C for 48 h. Finally, the optical density (OD) was measured at 578 nm. The percentage of fragmented DNA was calculated using the equation:

$$\text{DNA fragmentation\%} = \text{OD of supernatant} / [\text{OD of supernatant} + \text{OD of pellet}] \times 100.$$

2.8. 2. Comet assay (alkaline single-cell microgel electrophoresis)

Comet assay was carried out according to Singh et al. (1988). Briefly, 100 mg of brain samples was crushed in 1 ml ice cold phosphate buffer saline (PBS), stirred for 5 min and filtered. 100 µl of cell suspension were thoroughly mixed with 600 µl of low-melting agarose. Afterward, 100 µl of the mixture were spread on agarose pre-coated slides, left to solidify at 4°C, and then immersed in chilled lysing solution for 1 h at 4 °C. The slides were removed and placed in a horizontal electrophoresis chamber, filled with freshly prepared alkaline electrophoretic buffer for 20 min. After electrophoresis, the slides were gently washed in 0.4 M Tris–HCl buffer and stained with ethidium bromide. The DNA migration patterns of 100 cells for each sample were detected using fluorescence microscope, and images were captured by a Nikon CCD camera. The qualitative and quantitative extent of DNA damage in the cells was estimated using the Comet 5 image analysis software developed by Kinetic Imaging Ltd. (Liverpool, UK).

2.9. Quantitative real-time RT-PCR for iNOS Gene

The mRNA expression level of iNOS gene was assessed using real-time PCR standardized by co-amplification with the housekeeping GAPDH gene as an internal control. Total RNA was extracted from 100 mg of rat brain tissue using the Qiagen Rneasy Mini Kit according to the manufacturer's instructions. Total RNA concentration was measured spectrophotometrically (Thermo Scientific, USA). The purified RNA was reverse transcribed into cDNA and used for PCR with specific primers. The primers sequences were as follows: iNOS forward 5'- CCCTTCCGAAGTTTCTGGCAGCAG-3', iNOS reverse 5'- GGGCTCCTCCAAGGTGTTGCCC-3' and GAPDH forward 5'- ACCACAGTCCATGCCATCAC-3', GAPDH reverse 5'-TCCACCACCCTGTTG CTGTA-3'. Real-time PCR was performed in color for research Laboratory (Qiagen. Egypt). cDNA and 30 pg/ml of each primer were added to a SYBR Green qPCR Master Mix (Qiagen). The cDNA was amplified by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s and extension at 72 °C for 45 s. During the first cycle, the 95 °C step was extended to 1 min. The GAPDH gene was amplified in the same reaction to serve as the reference gene. Every sample was analyzed in triplicate. The gene expression

levels were calculated and determined according to the method described by Livak and Schmittgen (Livak and Schmittgen 2001).

2.10. Histopathological study

Liver and brain tissues were fixed in 10% formalin, embedded in paraffin, cut into 5-mm thick sections, stained with hematoxylin and eosin (H and E), and then examined by an experienced pathologist under binocular Olympus CX31 microscope (Banchroft et al. 1996).

2.11. Statistical analysis

All results are expressed as Means $\pm$ SD. Multiple group comparisons were performed by analysis of variance (ANOVA) followed by Dunnett's multiple comparisons post hoc test. Difference was considered significant when $p < 0.05$. SPSS® software (version 17.00 for Windows, Chicago, USA) was used to carry out these statistical tests.

3. Results

3.1. Effect on serum biochemical parameters

Administration of TAA was associated with marked increase in serum ammonia concentration to 188%, as well as serum activities of AST, ALT and ALP to 215%, 263% and 330 % respectively, as compared to normal control group.

Treatment of animals with PP (100 mg/kg) significantly decreased serum ammonia concentration by 62 % as well as serum AST and ALT activities by 86% and 61% respectively, while showed no effect on serum ALP. Treatment of animals with PP (200 mg/kg) significantly decreased serum ammonia concentration by 59 % as well as serum AST and ALT activities were decreased by 72% and 55% respectively. On the contrary, PP (200 mg/kg) couldn't ameliorate serum ALP activity. Treatment of animals with vitamin E (100 mg/kg) significantly decreased serum ammonia concentration by 64%, as well as AST, ALT and ALP activities by 69%, 59% and 42% respectively, as compared to hepatotoxic TAA group ($p < 0.05$, table 1).

3.2. Effect on oxidative stress parameters in liver and brain tissues

Administration of TAA caused a significant increase in both liver and brain MDA content to 176% and 228% respectively when compared to normal control group. Treatment of animals with PP (100 mg/kg) resulted in a significant decrease in both liver and brain tissue MDA contents by 64% and 49% respectively, compared to hepatotoxic TAA group. Treatment of animals with PP (200 mg/kg) resulted in a significant decrease in both liver and brain tissue MDA contents by 59% and 47% respectively, compared to hepatotoxic TAA group. Treatment of animals with vitamin E (100 mg/kg) resulted in a significant decrease in both liver and brain tissues contents of MDA to 63% and 105% respectively when compared to hepatotoxic TAA group ($p < 0.05$, table 2).

Likewise, administration of TAA caused a significant decrease in both liver and brain GSH contents to 58% and 55% respectively, when compared to normal control group. Treatment of animals with PP (100 mg/kg) resulted in a significant increase in both liver and brain tissue GSH contents by 158% and 150% respectively when compared to hepatotoxic TAA group. Treatment of animals with PP (200 mg/kg) resulted in a significant increase in both liver and brain tissues contents of GSH by 183% and 217% respectively when compared to hepatotoxic TAA group. Treatment of animals with vitamin E (100 mg/kg) resulted in a significant increase in both liver and brain tissue GSH contents by 183% and 167% respectively, when compared to hepatotoxic TAA group ($p < 0.05$, table 2).

3.3. DNA fragmentation percentage

The effect of TAA-induced HE on the brain DNA was evaluated by measuring the level of genomic DNA fragmentation percentage using the DPA assay (table 3). Compared to the normal control (group 1), TAA induced marked increases in the DNA fragmentation percentage (group 2). Group 3, treated with vitamin E showed a significant reduction in the DNA fragmentation. Besides, significant differences in DNA fragmentation were detected between PP-treated groups (4 and 5) and hepatotoxic control group (2).

3.4. Comet assay

Comet assay is a sensitive and well-validated technique used to assess DNA fragmentation which is a typical component of toxic DNA damage and apoptosis. According to our results, TAA significantly induced DNA damage compared to normal control group in rat's brain. This DNA damage was evidenced by the significant elevation in the comet parameters, presented as tail length (μm), tail DNA (%) and tail moment; the main indicative parameter for DNA damage (table 4). A large broom like tail and a small comet head were observed in hepatotoxic control rats (group 2) (figure 1). The length of the tail increases with the extent of DNA damage as observed in figure 1. On the other hand, co-administration of vitamin E as well as PP (group 3, 4 and 5) deteriorated the effect of TAA through significant reduction of the tail length, damaged DNA %, tail moment (table 4), as well as reducing the intensity of comet tail (figure 1).

3.5. iNOS Gene expression in brain

The expression response of iNOS gene in rat brains to TAA-induced HE was analyzed using qRT-PCR. iNOS was overexpressed in hepatotoxic control group (group 2), reaching 5.8-fold, compared to normal control (group 1). Treatment of rats with PP in a dose level of 100 mg/kg and 200 mg/kg resulted in reduction in the iNOS expression level to 3.92 and 3.3 folds, respectively, as observed in group 4 and 5 (figure 2). Oral administration of vitamin E suppressed iNOS expression level to 2.8 as detected in the group 3.

3.6. Histopathological examination of liver and brain

Liver sections prepared from the hepatotoxic control group showed severe congestion of blood vessels, activated Kupfer cells and enlarged hepatocytes with disappearance of sinusoids. PP administration resulted in improvement in the overall histopathological picture of the liver. Focal areas of coagulative necrosis were replaced and displaced by inflammatory cells especially around the central veins (figure 3).

Brain sections from hepatotoxic control rats revealed severe pathological lesions represented by significant demyelination, the presence of numerous astrocytes with

prominent nuclei, significant increase in vacuolization, edema and inflammation of the brain tissue with congestion of brain blood vessels. Focal gliosis was also detected. The cerebellum showed necrosis of Purkinje cells (figure 4). Interestingly, PP showed improvement in the overall histopathological picture of the brain, mild demyelination of neuropil and astrocytosis,

4. Discussion

Encephalopathy is a hallmark symptom in patients suffering from hyperammonemia which mostly developed as a result of liver pathology such as cirrhosis, liver failure, and some circulatory abnormalities with portosystemic shunting (Tarantino et al. 2009a). However, several non-hepatic causes of hyperammonemia have been also reported (Walker 2012).

In the current study, acute HE was induced in rats by TAA (300 mg/kg, i.p.) and proved to be a reliable and satisfactory model of HE. TAA eventually lead to significant impairment of hepatic tissue integrity, excretory and synthetic functions, along with significant increase in serum ammonia concentration as well as hepatic oxidative stress. Thioacetamide- S- dioxide, a highly reactive compound that resulted from TAA metabolism, binds to the tissue macromolecules causing hepatic necrosis (Reddy et al. 2004). Results of the current study showed an increased level of ammonia (table 1). Hyperammonemia, the main indicator of liver failure and consequently is the main etiology to develop encephalopathy, affects the mitochondrial function resulting in reduction of ATP synthesis and enhances free radicals production (Bachmann 2002). Moreover, elevation in the serum ALT, AST levels, and ALP activities in TAA-treated rats was recorded indicating membrane damage and releases of these enzymes into circulation. Meanwhile, brain oxidative stress biomarkers were significantly elevated. Our study emphasized the occurrence of liver and brain oxidative damage due to TAA administration, which is manifested by elevated MDA levels as well as decreased GSH levels in liver and brain (table 2). This data is in agreement with Bruck et al. (2004) and Mustafa et al. (2013). Moreover, the overall histopathological findings of liver and brain tissues in hepato-intoxicated rats showed significant damage to both organs. These

changes were also reported in previous studies (Abdelaziz et al. 2015; Farjam et al. 2012).

In accordance with our study, Túnez et al. (2005) reported that TAA corrupted the antioxidant defense and enhanced lipid peroxidation in liver and brain tissues. Similarly, Bastway Ahmed et al. (2010) confirmed the induction of oxidative stress through significant elevation of MDA levels and significant decline of GSH in liver rats after TAA administration.

Neurodegenerative diseases are associated with excessive generation of ROS, increased production of proinflammatory mediators and cytokine in addition to mitochondrial dysfunction which is a critical player in such conditions. The brain is considered highly vulnerable to oxidative stress because it consumes a high level of oxygen, contains high levels of polyunsaturated fatty acids and has low levels of antioxidant enzymes (Öztürk et al. 2008). In patients with HE, a spectrum of potentially reversible neuropsychiatric abnormalities could be obtained after exclusion of unrelated neurologic and/or metabolic abnormalities. Thus, the main target in order to manage HE is to prevent ROS damage and decrease the elevated blood ammonia level. In this concern, propolis was reported to have in vitro and in vivo neuroprotective properties through its antioxidant, anti-inflammatory and immunomodulatory actions (Farooqui and Farooqui 2012). As shown in the present study, PP significantly decreased serum ammonia concentration and decreased the elevated serum AST and ALT activities (table 1), significantly decreased brain and liver MDA content and elevated GSH concentrations as compared to hepatotoxic control group (table 2). The histopathological picture of both liver and brain tissues of rats treated with PP supported this data by improvement of the overall histopathological pictures (figure 3 and 4).

Chemically, PP mainly consists of resin (50%-60%), wax 30%-40%, essential oils (5%-10%), and pollen (5%), besides microelements (Marcucci 1995). PP also contains various organic compounds such as phenols, tannins, polysaccharides, terpenes, aromatic acids, and aldehydes (Nakamura et al. 2013).

Our results come in accordance with previous studies that reported the protective effect of PP against hepatotoxicity due to its high content of total phenolic compounds (Bhadauria, 2012; Saleh 2012). El-Mahalaway et al. (2015) reported a marked enhancement of biochemical parameters, histological changes of liver following PP administration. Moreover, PP possess strong antioxidant properties and acts as a free-radical scavenger and lipid peroxidation inhibitor. Furthermore, it increased reduced GSH concentration (Newairy and Abdou 2013). Likewise, PP improved the activity of hepatic microsomal metabolizing enzymes (Mahmoud and Mahmoud 2013).

Classically, the pathogenesis of most hepatotoxic agents based on their metabolism and formation of toxic compounds, mainly by cytochrome P450 (CYP450). Although, other pathway such as mitochondrial dysfunction and apoptosis have been considered to be further mechanisms (Tarantino et al. 2009b).

The hepatoprotectivity of PP was previously explained by its potent inhibitory effects on CYP2E1, a CYP450 isoform that plays a major role in the metabolism of endogenous compounds as well as xenobiotics (Ryu et al. 2016).

Administration of vitamin E counteracted TAA-induced hyperammonimia and normalized AST, ALT and ALP activities (table 1). Moreover, vitamin E restored the antioxidant capacity in both liver and brain (table 2). These data were further confirmed by the improved histopathological pictures of the liver and the brain (figure 3 and 4). Our findings are in agreement with those reported by Cuce et al. (2015). Vitamin E is a main non-enzymatic antioxidant which acts as a potent ROS scavenger (Traber and Atkinson 2007). Vitamin E is a chain-breaking antioxidant that prevents the propagation of free radicals in membranes and serum lipoproteins (Saad et al. 2014).

There is a massive evidence of a direct involvement of the cellular oxidative damage in the activation of apoptosis. Overproduction of oxidizing species results in mitochondrial dysfunction with loss of mitochondrial membrane potential, cytochrome *c* release from the mitochondria into the cytosol, triggering caspase activation and subsequent apoptosis (Tarantino et al. 2011).

DNA fragmentation is considered a marker typical and characteristic feature of apoptosis (Elmore 2007). According to our findings, HE induced by TAA significantly resulted in increased DNA fragmentation in brain (table 3). Comet assay was used to confirm the

results of DNA fragmentation. The comet assay is a highly sensitive method which can detect various lesions of DNA damage that affects the cell integrity accordingly causing cell death (Sreekumaran et al. 2005). This study revealed that TAA-induced HE significantly induced brain DNA damage manifested by the elevated tail moment and extensive bright comet tail compared to control group (figure 1). In this context, Afifi et al. (2016) observed increase in DNA fragmentation in the hepatocytes after TAA injection.

PP significantly reduced DNA fragmentation as detected at the two examined dose levels, in a dose dependent manner (table 3). The anti-oxidative property of phenolic compounds and flavonoids of PP might explain our results by way of inhibiting the formation of free radicals and reducing the oxidative DNA damage. These results are line with the findings of previous studies (Shalaby and Saleh 2011).

Hyperammonemia induces microglial activation and inflammation and contribute to the motor and cognitive alterations that occur during HE (Rodrigo et al. 2010). Growing data suggested the pathophysiological role of nitric oxide synthesized by iNOS in the brain (Yamada et al. 1999). Nitric oxide (NO) plays a relevant role in the development of HE as the excessive NO production in brain leads to cerebral vasodilatation which increases the capillary surface area and facilitates the diffusion of ammonia (Huang et al. 2007). On the other hand, hyperammonemia is accompanied with increased blood brain barrier permeability to the proinflammatory cytokines which induce the expression of iNOS.

According to our present study, the significant elevation of iNOS mRNA expression level in hepatotoxic group (figure 2) may be attributed to the neuroinflammatory reaction occurred in TAA intoxicated rats. This result was in agreement with previous reports detected the association between overexpression of iNOS and brain dysfunction (Ogaly et al. 2015; Rodrigo et al. 2010; Yamada et al. 1999).

PP was found to have inhibitory effect on iNOS (figure 2). These findings come in accordance with those of Song et al. (2002) who suggested that PP exerts its anti-inflammatory effect by inhibiting nitric oxide synthase gene expression and enzyme activity. The mechanism of iNOS inhibition included the action on the NF-kappa B sites in the iNOS promoter and hence iNOS gene downregulation and by the direct inhibition of the catalytic activity of iNOS.

PP was proven to have potent anti-inflammatory and immunomodulatory effects since it inhibited neutrophil infiltration, suppressed proinflammatory cytokines as tumor necrosis factor- α and interleukins. It also caused stimulation of macrophages and thus prompting specific and nonspecific immune defense mechanisms (Nakamura et al. 2013).

5. Conclusions

In light of all findings, the current study suggest that P can evidently inhibit hepatic encephalopathy, which might be related with improving hepatic function, decreasing serum ammonia, attenuating oxidative stress, and inhibiting DNA damage. One of the underlying mechanisms of Biopropilis hepatic and neuro protective effects may be its ability to down-regulate iNOS gene expression. PP could be a promising complementary treatment to combat hepatic encephalopathy.

6. Conflict of interest

Declared none.

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Table (1): Effects of biopropolis on serum biochemical parameters of TAA induced hepatic encephalopathy in rats.

Group	Ammonia ($\mu\text{g/dl}$)	AST (U/mL)	ALT (U/mL)	ALP (IU/L)
Group 1	108.7 ^b ±8.73	143.9 ^b ±4.25	102.8 ^b ±2.87	85.3 ^b ±6.18
Group 2	204.1 ^a ±14.38	308.9 ^a ±6.70	270.4 ^a ±6.90	281.7 ^a ±17.43
Group 3	130.7 ^b ±5.26	214.38 ^{a,b} ±18.48	160.48 ^{a,b} ±11.71	118.9 ^b ±12.64
Group 4	127.4 ^b ±2.76	265.8 ^{a,b} ±12.05	164.48 ^{a,b} ±4.79	290.7 ^a ±30.46
Group 5	120.6 ^b ±7.88	223.38 ^{a,b} ±18.92	149.58 ^{a,b} ±9.02	306.0 ^a ±28.50

Group 1: Normal control group received only saline and paraffin oil (5 ml/kg, p.o), **Group 2:** hepatotoxic control group received TAA (300 mg/kg, i.p.), **Group 3:** TAA plus vitamin E (100 mg/kg), **Group 4:** TAA plus biopropolis (100 mg/kg), **Group 5:** TAA plus biopropolis (200 mg/kg). Data are presented as mean $\pm$ SE, ($n=12$). Statistical analysis was carried out by one-way analysis of variance followed by Dunnett's test

^a $p < 0.05$: Statistically significant from normal control.

^b $p < 0.05$: Statistically significant from hepatotoxic control.

Table (2): Effects of biopropolis on liver and brain oxidative stress parameters of TAA induced hepatic encephalopathy in rats.

Group	Liver		Brain	
	MDA (nmol/g tissue)	GSH (μ mol/g tissue)	MDA (nmol/g tissue)	GSH (μ mol/g tissue)
Group 1	30.1 ^b $\pm$ 1.43	10.1 ^b $\pm$ 0.76	8.1 ^b $\pm$ 0.74	1.1 ^b $\pm$ 0.09
Group 2	52.9 ^a $\pm$ 1.12	5.9 ^a $\pm$ 0.23	18.5 ^a $\pm$ 0.82	0.6 ^a $\pm$ 0.04
Group 3	33.1 ^b $\pm$ 1.17	10.8 ^b $\pm$ 0.80	9.6 ^b $\pm$ 0.32	1.0 ^b $\pm$ 0.11
Group 4	33.8 ^b $\pm$ 0.63	9.3 ^b $\pm$ 0.66	9.1 ^b $\pm$ 0.69	0.9 $\pm$ 0.09
Group 5	31.5 ^b $\pm$ 1.25	10.8 ^b $\pm$ 0.19	8.7 ^b $\pm$ 0.26	1.3 ^b $\pm$ 0.14

Group 1: Normal control group received only saline and paraffin oil (5 ml/kg, p.o), **Group 2:** hepatotoxic control group received TAA (300 mg/kg, i.p.), **Group 3:** TAA plus vitamin E (100 mg/kg), **Group 4:** TAA plus biopropolis (100 mg/kg), **Group 5:** TAA plus biopropolis (200 mg/kg). Data are presented as mean $\pm$ SE, ($n=12$). Statistical analysis was carried out by one-way analysis of variance followed by Dunnett's test

^a $p < 0.05$: Statistically significant from normal control.

^b $p < 0.05$: Statistically significant from hepatotoxic control.

Table (3): Influence of biopropolis administration on the DNA fragmentation percentage in rats with Hepatic encephalopathy

	Group 1	Group 2	Group 3	Group 4	Group 5
DNA fragmentation %	14.69 ^a ±4.67	27.8 ^b ±2.34	18.26 ^a ±2.59	21.55 ^c ±2.11	19.14 ^c ±3.2

Group 1: Normal control group received only saline and paraffin oil (5 ml/kg, p.o),

Group 2: hepatotoxic control group received TAA (300 mg/kg, i.p.), **Group 3:** TAA plus vitamin E (100 mg/kg), **Group 4:** TAA plus biopropolis (100 mg/kg), **Group 5:**

TAA plus biopropolis (200 mg/kg). Values are expressed as the mean± S.D. Different superscript letters in the same row indicate significant differences ($p < 0.05$).

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Table (4) Tail length, tail intensity, and tail moment measured with comet assay in brain cells of rats treated with TAA and neuroprotective influence of biopropolis

Group	Comet %	Tail length (µm)	% DNA in tail	Tail moment
Group 1	15.4	12.6 ^a ±2.49	12.35 ^a ±0.95	0.91 ^a ±0.38
Group 2	25.9	19.6 ^b ±5.64	22.7 ^b ±2.53	3.6 ^b ±1.47
Group 3	17.6	11.6 ^a ±5.00	15.3 ^a ±5.18	1.50 ^a ±0.29
Group 4	21.1	16.7 ^c ±3.55	21.69 ^b ±2.68	2.9 ^c ±0.74
Group 5	18.4	14.8 ^a ±2.77	17.6 ^c ±2.25	1.67 ^a ±0.17

Group 1: Normal control group received only saline and paraffin oil (5 ml/kg, p.o), **Group 2:** hepatotoxic control group received TAA (300 mg/kg, i.p.), **Group 3:** TAA plus vitamin E (100 mg/kg), **Group 4:** TAA plus biopropolis (100 mg/kg), **Group 5:** TAA plus biopropolis (200 mg/kg). Values are expressed as the mean± S.D. Different superscript letters in the same column indicate significant differences (*p*< 0.05).

Figure captions

Figure 1: Apoptotic DNA damage detected by comet assay; (A) normal control group showed intact head without tail, (B) hepatotoxic control group received TAA (300 mg/kg, i.p.) showed a bright comet tailing, (C) group 3 received TAA plus vitamin E (100 mg/kg) showed decreased comet percent, (D) group 4 received TAA plus biopropolis (100 mg/kg) and (E) group 5 TAA plus biopropolis (200 mg/kg) showed the neuroprotective influence of biopropolis on brain DNA.

Figure 2: Real-time PCR quantitation of mRNA expression level of iNOS gene in brain tissue of different experimental groups. Group 1 (**G1**): Normal control group received only saline and paraffin oil (5 ml/kg, p.o), Group 2 (**G2**): hepatotoxic control group received TAA (300 mg/kg, i.p.), Group 3 (**G3**): TAA plus vitamin E (100 mg/kg), Group 4 (**G4**): TAA plus biopropolis (100 mg/kg), Group 5 (**G5**): TAA plus biopropolis (200 mg/kg). Values are expressed as mean $\pm$ S.E. Different superscripts letters are significantly different ($p < 0.05$).

Figure 3: Photomicrographs of liver sections (H and E X 400), prepared from a rat of (A) normal control group, showing normal architecture of the liver, (B) hepatotoxic control group showing severe congestion of blood vessels, activated Kupfer cells and enlarged hepatocytes with disappearance of sinusoids, (C) biopropolis (100 mg/kg) showing slight improvement in multiple areas of coagulative necrosis which was replaced or displaced by inflammatory cells; mainly neutrophils and macrophages, (D) biopropolis (200 mg/kg) showing improvement in the overall histopathological picture of the liver. Focal areas of coagulative necrosis were replaced and displaced by inflammatory cells especially around

the central veins, **(E)** Vitamin E (100 mg/kg) showing improvement in necrosis in the form of small areas of coagulative necrosis especially around the central veins.

Figure 4: Photomicrographs of brain sections prepared from a rat of **(A)** normal control group showing normal structure of the brain, **(B)** hepatotoxic control group with pathological lesions represented by significant demyelination, the presence of numerous astrocytes with prominent nuclei, significant increase in vacuolization, **(C)** biopropolis (100 mg/kg) showing improvement of demyelination of nerve fiber where nerve cells appeared slightly necrosed with neurophagia in which the glial cells surround the necrosed nerve cells, **(D)** biopropolis (200 mg/kg) showing improvement in the overall histopathological picture of the brain, mild demyelination of neuropil and astrocytosis, **(E)** Vitamin E (100 mg/kg), (H and E X 400) showing mild congestion of meningeal blood vessels.

Figure 1

Figure 2

Figure 3

Figure 4