

Research Article

A Biochemical Study of Agarwood on Methanol Injection in Rat

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Abstract

Agarwood has antioxidant, anti-inflammatory, neuroleptic and sedative effects along with other benefits. Reactive oxygen species (ROS) are produced during the detoxification of alcohols, xenobiotics and drugs, which generates oxidative stress, inflammation, apoptosis and injuries. The effect of Agarwood extracts on nitric oxide (NO), malondialdehyde (MDA), acetylcholinesterase (AChE), COX-2 (cyclooxygenase-2), LOX (lipoxygenase), caspase-3, TNF- α (tumor necrosis factor- α), monoamine oxidase (MAO) and DNAF (DNA fragmentation) levels in the liver and brain separately in rats treated and untreated with methanol was studied. The injection of rats with methanol caused an elevation in liver and brain NO, MDA, AChE, COX-2, LOX, TNF- α , Caspase-3, MAO and DNAF compared to the control. Treatment of animals with Agarwood extract pre, during and post-methanol administration improved the liver's and brain's biological parameters versus the methanol group. We therefore indicated that Agarwood diminished oxidative stress and apoptosis induced by methanol, and this may be based on a protective effect of Agarwood chemical compositions.

Keywords Agarwood, apoptosis, aquilaria, brain, injuries, methanol, rat

1. Introduction

Methanol is produced endogenously as a result of mediator metabolism. It is also already present in the diet, notably in storage fruit and vegetables and their juices as well as synthetically manufactured beverages, drinks and perfumes. Susceptibility to methanol at levels found in unnatural food would be expected to result in adverse effects if relied on by humans [1, 2]. Side effects may also arise from the intended or accidental ingestion of user products containing methanol, for example, antifreeze, brake fluid and window cleaning solutions.

Methanol is toxic if ingested, inhaled or exposed with dermally, and is readily incorporated by all roads and spread throughout body water undergoing extensive metabolism, however small amounts are eliminated

unchanged by the lungs and in the urine. Methanol toxicity may originally appear in the form of CNS depression, accompanied by an asymptomatic latent period metabolic acidosis and ocular toxicity that could end in blindness, and ultimately manifestations of toxicity coma and death [1, 2, 3].

The liver is the central section of metabolism for methanol, where it is oxidized to formaldehyde (HCHO), methanoic acid (H•COOH, formic acid) and eventually detoxified to carbon dioxide (CO₂) [1]. In humans and primates, the toxicity of methanol is mediated by metabolites and not the parent molecule. Formic acid is considered to be the critical toxicant in an animal with a reduced capacity to metabolize this lethal output toxicity, potentially forming major anion passage metabolic acidosis and neuronal toxicity [2, 4]. Undissociated formic acid immediately crosses the blood–brain barrier directly, leading to CNS toxicity. The best active alkaline therapy for CNS toxicity is to keep formic acid in the dissociated form [3].

Nowadays, natural food and herbs are the best alkaline medium to treat chronic and acute inflammation, as well as they are a wealth of antioxidant compounds. Agarwood (Aquilaria) is from trees local to Southeast Asia, and is aromatic while also being highly valuable heartwood belonging to the Thymelaeaceae family. A light pale color is considered good agarwood while the dark aromatic variety reflects an infected one. Agarwood has many beneficial health effects, including antimicrobial, antirheumatic, anti-convulsant, antiasthmatic, and acting as a carminative diuretic. Moreover, agarwood has laxative effects that accelerate gastrointestinal transit constipation as well as sedative effects and potent central nervous system depressant activities that may be based

on the influence of agarol and agarospiral, respectively [5, 6, 7]. As well, agarwood chemical compounds, like agarofuran, α -eudesmol and guaiol, exhibit anti-tumor effects, protects against brain injury and can be used in skin lightening products, respectively. Selinene has anti-inflammatory effects, and 2-(2-phenylethyl) chromone exhibits cytotoxicity against the human gastric cancer cell line [5].

Even though more investigative works have shown that agarwood is used in daily life in Saudi Arabia, there is insufficient scientific data. Moreover, both methanol and agarwood are featured in perfume composition. The goal of this study is to 1-Determine the effect of agarwood on a healthy liver and brain experiencing methanol's effects. 2-Study the chemoprevention of agarwood (AW) against liver and brain methanol toxicity. We also followed the changes in NO, MDA, ACHE, COX-2, LOX, caspase-3, TNF- α , MAO and DNAF levels in the liver and brain separately in rats treated and untreated with methanol.

2. Materials and Methods

2.1 Chemicals

Arachidonic acid, ethidium bromide, agarose, sulphanilamide, standard sodium nitrite, N-1-Naphthyl ethylene diamine, sodium dodecyl sulfate (SDS), thiobarbituric acid (TBA), tetramethoxypropan (TMP) and diethylene triaminopentaacetic (DTPA) were purchased from Sigma-Aldrich (USA). The caspase-3 assay kit was purchased from BioSource International, Inc. (Camarillo, USA). An AxyPrep. DNA gel extraction and purification kit was purchased from Montreal Biotechnologies Inc. (Dorval, Canada). Cayman's COX-2 activity assay (catalog kit no.760151) was purchased from (Cayman, USA). RayBio Rat TNF- α ELISA Kit (catalog no. ELR-TNF α -001C) was procured from (RayBio, USA). Proteinase K was obtained from (Finnymesoy. Keilaranta 16A, 02150 ESPOO, Finland). The monoamine oxidase kit was purchased from Cell Biolabs (catalog no. IncXPX-5006). The acetylcholinesterase kit was procured from Abcam (Cambridge, USA).

2.2. A collection of the Plant Materials and extraction of cured extract

AW chips [Cambodian agarwood] were collected from a local market in Saudi Arabia. Agarwood chips were crushed and extracted with absolute ethanol. After being soaked in a solvent for two days at room temperature ($28 \pm 2^\circ\text{C}$), the extract was concentrated with a rotary evaporator at $40\text{-}50^\circ\text{C}$. The ground plant (500 g) was extracted with absolute ethanol of AW yield (39 g) [5, 6, 7, 8].

2.3. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

GC-MS analysis was conducted for the ethanoic extracts using Agilent Technologies (G3440B). The ethanolic

extracts were re-dissolved in ethanol and filtered using a nylon 0.45- μm pore size membrane filter. The GC-MS analysis was performed using a general screening method [9]. The separation column was from Thermo Fisher Scientific (TR-5MS) and had the following properties: 30 m length, ID 0.25 mm and film thickness 0.25 μm . The carrier gas was helium and the flow rate was 1 ml/min. A total of 2 μl of each sample was injected into split less mode at an injection port with a temperature of 260°C . The GC thermal program started at 80°C and was held for 1.5 min. Next, the thermal program increased at the first ramp to 210°C at a rate of $30^\circ\text{C}/\text{min}$, and then the rate was slowed to $20^\circ\text{C}/\text{min}$ to reach the final temperature of 320°C , and this temperature was held for 11 min. The ion source in MS was electron ionization (EI), and the analysis was conducted in a scanning mode with electron energy of 70 eV. The ion source and transfer line temperatures were adjusted to 230°C . The composites of plant extract were identified by computer explorations in the commercial libraries of WILEY and NIST (National Institute of Standards and Technology).

2.4. Animals

Forty adult male Sprague-Dawley rats weighing 100-110 g were used in this study. All rats were examined for health status at 25°C , given standard diet and water daily for two weeks before handling. After acclimatization, rats were separated into four groups of 10 rats. All animal experiments were endorsed by the Ethics Committee of the Experimental Animal Care Society and related to the Three Rs (3Rs: Replacement, Reduction, Refinement) [10].

The control group (C): untreated rats.

(Methanol) group: The rats were injected with an intraperitoneal dose of 3 g kg^{-1} body mass (bm) methanol once per week for three weeks [10, 11, 12, 13]. The experimental period was 35 days.

(AW) group: The rats were treated orally with a daily dose of AW 100 mg kg^{-1} bm for 35 days [8]. The experimental period was 35 days.

(AW-methanol) group: Handled rats orally with a daily dose of AW 100 mg kg^{-1} bm for a week. The animals were treated with methanol (as mentioned before) at the starting of the second week in plus a daily dose of AW until 35 days had passed [8, 9, 10, 11, 12, 13]. The experimental period was 35 days.

After the experimental term, before rats were anesthetized with diethyl ether and sacrificed, feeding was stopped for 12 h. Liver and brain tissues were washed with a cold saline solution (0.9% NaCl), weighed and kept at -80°C until biochemical analysis.

2.5. Biochemical assay

2.5.1. NO level

NO concentration was determined spectrophotometrically

[14] The liver and brain tissues were separately homogenized in four volumes of cell lysate buffer (pH 7.5). The homogenate of the liver and brain were separately centrifuged at 10,000 g for one min at 4°C and the supernatant was saved at -30°C until being handled. One hundred μL of sample (liver, brain or standard sodium nitrite - 100 μM) combined with 1 ml of sulphanilamide was employed. Fifty μL N-1-naphthyl ethylene diamine was added, and then the solution was incubated at room temperature for 20 min. Subsequently, absorbance was recorded at 540 nm against a blank (as the buffer was scored alternatively to the sample).

2.5.2. Lipid peroxidation

We applied the calorimetric method for MDA level, the end product of lipid peroxidation, measurement [15]. Fifty microliters of liver and brain tissues were separately homogenated or the buffer was applied during the homogenization process (blank) incubated with an examined mixture that included (100 μL of 8.1% of SDS, 750 μL of 20% acetic acid, including HCl, 750 μL of 0.8% TBA and 300 μL of distilled water at pH 3.5). Then, the mixture was placed in a boiling water bath for 45 min. At room temperature, after cooling, 500 μL of distilled water and 2.5 mL of n-butanol/pyridine mixture (15:1 v/v) was added, stirred well and centrifuged for 10 min at 1780 g. We then estimated the absorbance of the pink color at 532 nm and fixed the concentration of MDA as nmol/g liver. We next applied different concentrations of TMP (20-300 nmol) as a standard and assayed the samples in a relevant.

2.5.3. Acetylcholinesterase

Rat liver and brain tissues were separately, quickly removed, placed on an ice-cold dish and washed with ice-cold buffer (0.5 M sodium phosphate, pH 7.5). The tissues were individually homogenized with buffer and centrifuged at 900 \times g for 10 min. The resulting supernatants were used as the enzyme source and the activity was determined by the colorimetric method according to Ellman (1961) [15, 16, 17]. Absorbance was measured at 410 nm through ELISA. Each sample was assayed in triplicate and AChE activity was established by direct comparison to the level of the control.

2.5.4. COX-2

The activity of the COX-2 enzyme in the liver and brain tissues was separately assayed according to Smith et al. (1998) [18]. In terms of the assay buffer, 120 μL was placed in a microplate reader. Then, 10 μL of the diluted heme and 40 μL of sample were added, mixed well and incubated at 25°C for 5 min. Next, 10 μL of SC-560 reagent (SC-560 in dimethylsulfoxide; SC-560 is a potent and selective COX-1 inhibitor) was added, mixed well and incubated at 25°C for 5 min. Thereafter, 20 μL of colorimetric substrate was added to every well of the plate reader we used. The reaction was started by adding 20 μL of arachidonic acid solution, mixing well and incubation

at 25°C for 5 min. The reaction rate was determined by estimating the absorbance at 590 nm against a blank using a microplate reader (Bio-Tex Instruments, Germany). The blank was assayed as the sample, but the inactive sample was used instead of a sample.

2.5.5. LOX

LOX enzyme activity in the liver and brain tissues were individually determined using a spectrophotometric method (using sodium arachidonate as the substrate) according to the process of Haining and Axelrod (1958) [19]. One unit of LOX enzyme was determined as the quantity of the enzyme catalyzing the synthesis of 1 μmol of hydroperoxyarachidonate (HPA) per min/mg protein under the test conditions. The extinction coefficient was equivalent to 25000 M/L.

2.5.6. Caspase-3

Caspase-3 activity was examined using a colorimetric kit according to the method of Talanian et al. (1997) [20]. Liver and brain tissues were separately homogenized in four volumes of cold cell lysis buffer (50 mM Tris-HCl buffer including 0.2M NaCl and 1% Triton X-100, pH 6.8) by a Teflon glass homogenizer. The homogenates were centrifuged at 44,720 g for 3 min at 4°C, and the supernatants were kept at -80°C. The supernatant (50 μL =150 μg protein) was placed in a microplate reader, and then 50 μL reaction buffer and five μL of 4 mM substrate were combined, stirred well and incubated at 37 °C in the darkness for 2 h. The reaction rate was established by measuring the absorbance of the produced yellow colour at 405 nanometers (nm) against a blank handling a microplate reader (Bio-Tek Instruments, Bad Friedrichshall, Germany). Fold elevate in caspase-3 activity was estimated by direct comparison to the level of the control.

2.5.7. TNF- α

The level of TNF- α in the brain and liver tissues was separately obtained using rat TNF- α ELISA kit according to Brouckaert (1993) [21]. The anti-Rat TNF-alpha precoated microplate was used in the determination of TNF-a concentrations. One hundred μL of sample (or standard) was added into the wells, then the well was capped and incubated for 2.5 h at room temperature. The solution was discarded, and wells were washed four times with wash buffer. One hundred μL of diluted biotinylated antibody was added to each well and incubated for 1 h at room temperature. The solution was discarded, and wells were washed four times with wash buffer. One hundred μL of prepared HRP-streptavidin solution was added to each well and incubated for 45 min at room temperature. The solution was discarded, and wells were washed four times with wash buffer. One hundred μL of TMB (3, 3', 5, 5'-tetramethylbenzidine) substrate reagent was added to each well and incubated for 30 min at room temperature in the dark. Fifty μL of (2 M H_2SO_4) was added to each well to stop the reaction. Absorbance was measured

directly at 450 nm against a blank using an ELISA reader (BioTek, USA).

2.5.8. MAO

Liver and brain MAO activity was separately assayed according to Tipton and Dawson (1968) as well as Youdim (1976) [22, 23]. MAO in the tissue homogenates reacts with its substrate, tyramine, and produces hydrogen peroxide (H_2O_2). The colorimetric probe reacts with the H_2O_2 to produce a red/pink-coloured product that is read by a microplate reader in the 540-570 nm range. Absorbance values are proportional to the MAO levels within the samples [22, 23].

2.5.9. DNA Fragmentation

DNA was isolated using a DNA purification kit. Then, DNA was isolated by electrophoresis on 1% agarose gel including ethidium bromide [24]. Brain and liver tissues were homogenized separately in (1:5 w/v) 50 mM Tris-HCl buffer containing 20% sucrose and 50 mM EDTA at pH 7.6. DNA was isolated using a DNA purification kit. Next, 15 μ g/lane of DNA was detailed by electrophoresis on 1% agarose gel containing 25 mL ethidium bromide at 5 V/1 cm for 2–3 h, following which it was visualized under UV light with a multiband transilluminator from Consort (Turnhout, Belgium).

2.5.10. Statistical analysis

Statistical analysis: All data are shown as means (X) \pm standard deviation (SD). Comparisons between the means of various groups were analyzed using the least significant difference (LSD) test. Variations were judged significant at $p < 0.05$. All statistical examinations were carried out using the statistical software, the Statistical Package for the Social Sciences (SPSS, Inc., Chicago, USA) v11.5.

3. Results

3.1. NO levels

The results for the brain tissue showed that the NO levels

in the control (C) were $3.4 \pm 0.10 \mu\text{m}$ lower than that in the methanol group ($22.3 \pm 0.02 \mu\text{m}$; $p < 0.05$). Brain NO levels in the AW group were $3.4 \pm 0.18 \mu\text{m}$ compared to C; $p < 0.05$. Brain NO levels in AW–methanol were $6.9 \pm 0.14 \mu\text{m}$ compared to methanol; $p < 0.05$ (Table 1). Meanwhile, the NO results in liver tissue showed the NO levels in C were $25.01 \pm 0.13 \mu\text{m}$ lower than that in the methanol group ($92.02 \pm 1.01 \mu\text{m}$; $p < 0.05$). Liver NO levels in the AW group were $25.09 \pm 1.03 \mu\text{m}$ compared to C; $p < 0.05$. Liver NO levels in the AW–methanol group were $39.21 \pm 0.02 \mu\text{m}$ compared to methanol; $p < 0.05$ (Table 1).

3.2. Lipid peroxidation (MDA)

The results for brain tissue showed that the MDA levels in C were $1.6 \pm 0.17 \text{ nmol/g}$ tissue lower than that in the methanol group $3.9 \pm 0.13 \text{ nmol/g}$ tissue; $p < 0.05$. Brain MDA levels in the AW group were $1.6 \pm 0.18 \text{ nmol/g}$ compared to C; $p < 0.05$. Brain MDA levels in the AW–methanol were $1.9 \pm 0.14 \text{ nmol/g}$ tissues compared to methanol; $p < 0.05$ (Table 1). Meanwhile, the MDA results in liver tissue demonstrated the MDA levels in C were $1.55 \pm 1.00 \text{ nmol/g}$ tissue lower than that in the methanol group at $15.44 \pm 1.02 \text{ nmol/g}$ tissue; $p < 0.05$. Liver MDA levels in the AW group were $1.55 \pm 0.02 \text{ nmol/g}$ tissue compared to C; $p < 0.05$. Liver MDA levels in AW–methanol were $3.00 \pm 0.17 \text{ nmol/g}$ tissue compared to methanol; $p < 0.05$ (Table 1).

3.3. AChE

The results for the brain tissue showed that the level of AChE in C was $0.8 \pm 0.06 \text{ mU/ml}$ lower than that in the methanol group $3.9 \pm 0.03 \text{ mU/ml}$; $p < 0.05$. Brain AChE levels in the AW group were $0.8 \pm 0.02 \text{ mU/ml}$ compared to C; $p < 0.05$. Brain AChE levels in AW–methanol were $1.4 \pm 0.02 \text{ mU/ml}$ compared to methanol; $p < 0.05$ (Table 1). Meanwhile, the AChE level results in liver tissue reflected the AChE levels in C were $0.4 \pm 0.01 \text{ mU/ml}$ lower than that in the methanol group $1.8 \pm 0.02 \text{ mU/ml}$;

Table 1: Different biochemical analyses.

Particulars	C	Methanol	AW –Methanol	AW
Brain				
NO (μm) in brain tissue	$3.4^a \pm 0.10$	$22.3^b \pm 0.02$	$6.9^c \pm 0.14$	$3.4^a \pm 0.18$
MDA levels (nmol/g tissue) in brain tissue	$1.6^a \pm 0.17$	$3.9^b \pm 0.13$	$1.9^c \pm 0.14$	$1.6^a \pm 0.18$
AChE (mU/ml) in brain tissue	$0.8^a \pm 0.06$	$3.9^b \pm 0.03$	$1.4^c \pm 0.02$	$0.8^a \pm 0.02$
COX-2 activities U/ml (nmol/min/ml) in brain tissue	$0.02^a \pm 0.012$	$0.08^b \pm 0.032$	$0.03^c \pm 0.024$	$0.02^a \pm 0.0045$
LOX activities ($\mu\text{mol/mg}$ protein) in brain tissue	$0.004^a \pm 0.001$	$0.012^b \pm 0.002$	$0.009^c \pm 0.012$	$0.004^a \pm 0.043$
Caspase-3 [OD at 405 nm] in brain tissue	$0.08^a \pm 0.003$	$0.12^b \pm 0.004$	$0.09^c \pm 0.004$	$0.08^a \pm 0.005$
TNF- α (pg/ml) in brain tissue	$14.01^a \pm 0.08$	$47.02^b \pm 4.03$	$20.87^c \pm 7.02$	$14.02^a \pm 8.01$
MAO ($\mu\text{U/ml}$) in brain tissue	$3.6^a \pm 0.17$	$13.2^b \pm 0.13$	$4.9^c \pm 0.15$	$3.6^a \pm 0.18$
Liver				
NO (μm) in liver tissues	$25.01^a \pm 0.13$	$92.02^b \pm 1.01$	$39.21^c \pm 0.02$	$25.09^a \pm 1.03$
MDA (nmol/g tissue) in liver tissue	$1.55^a \pm 1.00$	$15.44^b \pm 1.02$	$3.00^c \pm 0.17$	$1.55^a \pm 0.02$
AChE (mU/ml) in liver tissue	$0.4^a \pm 0.01$	$1.8^b \pm 0.02$	$0.9^c \pm 0.02$	$0.4^a \pm 0.07$
COX-2 activities U/ml (nmol/min/ml) in liver tissue	$1.58^a \pm 0.02$	$6.04^b \pm 0.10$	$2.28^c \pm 0.03$	$1.58^a \pm 0.03$
LOX activities ($\mu\text{mol/mg}$ protein) in liver tissue	$0.03^a \pm 0.002$	$0.09^b \pm 0.002$	$0.04^c \pm 0.002$	$0.03^a \pm 0.003$
Caspase-3 [OD at 405 nm] in liver tissue	$0.11^a \pm 0.01$	$0.79^b \pm 0.02$	$0.35^c \pm 0.01$	$0.11^a \pm 0.01$
TNF- α (pg/ml) in liver tissue	$23.22^a \pm 1.02$	$89.89^b \pm 3.03$	$33.87^c \pm 1.02$	$23.22^a \pm 1.20$
MAO activity ($\mu\text{U/ml}$) in liver tissue	$15.6^a \pm 0.4$	$73.2^b \pm 0.3$	$19.8^c \pm 0.5$	$15.6^a \pm 0.2$

$p < 0.05$. Liver AChE levels in the AW group were 0.4 ± 0.07 mU/ml compared to C; $p < 0.05$. Liver AChE levels in the AW–methanol were 0.9 ± 0.02 mU/ml compared to methanol; $p < 0.05$ (Table 1).

3.4. COX-2

The results for brain tissue showed that the level of COX-2 in C were 0.02 ± 0.012 U/ml (nmol/min/ml) lower than that in the methanol group at 0.08 ± 0.032 U/ml (nmol/min/ml); $p < 0.05$. Brain COX-2 levels in the AW group were 0.02 ± 0.0045 U/ml (nmol/min/ml) compared to C; $p < 0.05$. Brain COX-2 levels in AW–methanol were 0.03 ± 0.024 U/ml (nmol/min/ml) compared to methanol; $p < 0.05$ (Table 1). Meanwhile, the COX-2 level results in liver tissue showed that COX-2 levels in C were 1.58 ± 0.02 U/ml (nmol/min/ml) lower than that in the methanol group at 6.04 ± 0.10 U/ml (nmol/min/ml); $p < 0.05$. Liver COX-2 levels in the AW group were 1.58 ± 0.03 U/ml (nmol/min/ml) compared to C; $p < 0.05$. Liver COX-2 levels in AW–methanol were 2.28 ± 0.03 U/ml (nmol/min/ml) compared to methanol; $p < 0.05$ (Table 1).

3.5. LOX

The results for brain tissue showed that the level of LOX in C was 0.004 ± 0.001 μ mol/mg protein lower than that in the methanol group at 0.012 ± 0.002 μ mol/mg protein; $p < 0.05$. Brain LOX levels in the AW group were 0.004 ± 0.043 μ mol/mg protein compared to C; $p < 0.05$. Brain LOX levels in AW–methanol were 0.009 ± 0.012 μ mol/mg protein compared to methanol; $p < 0.05$ (Table 1). Meanwhile, the LOX levels in liver tissue showed they were 0.03 ± 0.002 μ mol/mg protein lower in C than in the methanol group at 0.09 ± 0.002 μ mol/mg protein; $p < 0.05$. Liver LOX levels in the AW group were 0.03 ± 0.003 μ mol/mg protein compared to C; $p < 0.05$. Liver LOX levels in the AW–methanol group were 0.04 ± 0.002 μ mol/mg protein compared to methanol; $p < 0.05$ (Table 1).

3.6. Caspase-3

The results for brain tissue showed that the enzyme levels of caspase-3 in C were 0.08 ± 0.003 μ mol/mg protein lower than that in the methanol group at 0.12 ± 0.004 μ mol/mg protein; $p < 0.05$. Brain caspase-3 levels in the AW group were 0.08 ± 0.005 μ mol/mg protein compared to C; $p < 0.05$. Brain caspase-3 levels in the AW–methanol group were 0.09 ± 0.004 μ mol/mg protein compared to methanol; $p < 0.05$ (Table 1). Meanwhile, the caspase-3 levels in liver tissue showed that caspase-3 in C was 0.11 ± 0.01 μ mol/mg protein lower than that in the methanol group at 0.79 ± 0.02 μ mol/mg protein; $p < 0.05$. Liver caspase-3 levels in the AW group were 0.11 ± 0.01 μ mol/mg protein compared to C; $p < 0.05$. Liver caspase-3 levels in AW–methanol were 0.35 ± 0.01 μ mol/mg protein compared to methanol; $p < 0.05$ (Table 1).

3.7. TNF- α

The results for brain tissue exhibited that the TNF- α

levels in C were 14.01 ± 0.08 pg/ml lower than that in the methanol group at 47.02 ± 4.03 pg/ml; $p < 0.05$. Brain TNF- α levels in the AW group were 14.02 ± 8.01 pg/ml compared to C; $p < 0.05$. Brain TNF- α levels in the AW–methanol group were 20.87 ± 7.02 pg/ml compared to methanol; $p < 0.05$ (Table 1). Meanwhile, the TNF- α results in liver tissue showed that the TNF- α levels in C were 23.22 ± 1.02 pg/ml lower than that in the methanol group at 89.89 ± 3.03 pg/ml; $p < 0.05$. Liver TNF- α levels in the AW group were 23.22 ± 1.20 pg/ml compared to C; $p < 0.05$. Liver TNF- α levels in the AW–methanol group were 33.87 ± 1.02 pg/ml compared to methanol; $p < 0.05$ (Table 1).

3.8. MAO

The results for brain tissue indicated that the enzyme levels of MAO in C were 3.6 ± 0.17 μ U/ml lower than that in the methanol group at 13.2 ± 0.13 μ U/ml; $p < 0.05$. Brain MAO levels in the AW group were 3.6 ± 0.18 μ U/ml compared to C; $p < 0.05$. Brain MAO levels in the AW–methanol were 4.9 ± 0.15 μ U/ml compared to methanol; $p < 0.05$ (Table 1). Meanwhile, the MAO results for liver tissue showed the MAO levels in C were 15.6 ± 0.4 μ U/ml lower than that in the methanol group at 73.2 ± 0.3 μ U/ml; $p < 0.05$. Liver MAO levels in the AW group were 15.6 ± 0.2 μ U/ml compared to C; $p < 0.05$. Liver MAO levels in AW–methanol were 19.8 ± 0.5 μ U/ml compared to methanol; $p < 0.05$ (Table 1).

3.9. DNA Fragmentation (DNAF)

Agarose gel electrophoresis showed very low or undetectable DNA laddering (DNAF) in the brain and liver tissues of the C group. The DNA intact band appeared to contract near the application point with no DNA smearing, suggesting no DNAF. Treatment with AW alone showed no DNA fragmentation in either brain or liver tissues. Otherwise, methanol administration resulted in massive DNA fragmentation compared to C. Treatment with AW pre-, during and post-methanol administration decreased DNA fragmentation compared to treatment with methanol alone in both liver and brain tissues (Figure 1).

3.10. GC-MS Analysis

Cambodian AW extract: The chemical composition of Cambodian AW chip extract was identified by GC-MS and the specific components that are unique to the extract are found in Table 2.

4. Discussion

Research has shown there are many adverse health effects from the consequences of alcohols contained in food and alcohol intake, including 61 various types of injury, illness or death. Alcohol induces diseases that liver cirrhosis, mental illness, and numerous kinds of cancer as well as pancreatitis. Alcohol consumption also leads to destruction and even loss of the fetus between pregnant women and is related to deviation from social

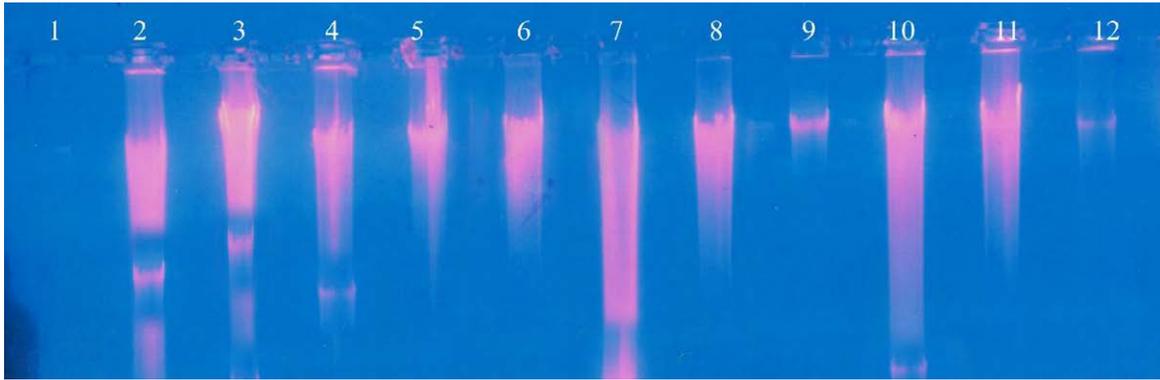


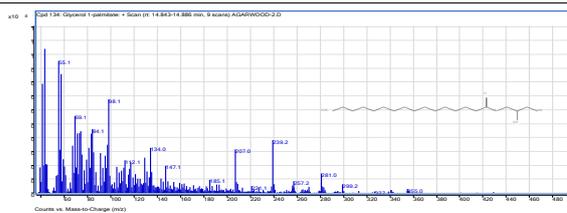
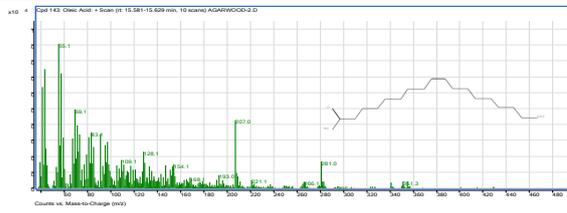
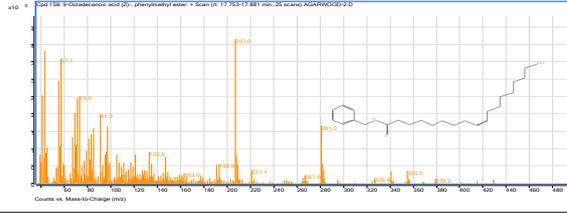
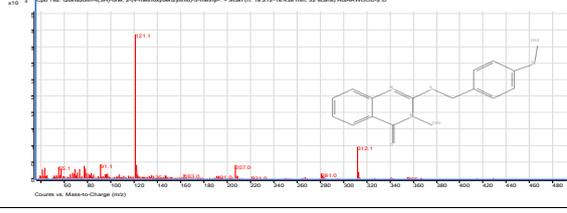
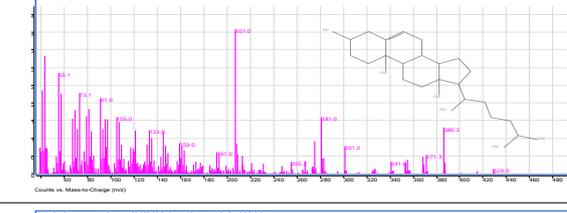
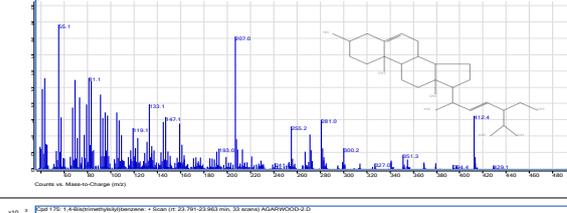
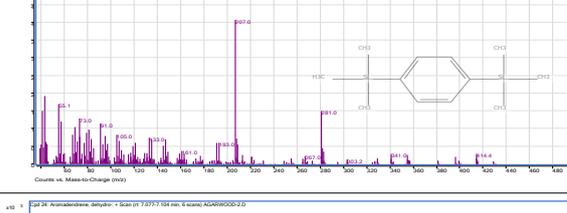
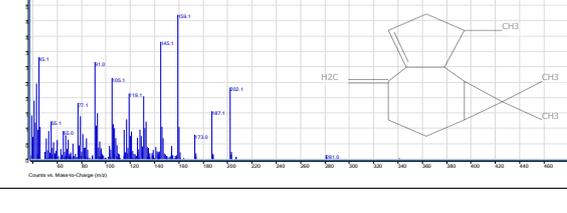
Figure 1: DNA fragmentation

DNA fragmentation (DNAF) in liver and brain tissues. DNA fragmentation separating in 1% agarose gel electrophoresis and visualized under UV. DNAF in liver tissue (lane 1 - control, lanes 2,3 - methanol, lanes 4,6 - AW-methanol, lane 5 - AW). DNAF in brain tissue (lanes 7,10 - methanol, lane 8 - control, lanes 9,11 - AW-methanol, lane 12 - AW). The DNA intact band seems to condense near the application point with no DNA smearing, suggesting no DNA fragmentation control (lane 1). Treatment with AW alone shows no DNA fragmentation. Otherwise, methanol administration resulted in massive DNA fragmentation compared to C. Treatment with AW pre-, during and post-methanol administration decreased DNA fragmentation compared to treatment with methanol alone in both liver and brain tissues.

Table 2: Results of the GC-MS analysis of AW.

Compounds	RT (min)	Peak Area (%)	Chromatogram
2-Butanone, 4-(4-methoxyphenyl)	8.318	1.99	
Doconexent	12.078	2.81	
Hexadecanoic acid, ethyl ester	11.714	7.62	
Phenol, 2,6-dimethoxy	7.002	0.22	
4-Isopropenyl-4,7-dimethyl-1-oxaspiro[2.5]octane	8.013	0.31	

4,5-di-epi-aristolochene)	8.211	0.37	
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Glycerol 1-palmitate	14.8645	0.9	
Oleic Acid	15.605	0.2	
9-Octadecenoic acid (Z)-, phenylmethyl ester	17.817	0.34	
Quinazolin-4(3H)-one, 2-(4-methoxybenzylthio)-3-methyl-	18.355	3.72	
Cholesterol	20.5395	1.02	
Stigmasterol	22.6465	2.36	
1,4-Bis(trimethylsilyl)benzene	23.877	0.61	
Aromadendrene, dehydro-	7.0905	0.33	

- RT: Retention times (minutes); PA: peak area (%).

- GC-MS analysis was carried for the ethanoic extracts applying Agilent Technologies (G3440B).

- The components of plant extract were characterized by computer simulations in commercial libraries of WILEY and NIST (National Institute of Standards and Technology).

values, such as drink-driving injuries and deaths, aggressive behavior, family confusion and reduced industrial impact [24, 25]. Methanol (CH_3OH) is a broadly used industrial solvent that is also recommended for use as an optional automotive fuel, thus boosting the risk for human exposure. Modern human exposure to CH_3OH necessarily happens via the ingestion of popular alcoholic beverages and fruit juices, use of cosmetics, dicing fluids, varnishes and perfumes. As CH_3OH is found relatively ubiquitously in our environment, it is frequently necessary to determine its toxic effects and methods for protection and treatment [24, 25, 26]. In an attempt to model this process, chemical induction of liver and brain injuries was initiated by methanol administration in our experimental study.

All harmful alcohol side effects that agree with our findings are a result of alcohol and its metabolites. Methanol metabolism yields formaldehyde and methanoic acid that leads to acidosis, liver and neuronal toxicity. As a modest inhibitor of cytochrome-c oxidase, formate may cause tissue oxygen use to be impaired; leading to anaerobic respiration with a following increased lactate generation that additionally contributes to acidosis. The relative susceptibility of alcohol dehydrogenase to ethanol is much greater than for methanol (20: 1). This diversity has been used therapeutically in cases of poisoning, where alcohol is given under medical supervision to diminish the formation of formic acid [1, 2, 3, 4].

Our results showed the effects of increasing NO, MDA, AChE, COX-2, LOX, caspase-3, TNF- α , MAO and DNAF levels in the liver and brain separately in rats treated with methanol compared with a control. They indicated that methanol itself or its metabolite-induced oxidative stress marked by an imbalance between pro-oxidant and antioxidant in the service of the pro-oxidants led to tissue injuries, inflammation and apoptosis in both the liver and brain tissues. Alcohol is metabolised and discharged outside the body by numerous metabolic mechanisms. Aldehyde dehydrogenase (ALDH), alcohol dehydrogenase (ADH), cytochrome P450 (CYP2E1) and catalase are the primary enzymes responsible for alcohol metabolism, and this depended on human gene varieties of that enzyme. The differences and variation of enzyme genetics have a role in influencing alcohol consumption and tissue damage as a result of alcohol and its metabolites. The outcomes of alcohol metabolism involve oxygen deficits (i.e., hypoxia) in the liver. As well, the interaction between alcohol metabolism by-products and other cell components leads to the formation of toxic compounds (i.e., adducts); formation of highly reactive oxygen-containing molecules (i.e., ROS) that attack cell membranes, proteins, lipids and DNA, ultimately bringing about cell death. Additionally, the change in the ratio of NADH to NAD^+ (i.e., the cell's redox state) as a result of alcohol metabolism results in tissue and fetal damage as along with impairment of other metabolic processes, cancer and medication synergies [24, 25,

26]. Alcohol metabolism occurs in the liver as well as extrahepatic tissues that do not carry ADH, including the brain, by the enzymes, cytochrome P450 and catalase. Alcohol metabolism occurs by both oxidative pathways, which either add oxygen or transfer hydrogen by ADH, cytochrome P450 and catalase along with nonoxidative pathways. In oxidative pathways, acetaldehyde from adducts in the brain, including brain signalling chemicals (i.e., neurotransmitter) like, dopamine, forms salsolinol that may contribute to alcohol addiction as well as DNA adducts, such as 1, N2-propanodeoxyguanosine, that causes cell death and is agreement with our results. Furthermore, the disposition of protein adducts in hepatocytes impairs protein secretion, which is proposed to have a function in hepatomegaly. The by-product is reduced nicotinamide adenine dinucleotide (NADH), which inhibits glucose production and breakdown of fat molecules as well as stimulates the production of fat molecules. Acetate produced from the oxidation of acetaldehyde escapes the blood to metabolize to CO_2 in numerous organs, including the heart, skeletal muscle and brain cells. The nonoxidative metabolism of alcohol increases production of fatty acid ethyl esters (FAEEs) in the liver and pancreas and are detectable in serum and other tissues after alcohol ingestion and continue to be present long after alcohol is eliminated and may have several side effects. There is another form of toxicity where phospholipase D (PLD) is the enzyme required for the second nonoxidative pathway, which ends with phosphatidyl ethanol that negatively metabolizes and accumulates at discernible levels following chronic consuming of large amounts of alcohol and may lead to serious side effects in both the liver and brain [24, 26].

Methanol itself or its metabolites lead to liver and brain injuries through an effect on pro-oxidant and free radical balance. NO has a significant role in neurotransmission, vascular regulation, immune response and apoptosis. NO immediately oxidizes to nitrite and nitrate that is used to quantify NO production. MDA is a primary oxidation product of peroxidized polyunsaturated fatty acids and enhanced MDA content is an essential indicator of lipid peroxidation [5, 30], in line with our findings. The present study has shown a significant elevation in MDA, NO, caspase-3 and DNAF levels after methanol administration as a result of the formation of lipid peroxides in liver and brain crude homogenates responding to the administration of methanol. The results may be based on certain enhanced generation of free radicals that stimulates peroxidation of native membrane lipids. Peroxidation of the mitochondrial membrane leads to a lack of cell integrity, rise in membrane permeability and alteration of Ca^{2+} homeostasis that contributes to cell death owing to a change in the inner membrane potential [26, 27, 28, 29, 30].

Certain studies have provided evidence there is neuronal cell loss after activation of caspase-3, which acts as both an initiator and executor of the apoptotic process

owing to stroke or toxins that lead to cell death. There is growing evidence that rapid cell death follows brain injury produced by toxicity over an extended period and is in agreement with our work. However, in the surrounding region, neurons remain viable for a prolonged period, perhaps for days, and there may be initiation of inflammation processes by local expression of cytokines, chemokines and adhesion molecules that appear in our results where there are increases in inflammatory factors, such as TNF- α , COX-2 and LOX, ending with inflammation [31].

The rise in TNF- α in our work was followed by elevation of COX-2 and LOX activity and NO levels in the methanol group compared to C in both the liver and brain. These results agree with another group's work that showed TNF- α induces inflammatory genes, such as COX-2, LOX and iNOS [26, 27, 28, 29, 30]. NO radicals elevate the production of PGE2 by direct activation of COX in liver tissue. The investigators found that COX-2 gene expression in the brain contributed to cerebral ischemic damage and toxic administration [26, 27, 28, 29, 30]. Another investigator determines that COX-2 gene expression in the brain contributed to cerebral ischemic damage and toxic administration. In the CNS, epidermal growth factor (EGF) is associated with brain diseases, such as Parkinson's disease and schizophrenia, whereby it enhances the expression of inducible prostaglandin synthetase [cyclooxygenase 2 (Cox-2)] and triggers a variety of inflammatory processes that produce a cyclooxygenase-2 inhibitor ameliorate behavioral impairments [32, 33, 34, 35, 36]. Lipoxygenases are enzymes that include cyclooxygenases (COX) and can introduce oxygen into the molecule of arachidonic acid and through synthesizing inflammatory eicosanoids: leukotrienes [due to 5-lipoxygenase (5-LOX) activity] and prostaglandins (via COX activity). It seems that 5-LOX is expressed in central nervous system neurons and may play a part in neurodegeneration 5-LOX-triggered apoptosis cell death agrees with our results where there is an increase in COX-2, LOX, caspase-3 and DNAF in the methanol group compared to controls in the brain. This indicated that methanol leads to apoptosis and inflammation in brain tissue. 12/15-lipoxygenase is raised in Alzheimer's disease and there is reasonable involvement in brain oxidative stress. In addition, characteristic patterns of 5-LOX in the human brain are present during traumatic injury and astrocytoma [32, 33, 34, 35, 36].

Neurotransmitters are endogenous chemical carrier that enables neurotransmission. They transmit signals beyond the synapse, such as a neuromuscular junction, from one neuron to another in different types of cell bodies. Neurotransmitters include amino acids, gasotransmitters, monoamines, trace amines, peptides, purines, acetylcholine (ACh), anandamide, etc. Further, they play a significant role in shaping everyday life

and functions, where increases or decreases in their concentrations can lead to serious illness. MAOs catalyze the oxidative deamination of neurotransmitter monoamines. AChE is an enzyme that catalyzes the distribution of ACh and of other choline esters that work with neurotransmitters primarily. Remarkably large or moderate levels of MAOs or AChE in the body is correlated with schizophrenia, depression, attention disorder, migraines and irregular sexual maturation, and is also associated with Alzheimer's and Parkinson's diseases, etc. [37, 38, 39, 40, 41, 42, 43, 44]. Our results showed an elevation in liver and brain AChE and MAO in rats treated with methanol compared to the control in line with recent reports. Previous studies have shown that methanol increases glutamate, aspartate, glutamine, taurine, 5-hydroxytryptamine and 5-hydroxyindoleacetic acid concentrations in the hippocampus. Furthermore, it increases 5-hydroxytryptamine, indicating that chronic methanol administration produces a collection of aspartate, an excitotoxic amino acid, in the optic nerve, resulting in methanol neurotoxicity and the development of optic neuropathies that may affect the balance of MAO and AChE [45, 46, 47], once again agreeing with our findings.

Our results also determined that an elevation in liver and brain AChE and MAO in rats treated with methanol compared to the control is in line with another study that reported alcohol consumption led to an imbalance in neurotransmitters. That work also reported that AChE was found at mainly neuromuscular intersections and in chemical synapses of the cholinergic type, where its activity assists in terminating synaptic transmission. This relates to the carboxylesterase family of enzymes. Researchers have determined that the liver is an important origin of serum AChE, and sequentially changing AChE levels may be helpful biomarkers for liver cirrhosis and brain diseases, such as Alzheimer's and others [47, 48, 49, 50]. Excessive alcohol consumption leads to abnormalities in the central nervous system, especially during pregnancy, specifically *via* reduced brain size (microencephaly), growth retardation and facial dysmorphism in newborn children. Methanol may damage the developing brain by affecting neurogenesis, migration or survival of cells. Neurons are more susceptible to methanol-induced apoptotic cell death during synaptogenesis, also known as the brain growth spurt. Methanol—and not its metabolites—is the key toxicant, with features of central nervous system depression a common finding in animals studied—there may be the formation of DNA adducts causing cell death in the liver and brain as discussed before [49, 50].

AW is used in Saudi Arabia and all across the broader Middle East. Our results showed a significant decline in liver and brain NO, MDA, ACHE, COX-2, LOX, TNF- α , Caspase-3, MAO and DNAF in AW-methanol compared to the methanol separately. While there is no significant

change in the levels of NO, MDA, ACHE, COX-2, LOX, TNF- α , Caspase-3, MAO and DNAF in rats treated with AW alone in both liver and brain separately as compared to a control group, this indicated that AW has selective roles in their effects. Oxidative stress is a part of many pathological maladies covering neurodegenerative diseases and inflammation. The first source of ROS is LOX) and COX-2—enzymes capable of the metabolism of arachidonic acid and different polyunsaturated fatty acids that may be an adaptive process of fighting excessive damage toward toxic methanol effect. LOX inhibitors have a shielding effect in inflammatory and neurodegenerative diseases because of their anti-inflammatory and antioxidant activity. This finding explains our result where the antioxidant scavenger free radical and the inhibitory effect of AW chemical composition on LOX, MDA, NO and COX-2 consequently ameliorate liver and brain apoptosis, caspase-3, DNAF and lipid peroxidation toxicity [50, 51, 52].

Researchers have reported that inhibition and repression of TNF- α action within the CNS reduced inflammation and apoptosis in rats and this agrees with our results for AW-methanol [53]. Our work indicated that AW decreases apoptosis and inflammation, and this was evident in inhibition of TNF- α , ACHE and MAO in both liver and brain tissue induced with methanol. The report indicated that β -caryophyllene, essential AW oil, can suppress metastasis of colon cancer and have an anti-inflammatory effect that makes AW able to manage inflammation-related diseases. Understanding of the chemical components of the AW is helpful in the discovery of their therapeutic potential. The essential bioactive parts of these plants are alkaloids, tannins, flavonoids and phenolic compounds, such as agarofuran, α -eudesmol, guaialol and selinene, which have excellent anticancer activity and CNS antidepressant activities [5, 6, 7, 52, 53, 54]. Other findings have suggested that a natural AChE inhibitor helps in treatment of Alzheimer's disease. This previous report agrees with our results where there was inhibition of AChE activity in rats treated with AW before, during and post-methanol injection as compared to methanol, thus it is safe for the brain to use natural AW perfume instead of other methanol containing fragrances [54, 55, 56, 57, 58].

Some of the herbal therapeutic targets distinguished in the control of neurodegenerative diseases include MAO, AChE, butyrylcholinesterase (BChE) and oxidative stress. The use MAO inhibitors and their mechanism of action in the control of depression and other neurodegenerative maladies, such as Alzheimer's disease and Parkinson's disease, encouraged us to study AW extract as a MAO inhibitor [52, 53, 54]. AW products and their chemical compositions are available and recognized as complementary strategies for health improvement. Aquilaria, including AW, has been utilised in the treatment of various sorts of pain, cough and anaphylaxis for years in Asia, especially Saudi Arabia.

AW studies showed there to be an inhibition of histamine release from mast cells and suppressing the immediate hypersensitivity reaction, an anti-inflammatory and analgesic factor. It was also reported that AW has no toxicity [50, 51, 52, 53].

A pharmacological impact on the CNS may be managed by oral administration or abdominal doses of AW owing to jinkoh-eremol, agarospirol, and b-santalols, dehydrocostus lactone and costunolide, chemicals isolated from AW. AW's chemical composition affects psychosomatic brain disease caused by stress and depression. AW contents, jinkoh-eremol and agarospirol, have a neuroleptic impact on the CNS in mice. AW content and extracts reduce high-protein, high-fat-diet-induced intestinal putrefaction toxins and inflammation in mice [58, 59, 60]. AW aids in distribution of MAO and AChE in neurodefect cases, like Alzheimer's disease. Further, the inhibition of MAO and AChE may diminish β -amyloid deposition in the future. Our results suggested that AW composition can act both MAO and AChE inhibitors and may have promising characteristics in the treatment of Alzheimer's and Parkinson's diseases [46, 47, 48, 60].

The chemical composition of Cambodia AW chip extract identified by GC-MS in our result have beneficial effects. AW aromatic compound, doconexent, hexadecanoic spiro derivative compounds, aristolochene, aromadendrene, octadecanoic acid and quinazolin are natural components, and it could be a potential source of bioactive phytochemicals compounds. Most of the compound identified in AW is established as antioxidant, anticancer, antimicrobial, and antidiabetic activity compounds [62, 63, 64]. Hexadecanoic derivative compound identified in AW improves mice learning ability. Other components, such as doconexent derivatives, are related to omega-3 fatty acid, oleic acid, cholesterol, stigmasterol and plant sterols as the primary structural component of hormones and the human brain, cerebral cortex, skin, and retina thus play an essential role in their metabolism, brain development and function. It can process a wide array of applications in food, pharmaceuticals and cosmetic industries either as anti-microbial agents, natural flavouring agents or as critical ingredients in skin care and cosmetic products. Meanwhile, another compound identified in AW extract is a benzenoid compound. This mixture would be beneficial for anti-glucocorticoid therapy, and it was suggested as a factor that increases consciousness of rats and their studying ability, as well as having an anti-diabetic and anti-obesity potential [59, 60]. Hexadecanamide AW component and palmitoylethanolamide (PEA) derivatives reported as nuclear factor agonists and have the affinity to cannabinoid-like G-coupled receptors. Several results have shown there is an imbalance of the endocannabinoid system (ECS) and modifications in the levels of PEA study in acute and chronic inflammation. For instance, during β -amyloid-induced neuroinflammation, the deregulation

of cannabinoid receptors and its endogenous ligands follows the development and progression of a disease. PEA has been shown to possess anti-inflammatory, anti-nociceptive, neuroprotective and anticonvulsant characteristics [60, 61, 62, 63, 64, 65]. Our results suggested that AW composition can have both brain and liver effects that are beneficial and may have promise in the treatment of brain and liver conditions.

5. Conclusion

The inhibition of NO, MDA, ACHE, COX-2, LOX, TNF- α , Caspase-3, MAO and DNAF level of brain and liver tissue were restored to near control levels before, during and post-methanol administration could make AW a healthy dietary midpoint for the prevention/management of liver and neurodegenerative diseases.

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7. Author Disclosure Statement.

"No competing financial interests exist."

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