Histopathologic Changes in Liver and Kidney Tissue from Chronic Unpredictable Mild Stress-induced Stress-Depression Male Sprague-Dawley Rats Supplemented with Acacia Honey

Anis Syamimi Mohamed[1], Abdah Md Akim[2], Kalavathy Ramasamy[3], Mizaton Hazizul Hasan[1], and Zolkapli Eshak[1]*

[1] Department of Pharmacology and Chemistry, Faculty of Pharmacy, Universiti Teknologi MARA (UiTM), Malaysia.
[2] Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Malaysia.
[3] Department of Pharmaceutical and Life Sciences, Faculty of Pharmacy, Universiti Teknologi MARA (UiTM), Malaysia.

Abstract

Stress-depression disorder comprised of 29 % and 12 % among adults and children in Malaysia, respectively. It involves a series of pathological stages associated with weight loss and anhedonia. Previous studies on stress-depression model mainly focused on changes at molecular level. Therefore, this study aims to investigate the sucrose preference activity, body weight changes and histopathological changes in kidney and liver of stress-depression disorder induced-rats by chronic unpredictable mild stress (CUMS) model supplemented with acacia honey (AH). Eighteen male Sprague-Dawley rats were divided into three groups [(1) normal control (NC), (2) CUMS-induced stress-depression and (3) CUMS supplemented with AH] and were subjected to sucrose preference test (SPT). The body weight was recorded weekly whereas the liver and kidney were collected at the end of the experiment. The results showed CUMS-induced stress-depression rats (28.60 % ± 1.86) had a significant decrease in the percentage of body weight whereas CUMS supplemented with AH demonstrated no significant changes (31.34 % ± 1.88, $p > 0.05$) compared to NC (38.60 % ± 2.67). SPT demonstrated a significant increase in the sucrose preference ratio for CUMS supplemented with AH group (83.67 % ± 1.20) compared to CUMS-induced rats (44.33 % ± 12.17). CUMS-induced rats had abnormal histopathology for kidney and liver compared to NC and AH supplemented groups. In conclusion, AH improved body weight, sucrose preference ratio and protect the liver and kidney of the stress rats against abnormal histopathological changes.

Keywords: Acacia honey, Histopathology, CUMS-induced stress-depression model.

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*Corresponding author: Zolkapli Eshak; email: zolkapli_eshak@puncakalam.uitm.edu.my

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Introduction

Mental health disease is the second leading disability in the world. It accounted for 15 % of the global disease burden [1] and gradually increases due to the change in lifestyle that influences many aspects such as social competition, employment and interpersonal communication [2]. In Malaysia, Health Morbidity Survey reported that approximately 30 % of adults citizen and 12 % of children experienced mental health issues such as anxiety and depression [3]. Major risk factors for mental health diseases are related to stress disorder [1] that linked to other morbidity diseases such as obesity, depression, hypertension and cancer [4–6].

Stress-depression is clinically characterized by a continuous depressed mood, inability to feel pressure and anxiety [6]. Thus far, the best treatment option for patients with stress-depression is a combination of antidepressants and psychotherapy which is prescribed based on their symptoms and illness [7]. However, the antidepressants have various side effects such as weight gain, constipation, dizziness and headache leading to patients incompliant to the treatments [8]. Apart from that, the drugs are not suitable for patients with chronic heart disease and are known to cause liver injury [9] and increase the risk of bone fracture [10]. Therefore, it is important to investigate for a potential supplement that can significantly reduce stress-depression disorder by focusing more on natural products.

Malaysians usually consume honey for general well-being and as an alternative supplement to treat various types of diseases such as cancer [11,12] and obesity [13]. Honey is preferred over other supplements as it has a pleasant taste and available all year round. A preliminary study shows that Acacia honey (AH) able to reduce stress-depression disorder. However, the study only emphasizes on physical parameters [14]. Whereas, other studies focused on biochemical analysis [2, 10]. Due to limited scientific publications, this study was designed to investigate the effect of AH on stress-depression disorder by using CUMS model through structural changes of the kidney and liver. This study hypothesized that AH protects the kidneys and liver during stress-depression disorder induced by CUMS model.

Materials and Methods

Materials

All chemical and reagent used was purchased from GIBCO (Waltham, USA) and Sigma-Aldrich (St. Louis, USA) unless otherwise stated.

Methods

Honey collection

In this study, AH used was obtained from a local Malaysian beekeeper in Kluang, Johor (July 2017) and stored in the dark at 2 - 4°C prior to the study. The physicochemical test was conducted to determine the authenticity and quality of the AH [16].

MTT assay

The WRL-68 cell line was seeded (2.0 x 10^4 cells/ well in 100 µl of DMEM supplemented with 10% foetal bovine serum and 1% Penicillin-streptomycin) in a 96 well plate. The cell was then subjected to a series of AH dilution (0 - 100 % v/v) for 24, 46 and 72 hrs. The MTT solution (1.6 mg/mL) was then added (50 µL/ well) to each well and incubated for 4 hrs in the dark (5 % CO_2 atmosphere, 37 °C). Subsequently, the solution was discarded, replaced with DMSO (200 µL) and analyzed at 520 nm by using a microplate reader (Perkin Elmer, USA).

Experimental animal

The procedures were conducted in accordance to the Animal Research and Ethics Committee of the Universiti Teknologi MARA (UiTM), Puncak Alam Campus, Faculty of Pharmacy [UiTM CARE: 237/2/2018 (6/4/2018)]. The *in-vivo* study was conducted in accordance to Abidin et al. [14] with slight modifications. In brief, Sprague-Dawley male rats (150-170g) age between 8 to 12 weeks were obtained from Laboratory Animal Facility and Management (LAFAM) and acclimatized for 7 days (3rd floor LAFAM). The male rats were used due to inconsistent results obtained by our research group using female rats [13]. Prior to the experiments, the randomly selected rats were caged individually and fed with rat chow and reverse osmosis water *ad libitum* while being maintained under standard laboratory conditions (24 °C ± 1, 45 % ±15 relative humidity and 12h light/dark cycle).

The rats were divided into three group; (1) normal control (NC, n = 6), (2) CUMS-induced stress-depression (CUMS, n = 6) and (3) CUMS supplemented with AH (CUMS + AH, n = 6). The rats were subjected to CUMS with random stressors daily. Stressors were randomized by using Research Randomiser software version 4.0. Stressors include overnight illumination, cage tilting, forced swimming, white noise, damp bedding and restricted movement [11, 14].

The body weight, food and water intake were recorded weekly throughout the experimental period. Apart from that, the rats were subjected to sucrose preference test (SPT) to investigate the anhedonia behaviour on day 28. SPT was conducted according to Zhang et al. [17] with slight modification. In brief, the rats were provided with RO water and 1% sucrose solution for 24 hours. Sucrose preference was calculated as a ratio volume of sucrose solution consumption to the volume of total fluid intake. The rats are classified as anhedonia if sucrose preference is less than 65 % after continuous stress application [18].

On day 30, the rats were euthanized by overdose injection of sodium pentobarbital (100mg/mL). The rats were subjected to cardiac puncture for hematology analysis and haemolysis assay while serum was used to measure the liver and kidney enzymes. The liver and kidney were fixed in 10% buffered formalin.

Histopathological analysis

The fixed liver and kidney were embedded in paraffin, sectioned (5 µm), stained with Hematoxylin and Eosin (H & E) and mounted by using DPX. The slides were viewed under a compound microscope (Leica, Germany) and the representative images were examined. Changes observed in the liver and kidney sections were graded as follows: (0), (1), (2), and (3) indicating no changes, mild, moderate and severe changes, respectively.

Haemolysis assay

The haemolysis assay was conducted according to Okoko and Ere [19] to investigate the protective effect of AH against free radicals, hydrogen peroxide (H_2O_2). In this experiment, the blood collected from each group was spun at 4000 rpm at 4 °C for 10 min. The plasma and buffy coat were discarded followed by the washing of erythrocytes with phosphate-buffered saline, PBS (0.2 M, pH 7.4). The packed cells were re-suspended in PBS to produce 5 % erythrocytes suspension. The mixtures were then incubated with or without H_2O_2 (10 mM, 0.5 mL) for 3 hrs at 37°C. Subsequently, PBS (6 mL) was added into a test tube containing the mixtures, spun at 2000 g for 10 min at 4 °C. The absorbance of the mixture was analyzed at 540 nm using SmartSpec^{TM} Plus spectrophotometer (Bio-Rad, California).

Statistical analysis

Results obtained were presented as the mean and standard error of mean (SEM) after three or more independent experiments were performed. The values were considered to be parametric and analyzed using two-way ANOVA. A mean difference was considered significant when $p < 0.05$. Statistical analysis was performed using GraphPad Prism version 8.0 (GraphPad Sofware, San Diego, California).

Results and Discussions

Effect of Acacia honey on normal Liver Cell Line (WRL-68)

Figure 1 represent the survival rate and log-dose response curve of MTT [3-(4,5-dimethyltthiazol-yl)-2,5-diphenyltetrazolium bromide] assay for WRL68 cells in response to AH. The figure showed significant decrease in survival rate of cells ($p < 0.05$) were noted at dose 1.56 (68.67 % ± 7.67), 3.13 (35.33 % ± 2.33), 6.25 (18.00 % ± 2.08), 12.50 (8.00 % ± 0.58), 25.00 (4.67 % ± 0.33), 50.00 (5.00 % ± 0.58) and 100.00 % (6.00 % ± 0.58) compared to the untreated group after 24 hrs of AH exposure. No significant different was observed at concentration of 0.10 (95.33 % ± 1.33), 0.20 (88.33 % ± 10.87), 0.40 (82 % ± 11.27) and 0.80 % (81.33 % ± 7.97) ($p > 0.05$). In addition, similar pattern was observed for 48 and 72 hrs of treatment with AH.

Based on the current data, it can be concluded that the reactions were time and dose-dependent manner. The inhibition of 50% cell viability (IC_{50}) for WRL-68 cells was obtained at 2.48, 2.53, and 1.26 % of AH honey treatment after 24, 48 and 72 hrs (Figure 1), respectively. The cytotoxic effect of honey is varied between the type of honey and cell type [12]. Previous studies reported that *Heterotrigona itama sp.* honey IC_{50} was at 10 % for malignant glioma cells [20] while 6.31% and 5.00 % for MCF-7 cells treated with Gelam and Tualang honey [12], respectively.

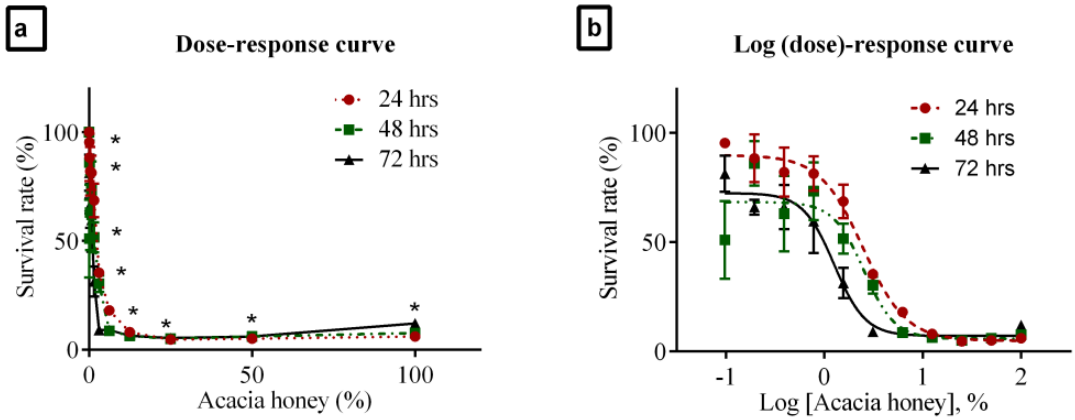

Figure 1: Response Curves of WRL-68 Cell Line Treated with Acacia Honey Using MTT Assay. (a) A dose-response curve was created by GraphPad prism software and subsequently transformed to obtain the sigmoidal shape of (b) log (dose)-response curve. This curve was used to determine the IC_{50} of Acacia honey. *Indicates statistical significance ($p < 0.05$) compared with untreated comparing by using 2-way ANOVA, with Bonferroni post hoc test, n = 3 independent observations).

Body Weight Changes, Food, Water and Sucrose Intake

In this study, the percentage of body weight shows a significant increase throughout the experimental period for all groups ($p < 0.05$). In comparison on day 28, CUMS-induced rats (CUMS group) exhibited (28.60 % ± 1.86) the lowest body weight percentage compared to NC group (38.60 % ± 2.67, $p < 0.05$). Supplementation with AH (CUMS + AH group) increases the percentage of body weight changes (31.34 % ± 1.88). No significant changes were observed between CUMS + AH group when compared with NC group ($p > 0.05$) (Figure 2a).

The current data was coherent with previous studies by Abidin et al. [14] and Jaime et al. [21]. Body weight changes during stress can be interpreted as depressive-like states in rats mimicking one of the symptoms of the human disorder (*viz.* anxiety disorders). Continuous stress can lead to low weight gain due to hyperglycemia and insulin resistance [14]. Hyperglycemia is the immediate response to stress as it becomes the source of energy. High glucose level in the blood increases the activities of the hepatic enzyme as well as insulin to maintain homeostasis [22]. However, prolonged exposure to stress lead to insulin resistance and failure to tolerate glucose [22] which activates lipolysis [23] and subsequently reduces bodyweight.

No significant changes were observed in all groups from week 1 to week 3 for food intake. Apart from that, CUMS group (477.50 g ± 19.50) was demonstrated to have significantly low food intake when compared to NC group (563.50 g ± 4.50). However, CUMS + AH group has a significant increase in food intake (581.00 g ±7.00) on week 4 (Figure 2b). For water intake, the data revealed no significant changes between groups (Figure 2c) throughout the experiment ($p > 0.050$). Stress response has been reported to activate corticotrophin-releasing factor (CRF), which binds to CRF1 and CRF2 receptors resulting in decreasing activity (exploration), appetite and body weight while increasing despair as well as disrupting sleep due to the poor food intake [22].

Anhedonia is the main symptom of major depressive disorder whereby it is defined as a loss of interest or inability to feel pleasure. These symptoms are usually associated with "social withdrawal" [24]. SPT is a test used to investigate the behavioral response of the animal to a reward. In this study, CUMS group (44.33 % ± 12.17) has low sucrose intake compared to NC group (78.00 % ± 3.21). However, the opposite effect was noted in CUMS + AH group (83.67 % ± 1.20) ($p < 0.05$). The reduction of sucrose consumption during stress indicates the presence of anhedonia in CUMS model which is similar to other previous studies [11, 15, 19]. Stress response was reported to cause oxidative damage to the animals by activating various pathways [26,27]. As honey is known to have high antioxidant properties [13,16,28,29], it is shown to reduce the level of plasma corticosterone and adrenocorticotropic levels to baseline or suppress HPA mobilization in stress rats [26].

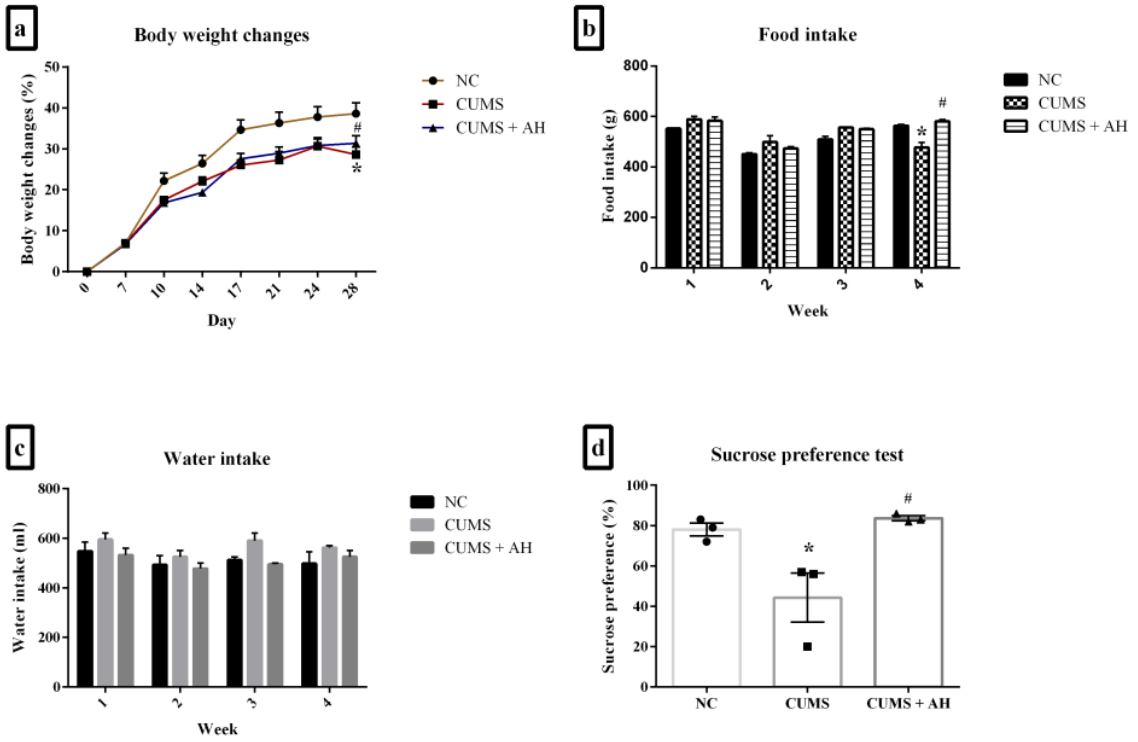

Figure 2: Bodyweight changes, food, water and sucrose intake. On day 28, the CUMS-induced rats show a significant reduction ($p < 0.05$) in bodyweight percentage, food and sucrose intake. Supplementation with AH on CUMS-induced stress-depression rats significantly increases bodyweight percentage, food and sucrose intake ($p < 0.05$). *Indicates statistical significance ($P < 0.05$) compared with NC. # Indicates statistical significance ($P < 0.05$) compared with CUMS comparison by using 1-way ANOVA or 2-way ANOVA, with Bonferroni post hoc test, n = 6 independent observations).

Blood haematology and biochemistry

Biochemistry analysis revealed a significant decrease of AST level in CUMS + AH group when compared to CUMS group ($p < 0.05$). Apart from that, the level of AST for CUMS + AH group was found to be similar to NC group (Table 1). The level of AST in the blood is used as a quantitative evaluation for liver function. Study by Zhai *et al.* [30] uses the AST level as an indicator to prove the protective effect of chicken meat extract against restraining stress-induced liver damage in mice. Multiple studies reported that stress can act as oxidative stress

which in turn damaging the structure of the liver [20-22]. Haematology analysis shows no significant changes between groups as presented in Table 2.

Table 1: Biochemistry level in rats.

Parameters	NC	CUMS	CUMS +AH
Na^+ (mmol/L)	135.60 ± 3.19	138.00 ±1.90	140.16 ± 1.08
K^+ (mmol/L)	7.28 ± 0.41	6.93 ± 0.29	7.42 ± 0.73
Cl^- (mmol/L)	95.40 ± 1.81	96.67 ± 1.45	98.00 ± 0.81
ALP (U/L)	92.20 ± 8.64	116.67 ± 10.37	99.50 ± 8.66
AST (U/L)	167.80 ± 6.12	213.67 ± 32.90 *	183.17 ± 30.24
GGT (U/L)	4.67 ± 1.67	12.00 ± 10.00	14.00 ± 0.00
Glucose (mmol/L)	13.02 ± 1.54	15.30 ± 1.99	13.03 ± 0.70
Total Billirubin (mmol/L)	3.78 ± 0.23	4.02 ± 0.16	3.95 ± 0.15
Creatinine (mmol/L)	45.60 ± 1.69	47.50 ± 3.24	48.50 ± 5.27
Urea (mmol/L)	5.84 ± 0.43	8.10 ± 0.75	7.90 ± 0.61
ALT (U/L)	49.20 ± 6.35	72.17 ± 11.37	50.17 ± 4.70

*Indicates statistical significance ($P < 0.05$) compared with NC comparison by using 2-way ANOVA, with Bonferroni post hoc test, n = 6 independent observations).

Table 2: Haematology level in rats.

Parameters	NC	CUMS	CUMS +AH
WBC ($x10^9$/L)	7.37 ± 0.36	4.28 ± 0.04	3.76 ± 0.35
RBC ($x10^{12}$/L)	8.4 ± 0.09	8.01 ± 0.19	8.53 ± 0.11
Hb (g/L)	154.33 ± 0.67	145.00 ± 2.31	154.00 ± 1.16
PCV (L/L)	0.45 ± 0.02	0.47 ± 0.01	0.50 ± 0.01
MCV (fL)	54.00 ± 1.53	57.67 ± 0.33	58.00 ± 0.58
MCHC (g/L)	230.43 ± 14.48	312.67 ± 1.43	311.00 ± 0.58
PLT ($x10^9$/L)	786.50 ± 54.50	926.00 ± 75.00	867.50 ± 11.50

Effect of hydrogen peroxide on blood haemolysis of the rats

A significant decrease in blood haemolysis was observed in CUMS (70.48 % ± 2.42) and CUMS + AH group (14.30 % ± 2.96) compared to NC group (80.82 % ± 4.636). The data show the protective effect of AH against H_2O_2 (Figure 3). This is due to the presence of high phenolic and flavonoid content in AH as demonstrated in our previous study [33]. Apart from that, AH was demonstrated to have a high scavenging capacity [33]. The protective effect of AH was due to the binding of the flavonoids to the blood cell membrane preventing H_2O_2 from attacking the erythrocyte membrane [34].

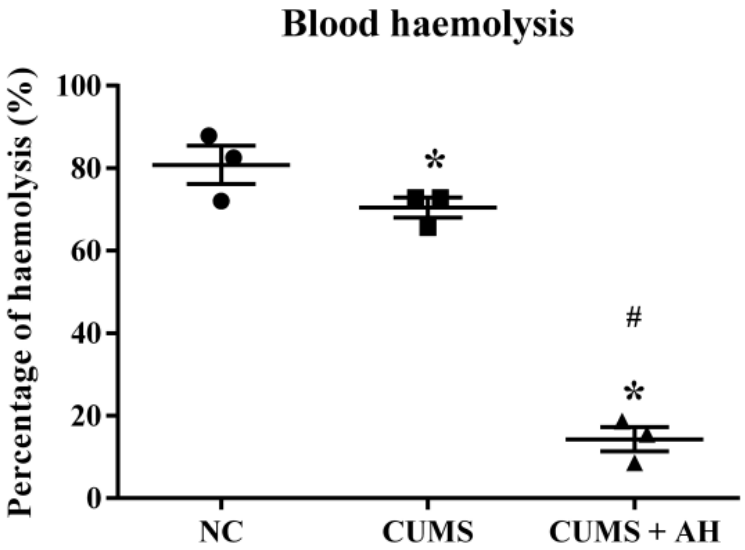

Figure 3: Percentage of blood haemolysis-induced by H_2O_2 after 28 days supplemented with Acacia honey. AH protects the blood against H_2O_2-induced lysis indicated by a low percentage of haemolysis in CUMS + AH group compared to CUMS group. *Indicates statistical significance (P < 0.05) compared with NC group. # Indicates statistical significance (P < 0.05) compared with CUMS group comparison by using 1-way ANOVA, with Bonferroni post hoc test, n = 3 independent observations).

Histopathology changes of liver and kidney

The results of histopathology changes in liver tissues are summarized in Table 3. The liver tissue of NC group demonstrated a normal structure of central vein (CV), sinusoidal (S) and Kupffer cell (KC) as shown in Figure 4 (a). Histological liver examination of CUMS group reveals the presence of cytoplasmic vacuolation (Figure 4b). Previous study has reported that an increase in serum AST, ALT and GGT levels reflects the loss of structural integrity of the liver [35] which is in alignment with the current findings. AST is released into the bloodstream due to hepatocellular degeneration and necrotic changes leading to the elevation of serum AST level [35]. However, in this study, CUMS group show the presence of mild cytoplasmic vacuolation without any hepatocellular degeneration and necrotic changes. Cytoplasmic vacuolization in mammalian cells can be transient or irreversible. The transient vacuolization can only be observed during the exposure to an inducer and reversibly affects the cell cycle and migration. Meanwhile, cytoplasmic vacuolation was not observed in CUMS+AH group (Figure 4c). These findings suggest that AH honey may have protective effects towards stress-induced hepatocellular damage.

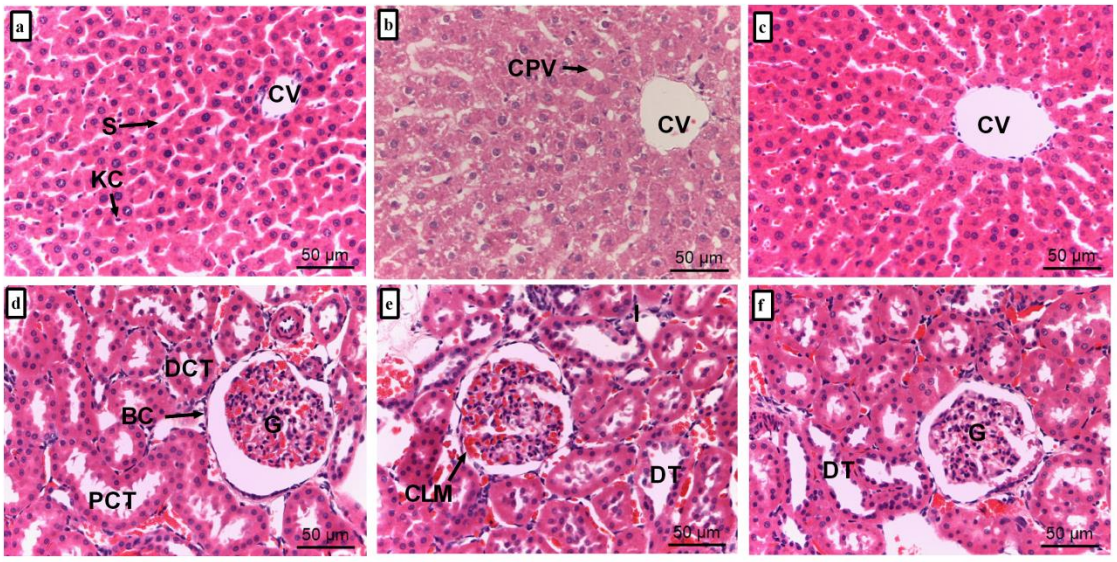

Figure 4: Histological section of liver and kidney tissue of group (a, c) normal control, (b, e) CUMS-induced stress rats and (c, f) CUMS-induced stress rats with AH. CV, central vein; S, sinusoidal; KC, Kupffer cell; CPV, cytoplasmic vacuolation; G, glomerulus; BC, bowman's capsule; PCT, proximal convoluted tubules; DCT, distal convoluted tubules; CLM, cell lining missing; DT, dilated intertubular capillaries; I, inflammation.

Meanwhile, histology of kidney tissue for NC group reveals intact glomerulus (G), bowman's capsule (BC), proximal (PCT) and distal convoluted tubules (DCT). However, the CUMS group demonstrated the presence of dilated intertubular capillaries, inflammation and absence of cell lining (Figure 4e). Similar finding was observed in CUMS+AH group (Figure 4f) with significantly reduce abnormal morphology characteristics ($p < 0.05$) compared to CUMS group. The presence of abnormal structure in stress rats was associated with the overproduction of reactive oxygen species [36] leading to activation of IL-1 [37], IL-6 [37], COX-2 [38] and TNF-α [38]. The protective effect of honey on renal tubular has been shown previously against cadmium through the ability of honey in reducing lipid peroxidation and increasing tissue levels of glutathione and glutathione peroxidase activity [36].

Table 3: Lesion scores of kidney and liver

Organ	Histopathology observation	NC	CUMS	CUMS +AH
Liver	Cytoplasmic vacuolation	0	1.40 ± 0.25 *	0.40 ± 0.25 #
Kidney	Cell lining missing	0	1.20 ± 0.37 *	0.20 ± 0.20 #
	Dilated inter tubular capillaries	0	1.40 ± 0.51 *	0.60 ± 0.25
	Inflammation	0	2.20 ± 0.20 *	0.60 ± 0.25 #

*Indicates statistical significance ($P < 0.05$) compared with NC. # Indicates statistical significance ($P < 0.05$) compared with CUMS comparison by using 2-way ANOVA, with Bonferroni post hoc test, n = 5 independent observations).

Conclusion

This study shows that AH has a protective role against CUMS-induced stress-depression disorder as its improved body weight, sucrose preference ratio and has a protective effect against abnormal histopathological changes in the liver and kidney. The mechanism of AH in reducing stress-depression disorder mediated by neurotransmitters, hormones and inflammatory cytokines are required for further study.

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Author contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure of conflict of interest

The authors have no disclosures to declare.

Compliance with ethical standards

The work is compliant with ethical standards.

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