Available online at www.ijpsdr.com

International Journal of Pharmaceutical Sciences and Drug Research
2016; 8(1): 21-29

Research Article

ISSN: 0975-248X
CODEN (USA): IJPSPP

Protective Properties of Flavonoid Extract of Coagulated Tofu (Curdled Soy Milk) Against Acetaminophen-Induced Liver Injury in Rats

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ABSTRACT
The total flavonoid contents of the various coagulated tofu and the hepatoprotective potential of all tofu flavonoid extracts were investigated. Tofu was prepared from locally sourced coagulants (steep water, alum, lemon, and lemon peel ash extract). Total flavonoid contents of all coagulated tofu were investigated as established *in vitro* flavonoid assay. The hepatoprotective activities of tofu flavonoid extracts against acetaminophen-induced hepatic cell toxicity in rats was also investigated in this study. The activity was analyzed by assessing the levels of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH). The concentrations of the serum sugar, total protein, albumin, and cholesterol as well as prothrombin time (PT) of experimental rats with histopathological analysis were also conducted. The range of the total flavonoid contents of tofu was 4.3-6.4 mg/g. Tofu flavonoid extracts significantly reduced the activities of serum AST, ALT, ALP, and LDH; total cholesterol, and sugar levels, but total protein and albumin concentrations increased compared to acetaminophen-intoxicated rats. Also, the prothrombin time prolongation of serum in acetaminophen intoxicated rats was reduced. Histology of the liver tissue demonstrated that tofu flavonoid extracts inhibited the acetaminophen-induced hepatic cell necrosis, decreased inflammatory cell infiltration and accelerated hepatocellular regeneration. Therefore, all tofus exhibited high total flavonoid contents, and the tofu supplement in human diets is highly recommended as it can be used as a functional food to prevent liver injuries.

Keywords: Acetaminophen, coagulant, flavonoid, hepatoprotection, flavonoid, tofu.

INTRODUCTION

A common cause of liver damage is by hepatotoxicity induced via drugs. Approximately, drugs account for almost half of acute liver failure cases, which resulted in all forms of acute and chronic liver diseases. [1-2]

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Received: 08 December, 2015; **Accepted:** 05 January, 2016

Most of the drugs currently in use show side effects which aid in liver injury and are becoming a well recognize a health issue for the fact that the liver is the major site of drug metabolism. [2]

Acetaminophen (paracetamol), a commonly used and safest antipyretic and analgesic drug sold over the counter exhibited weak anti-inflammatory activity and known to cause the necrosis of the centrilobular cells. [3] This occurred upon acute paracetamol overdose after metabolized to its active metabolite, N-acetyl-phenyl benzoquinone (NAPQI), which can bind, covalently with proteins. Upon acute overdoses of paracetamol,

the active metabolite formed inhibits the defense system antioxidant activities, which causes hepatic necrosis. [4] Paracetamol liver toxicity is now a reliable mode of investigation employed by researchers in assessing the potential activities of drugs. [2] In order to have a sustainable therapeutic model to prevent the liver injury induced by drugs, production of foods or their products with valuable protecting molecules that can trap reactive oxygen species and also provide a protection against damaged by oxidative stress is very important.

Traditionally, tofu is produced by curdling of fresh hot soy milk using steep waste water from pap production (Nuwan baka), alum, lemon, and lemon peel ash extract (Suaka) in the Northern part of Nigeria. Tofu has been demonstrated to show potential compounds that are valuable antioxidants and protecting molecules, which can scavenge or destroy free radicals and subsequently protect us from damage due to oxidative stress. [5-6] Tofu is a phenolic-rich soybean product accepted for consumption worldwide, mostly in Asian countries. [7] It was suggested that tofu produced from synthetic and non-synthetic coagulants [6] and locally sourced (steep water) coagulant [6] possessed potential compounds with potent antioxidant activities. Yakubu *et al*. [5] have suggested that steep water and alum coagulated tofu gave higher total phenol and polyphenol extracts with valuable antioxidant activities. It was also demonstrated to prevent acetaminophen-induced liver damage in rats [5] and inhibited hypocholesterolemic effects in rats. [8] Yakubu *et al*. [5] and Oboh *et al*. [8] have concluded that antioxidant activities displayed by coagulated tofu in combating hypercholesterolemia and acetaminophen-induced oxidative stress, respectively, could be associated with the presence of protective phytochemical compounds alone, such as flavonoids.

Flavonoids are a group of natural compounds that occur in crops, fruits, and vegetables. [9] They are dominantly found in crops, fruits, and vegetables in the form of glycosides. [10] The glycoside forms of flavonoids in plants are metabolically active, which possesses higher antioxidant activities, and get absorbed faster in the mucosal layers of the small intestine than vitamin C and E. [11] Alfa *et al*. [12] have demonstrated the high antioxidant ability of flavonoids than that of vitamins C and E, which act to prevent free radical generation. The oxidation of lipid membrane could be prevented by flavonoids. [12] The potent antioxidant activities of flavonoids are associated with scavenging the effect of lipid peroxidation, hydroxyl radicals, and superoxide ions. [13] Flavonoids have been shown to exhibit potent anti-inflammatory, hepatoprotective, antiviral, anticarcinogenic, and anti-allergic activities. [14] Higher ascorbic acid, phenolic acid, polyphenol, and flavonoids were demonstrated in coagulated tofu. [6] Hence, the production of foods with a sustainable therapeutic property to prevent the liver injury induced by drugs must be a focus area of research. Therefore, this study is seeking to investigate the total flavonoids content and effects of flavonoid extract from tofu produced using locally sourced coagulants on the acetaminophen-induced liver damage in rats.

MATERIALS AND METHODS

Soybeans and local coagulants: Alum and *C. limonun* (lemon) were procured from a local market. Steep water (waste water from pap production), and lemon peel ash extract were given by a domestically processed pap. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), total protein, albumin, sugar, and cholesterol kits were procured from Randox Laboratories Ltd., UK. Prothrombin time kit was procured from Quimica Clinica Aplicada, S.A., Spain. All other chemicals were of analytical grade and were procured from Sigma Co. (St. Louis, Missouri, USA).

Animals

Sixty of three-week old strain albino rats with 50 g, as average weights were used in this study. They were obtained from the animal house of the College of Medicine, University of Ilorin, Kwara State, Nigeria. The animals were housed in wire mesh cages in the laboratory under ambient temperature and 12 h light and dark cycle. They were fed with commercial rat pellets (Neimeth Livestock Feeds Ltd., Ikeja) and water *ad libitum* and allowed to acclimatize for 2 weeks. Animal experiments were conducted in accordance with the internationally accepted principle for laboratory animal use and care. [15]

Preparation of coagulant solutions

Alum solution (0.2% w/v) was prepared as the coagulant. A fresh weight of lemony fruits (20 g) was sucked in 50 mL of distilled water for 20 min and ground into slurries using pestle and mortar. The slurries were filtered using cheese cloth and then the final volume of the filtrate was made up to 100 mL with distilled H_2O. Also, about 100 mL of steep waste water and lemon peel ash extract were collected directly from the domestically processed pap, separately, and all solutions were served as local coagulants.

The Acidity of coagulants

An aliquot of each filtrate prepared from local coagulants was titrated against 0.1 NaOH with methyl orange indicator. The acidity of each coagulant was determined and expressed as the % acidity. [15] The acidity of each filtrate was adjusted with the distilled H_2O to 2% as the final % acidity of all filtrates from local coagulants. [16]

Preparation of tofu

The cleaned soybean (150 g) was soaked in 5 L of H_2O at 28°C for 12 hours. The soaked soybeans were re-washed thoroughly, the outer cover of soybeans was removed, and ground with H_2O (1:8) in a blender. The soybean slurry obtained was then heated slowly at 85°C for 60 min with constant stirring. Then, the hot slurry

was filtered using cheese cloth to separate soy milk from the residue (Akara). About 200 mL of cold soy milk at 60°C were measured into five separate 500 mL labeled (1, 2, 3, and 4) beakers. Into beaker (1), 10 mL of the prepared steep water was added, also10 mL of alum, lemon, and lemon peel ash solution were separately added to beakers (2, 3, and 4), respectively. Then, after the addition of respective coagulants, soy milk were stirred for 5 min and kept for 15 min without disturbing for the coagulation to take place. The coagulated soy milk in each beaker was filtered, separately, using cheese cloth and the curdled soy milk (tofu) was pressed with 1 kg weight for 1 hour. The prepared tofu cake was then kept in a refrigerator (4^{o}C) for the subsequent analysis.

Preparation of tofu crude extracts

The fresh soy milk and tofu extraction for total flavonoid content was determined following the method of Komolafe et al.[17] with some modification. Briefly, about 50 g of each tofu of different coagulant was measured, separately, in 100 mL of 80% methanol, homogenized using a pestle and mortar at room temperature, transferred, separately, into a covered flask, and then centrifuged at 16000 × g for 5 min. The supernatant (extracts) of each coagulated tofu were evaporated using a rotary evaporator at 45 ^{o}C to dryness and the dried extracts were stored in bottles covered with an aluminium firm, separately, at -80 ^{o}C for the subsequent analyses.

Total flavonoid content assay

The total flavonoid content was assessed by aluminium chloride colorimetric method. [18] About 1000µL of each coagulated tofu extract was transferred into test tubes containing 4 mL of distilled water, separately, which were further top up with 300µL of 5% sodium nitrite, individually. Then, at the interval of 5 and 6 min, about 300µL of 10% aluminium chloride and 2000µL of 1 M sodium hydroxide were added to all mixtures, respectively. Then, the volume of mixture in each tube was increased to 10 mL by the addition of distilled water, mixed thoroughly using a vortex machine, and the absorbance was measured at 510 nm using the spectrophotometer. Rutin was used as standard and the total flavonoids content was expressed as mg rutin equivalents/g tofu (mg RE/g FW).

Preparation of flavonoid extracts

About 50 mg of methanolic extracts of each coagulated tofu was dissolved in 20 mL of 1% H_2SO_4 in a 50 mL tubes, separately, and then heated on a water bath for 30 min. After heating, the mixtures were placed on the ice for 15 min, cooled solutions were centrifuged at 16000 × g for 5 min, and the pellets were dissolved, separately, in 100 mL of warm 95% ethanol at 50°C. Then, the final solutions were separately concentrated to dryness by a rotary evaporator. [19] About 30 mg of each dried extract was dissolved in 1 mL DMSO (stock extract) and 250µg of each stock flavonoid extract containing 1% DMSO was prepared in 100 mL corn oil, which was served as dosage to be administered to experimental animals by oral gavage.

Experimental Design

Animals were weighed and randomly divided into eleven groups of six rats per group (n=6) and treated as follows:

- Group I (Control): 1 mL/g of corn oil only
- Group II : 1 mL/g corn oil + 100 mg/g Acetaminophen (ACE)
- Group III: 1 mL of 250µg/g steep water tofu flavonoid extract + 100 mg/g ACE
- Group IV: 1 mL of 250µg/g alum tofu flavonoid extract + 100 mg/g ACE
- Group V: 1 mL of 250µg/g lemon tofu flavonoid extract + 100 mg/g ACE
- Group VI: 1 mL of 250µg/g lemon peel ash flavonoid extract + 100 mg/g ACE
- Group VIII: 1 mL of 250µg/g steep water tofu flavonoid extract only.
- Group IX: 1mL of 250µg/g alum tofu flavonoids extract only
- Group X: 1 mL of 250µg/g lemon tofu flavonoid extract only
- Group XI: 1 mL of 250µg/g lemon peel ash tofu flavonoid extract only

The duration of experimental treatment was 14 consecutive days and it was served once daily by oral administration.

Measurements

The weight of rats per group was measured prior to the experiment and during the period of experiments, the weight of rats was measured at 7th and 14th day, respectively. This was determined using a weighing scale (OHAUS MODEL Cs 5000, CAPACITY 500 × 2 g) by placing a container on the scale, with the balance adjusted to zero, after which the rats in each group were placed into the container and the measurement was taken. [20] The percentage (%) total mean weight gain (TMWG) was determined as 100 × (Final weight – Initial weight) /Initial weight. [21] Thereafter, on the 15th day, the animals were killed by cervical dislocation, blood was collected by cardiac puncture, and the liver tissues were harvested for biochemical and histological evaluation.

Liver function assay

The blood collected was made to clot to have the serum upon centrifugation at 3000 rpm for 15 min. Then, the activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) in the serum were accessed using assay kits procured from Randox Laboratories Ltd., UK, following the guidelines of the manufacturer. In addition, the serum chemistry level (total protein, albumin, sugar, and cholesterol) was estimated using assay kits procured from Randox laboratories Ltd., UK, following the instructions given by the manufacturer.

Assay of prothrombin time

The prothrombin time is the time needed for the formation of a fibrin clot upon addition of tissue factor (thromboplastin), phospholipid, and calcium to decalcify the platelet poor plasma, which was done by the assay kit procured from Quimica Clinica Aplicada, S.A., Spain, going by the instructions of the manufacturer.

Histopathology

The liver tissue of each treatment was excised, trimmed of fat and other connective tissues and prepared for histological studies. The tissues were fixed in 10% phosphate-buffered neutral formalin, dehydrated in graded (50–100%) alcohol and embedded in paraffin. Then, thin sections (5 nm) were cut and routinely, stained with hematoxylin and eosin (H & E) stain for the microscopic examination.

Statistical analysis

Data were presented as mean ± SD (n=6). Statistical differences were done by one-way analysis of variance (ANOVA) and the Newman–Keuls comparison test using GraphPad software. The significance level was set at $p < 0.05$.

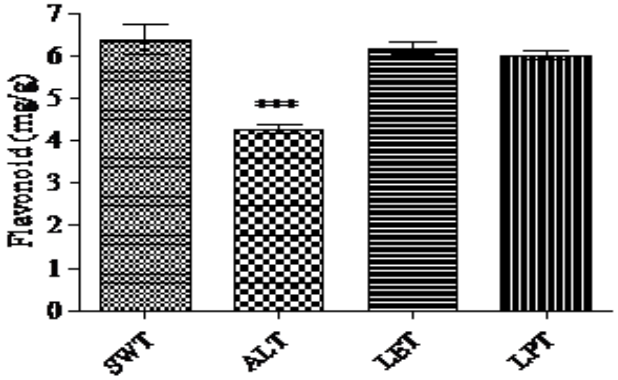

Fig. 1: Total flavonoid contents of tofu (Curdled soy milk) using different coagulants. Values are presented in means ± SD (n=3). SWT: Steep water coagulated tofu, ALT Alum coagulated tofu, LET: Lemon coagulated tofu, LPT: Lemon peel ash extract coagulated tofu. Significantly different was set at $p<0.05$.

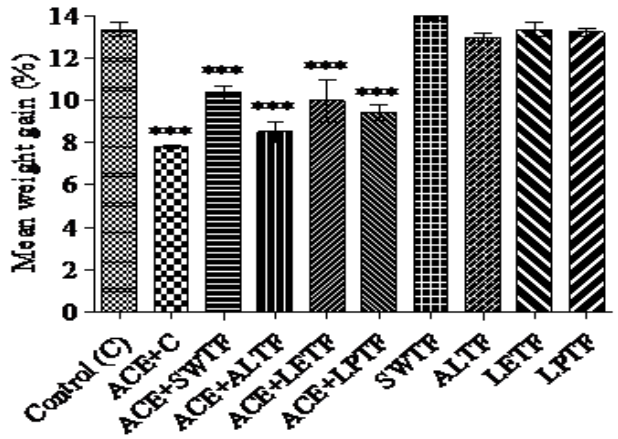

Fig. 2: The mean weight gain of the treated albino rats. Values are presented in percentage mean ± SD (n=6). C: Corn oil, ACE: Acetaminophen, SWTF: Steep water coagulated tofu flavonoid extract, ALTF: Alum coagulated tofu flavonoid extract, LETF: Lemon coagulated tofu flavonoid extract, LPTF: Lemon peel ash coagulated tofu flavonoid extract. Significantly different was set at $p<0.05$.

RESULTS

Total flavonoid contents

The total flavonoid contents in coagulated tofu using locally sourced coagulants are presented in Table 1. The steep water coagulated tofu showed the higher total flavonoid contents (6.4 ± 1.2 mg/g) follows by lemon coagulated tofu (6.2 ± 1.0 mg/g), and lemon peel ash extract coagulated tofu (6.0 ± 1.2 mg/g), which are not differed significantly ($p<0.05$) compared to each other (Fig. 1). However, significantly decreased ($p<0.05$) of total flavonoid content was observed in alum coagulated tofu compared to others (Figure 1).

Total mean weight gain of rats

The result of the mean weight gain of rats is presented in Figure 2. Acetaminophen-induced toxicity effect was shown in rats as indicated in the significant reduction ($p<0.05$) in mean weight gain of rats fed acetaminophen and corn oil (ACE+C) (7.8%), acetaminophen and steep water tofu flavonoid extract (ACE+SWTF) (10.4%), acetaminophen and alum tofu flavonoid extract (ACE+ALTF) (8.5%), acetaminophen and lemon coagulated tofu flavonoid extract (ACE+LETF) (10%), and lemon peel ash coagulated tofu flavonoid extract (ACE+LPTF) (9.4%) compared to the control (13.4%). However, no significant difference ($p<0.05$) of mean weight gain was observed in rats treated with 250µg/mL of steep water coagulated tofu flavonoid extract (SWTF) (14%), alum coagulated tofu flavonoid extract (ALTF) (13%), lemon coagulated tofu flavonoid extract (LETF) (13.4%), lemon peel ash tofu flavonoid extract (LPTF) (13.2%) compared to control (Figure 2).

Serum enzyme markers

Table 1 shows the result of serum enzyme markers in rats studied. It shows that rats treated with ACE+C had the serum enzyme markers elevated significantly ($p<0.05$) with AST (90.6 mg/dL), ALT (28.6 mg/dL), ALP (110.6 mg/dL), and LDH (97.5 mg/dL) compared to AST (54.7 mg/dL), ALT (15.4 mg/dL), ALP (65.5 mg/dL), and LDH (56.4 mg/dL) observed in normal control (Table1). The protective effects of the coagulated tofu flavonoid extract were observed by a significant decrease ($p<0.05$) in AST (65.4-68.8 mg/dL), ALT (18.2-18.8 mg/dL), ALP (84.7-86.1 mg/dL), and LDH (82.5-83.2 mg/dL) in rats treated with 2 mg/mL ACE and 250µg of tofu flavonoid extract, simultaneously, compared to AST (90.6 mg/dL), ALT (28.6 mg/dL), ALP (110.6 mg/dL), and LDH (97.5 mg/dL) in ACE+C-treated rats (Table 1). Similarly, protective effects of tofu flavonoid extracts were detected by no significant difference ($p<0.05$) in AST (52.6-53.7 mg/dL), ALT (14.2-14.7 mg/dL), ALP (64.0-65.1 mg/dL), and LDH (55.4-56.1 mg/dL) in rats treated with coagulated tofu flavonoid extract alone compared to AST (54.7 mg/dL), ALT (15.4 mg/dL), ALP (65.5 mg/dL), and LDH (56.4 mg/dL) in the normal control (Table 1).

Serum chemistry

The result of serum chemistry levels in rats studied is shown (Table 2). It reveals that rats treated with 100

mg/g of ACE+C showed significantly higher (p<0.05) of serum glucose levels (123.5 mg/dL) and cholesterol (175.6 mg/dL) compared to normal control (65.5 and 66.4 mg/dL), respectively (Table 2). On the contrary, the total protein (1.6 mg/dL) and albumin (0.82 mg/dL) levels in rats treated with 100 mg/g of ACE+C were reduced significantly (p<0.05) compared to the normal control (5.5 and 3.6 mg/dL), respectively (Table 2). However, the protective effects were observed in rats treated with 100 mg/g acetaminophen and 250µg/mL coagulated tofu flavonoid extract, simultaneously, by a significant decrease (p<0.05) in serum glucose ranged from 85.5-88.4 mg/dL and cholesterol with 86.7-89.4 mg/mL compared to glucose (123.5 mg/dL) and cholesterol (175.6 mg/mL) in ACE+C-treated rats (Table 2). Also, the protective effects were observed by a significant increase (p<0.05) in serum total protein ranged from 2.3-2.5 mg/dL and albumin ranged from 1.5-1.7 mg/dL compared to total protein (1.6 mg/dL) and albumin (0.82 mg/dL) in ACE+C-treated rats (Table 2). Similarly, the biological benefits of the coagulated tofu flavonoid extract were shown by a no significant difference (p<0.05) in the serum glucose (63.7-65.3 mg/dL), total protein (5.7-6.4 mg/dL), albumin (3.6-4.2 mg/dL), and cholesterol (63.5-65.1 mg/mL) compared to serum glucose (65.5 mg/dL), total protein (5.5 mg/dL), albumin (3.6 mg/dL), and cholesterol (66.4 mg/mL) of the normal rats (Table 2).

Prothrombin time

Figure 3 presents the result of serum prothrombin time in acetaminophen-intoxicated rats. The prothrombin time in rats treated with acetaminophen was significantly elevated (p<0.001) with ACE+C (18.5 s), ACE+SWTF (15.4 s), ACE+ALTF (15.7 s), ACE+LETF (15.1 s), and ACE+LPTF (15.0 s) compared to normal control (9.6 s). However, the protective effects were observed in rats treated with coagulated tofu flavonoid extract by a significant decrease (p<0.05) of prothrombin time with SWTF (10.0 s), ALTF (9.9 s), LETF (9.8 s), and LPTF (10 s) compared to control (9.6 s) (Figure 3).

Table 1: Serum enzyme markers of the acetaminophen-intoxicated rats.

Treatment	AST (mg/dL)	ALT (mg/dL)	ALP (mg/dL)	LDH (mg/dL)
ACE+C	90.6 ± 1.2^a	28.6 ± 1.4^a	110.6 ±1.2^a	97.5 ± 1.5^a
Control (C)	54.7 ± 1.4^c	15.4 ± 1.2^c	65.5 ± 1.4^c	56.4 ± 1.5^c
ACE+SWTF	65.4 ± 1.5^b	18.6 ± 1.2^b	85.5 ± 1.5^b	82.5 ± 1.2^b
ACE+ALTF	68.7 ± 1.2^b	18.8 ± 1.4^b	86.1 ± 1.4^b	83.1 ± 1.4^b
ACE+LETF	66.5 ± 1.0^b	18.2 ± 1.5^b	84.7 ± 1.2^b	83.0 ± 1.4^b
ACE+LPTF	67.6 ± 1.5^b	18.4 ± 1.2^b	85.1 ± 1.4^b	83.2 ± 1.5^b
SWTF	52.7 ± 1.5^c	14.5 ± 1.4^c	64.6 ± 1.4^c	55.6 ± 1.5^c
ALTF	53.7 ± 1.2^c	14.7 ± 1.5^c	65.1 ± 1.2^c	56.1 ± 1.2^c
LETF	52.9^d	14.2 ± 1.2^c	64.0 ± 1.5^c	55.4 ± 1.2^c
LPTF	52.6^d	14.4 ± 1.4^c	64.3 ± 1.2^c	55.5 ± 1.5^c

Values are presented in mean ± SD (n=6) and means with the same superscript letter(s) along the same column are not significantly different (p<0.05). C: Corn oil, ACE: Acetaminophen, SWTF: Steep water coagulated tofu flavonoid extract, ALTF: Alum coagulated tofu flavonoid extract, LETF: Lemon coagulated tofu flavonoid extract, LPTF: Lemon peel ash coagulated tofu flavonoid extract.

Table 2: Serum chemistry of the acetaminophen-intoxicated rats.

Treatment	GLU (mg/dL	TP (mg/dL)	ALB (mg/dL)	CHL (mg/dL)
ACE+C	123.5 ±1.2^a	1.6 ± 1.2^c	0.82 ± 1.2^c	175.6 ±1.2^a
Control (C)	65.5 ± 1.4^c	5.5 ± 1.3^a	3.6 ± 1.5^a	66.4 ± 1.5^c
ACE+SWTF	86.7 ± 1.4^b	2.3 ± 1.4^b	1.5 ± 1.5^b	89.4 ± 1.4^b
ACE+ALTF	88.4 ± 1.5^b	2.4 ± 1.5^b	1.5 ± 1.4^b	86.7 ± 1.2^b
ACE+LETF	85.5 ± 1.5^b	2.5 ± 1.5^b	1.7 ± 1.2^b	87.4 ± 1.2^b
ACE+LPTF	86.4 ± 1.2^b	2.5 ± 1.2^b	1.6 ± 1.3^b	87.2 ± 1.5^b
SWTF	64.5 ± 1.5^c	5.7 ± 1.2^a	3.8 ± 1.4^a	63.5 ± 1.4^c
ALTF	65.3 ± 1.2^c	6.1 ± 1.5^a	3.6 ± 1.2^a	64.5 ± 1.2^c
LETF	63.7 ± 1.2^c	6.4 ± 1.4^a	4.2 ± 1.5^a	64.8 ± 1.4^c
LPTF	64.1 ± 1.5^c	6.3 ± 1.5^a	4.0 ± 1.5^a	65.1 ± 1.4^c

Values are presented in mean ± SD (n=6) and means with the same superscript letter(s) along the same column are not significantly different (p<0.05). GLU: Glucose, TP: Total protein, ALB: Albumin, CHL: Cholesterol, C: Corn oil, ACE: Acetaminophen, SWTF: Steep water coagulated tofu flavonoid extract, ALTF: Alum coagulated tofu flavonoid extract, LETF: Lemon coagulated tofu flavonoid extract, LPTF: Lemon peel ash coagulated tofu flavonoid extract.

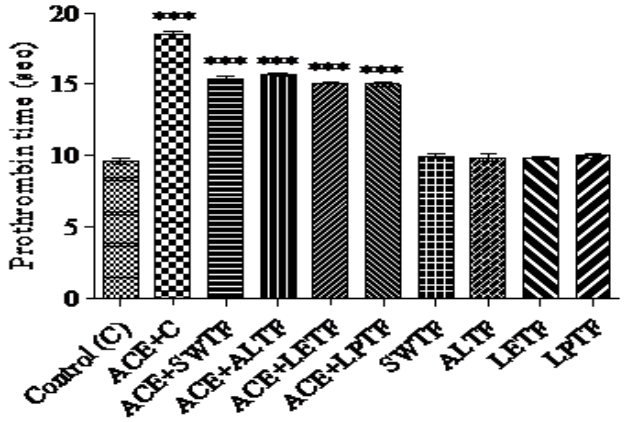

Fig. 3: Serum prothrombin time of acetaminophen-intoxicated rats. Values are presented in mean ± SD (n=6). C: Corn oil, ACE: Acetaminophen, SWTF: Steep water coagulated tofu flavonoid extract, ALTF: Alum coagulated tofu flavonoid extract, LETF: Lemon coagulated tofu flavonoid extract, LPTF: Lemon peel ash coagulated tofu flavonoid extract. ***p<0.001: Statistically significant in the normal control.

Histopathology

Figure 4 shows the microscopic examination of the liver sections from rats studied. Normal hepatic architecture with an indication of no pathological changes was observed in the liver sections from normal control (Figure 4A). Some patterns of necrosis and inflammatory across the hepatic cells were observed in rats intoxicated with 100 mg/g of acetaminophen (Figure 4B). However, less hepatic cell damage, which is a sign of protective activities of coagulated tofu flavonoid extracts were observed in the liver of rats treated with 100 mg/g of acetaminophen and 250µg/g of coagulated tofu flavonoid extracted, simultaneously, compared to ACE+C- treated rats (Figure 4C, D, E, F). Similarly, a biological benefit of the flavonoid extracts was demonstrated in the liver of rats treated with 250 µg/g of coagulate tofu flavonoid extracts, as indicated by normal hepatic features compared to the normal (Figure 4G, H, I, J).

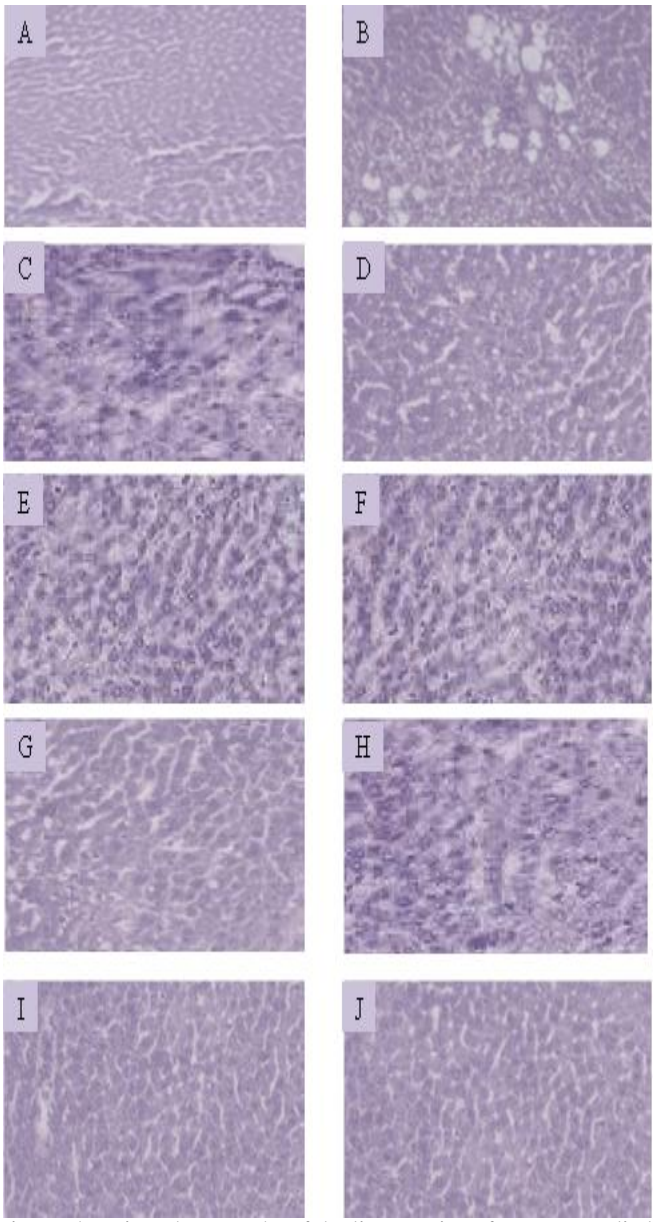

Fig. 4: The microphotographs of the liver sections from rats studied. A: Control (corn oil), B: 100 mg/g of acetaminophen, C: 100 mg/g of acetaminophen and 250µg/g of steep water tofu flavonoid extract, D: 100 mg/g of acetaminophen and 250µg/g of alum tofu flavonoid extract, E: 100 mg/g of acetaminophen and 250µg/g of lemon tofu flavonoid extract, F: 100 mg/g of acetaminophen and 250µg/g of lemon peel ash tofu flavonoid extract, G: 250µg/g of steep water tofu flavonoid extract, H: 250µg/g of alum tofu flavonoid extract, I: 250µg/g of lemon tofu flavonoid extract, J: 250µg/g of lemon peel ash water tofu flavonoid extract.

DISCUSSION

The total flavonoid contents and its protective ability of coagulated tofu produced from locally sourced coagulants against acetaminophen-induced hepatic injury in rats were investigated. Flavonoids (a polyphenolic compound) react to form complexes with metallic ions (iron, zinc, copper) and thereby reduced their absorption. [22] It reduces the nutrient absorption; however, high levels of these metals (cations) in the body can promote oxidative damage to the cell membrane and cellular DNA. [22] Flavonoid functions as a potent neutralizer of free radical species in the body. [22] The total flavonoids of coagulated tofu as presented in Figure 1 demonstrated that all tofu exhibited high flavonoid contents, which are not significantly different from each other. These findings are well compared to the value of the flavonoid content of *C. ternatea* flower extract. [22] This report is supported by the observation of [5] that the antioxidant activity exhibited by coagulated tofu could be responsible for their polyphenol contents.

Figure 2 reveals a significant decrease in total weight gain of rats intoxicated with 100 mg/g of acetaminophen compared to normal control. This decrease in weight gain could be associated with the acetaminophen-intoxication that produced oxidative stress in rats. The oxidative stress generated in rats intoxicated with acetaminophen could also be responsible for the loss in body weight gain. The protective activity of tofu flavonoid extracts was confirmed by a significant increase (p<0.05) of body weight gain in rats given acetaminophen and tofu flavonoids, simultaneously, compared to ACE-intoxicated rats. The body weight gain exhibited by rats given a combination of acetaminophen and tofu flavonoids could be associated with the highest antioxidant activity of tofu flavonoid extracts. This finding is supported by the report of Yakubu *et al.* [5] that rats fed with alum and steep water coagulated tofu, separately, for 14 days gained in body weight over the acetaminophen intoxicated rats. Likewise, the protective ability of tofu flavonoid extracts was also shown as an indication of no significant differences (p<0.05) in body weight gain in rats orally given tofu flavonoid extracts compared to the normal control. This could be due to the high antioxidant activity of tofu flavonoid extracts, considering the high total flavonoid contents as confirmed earlier in this study. Yakubu *et al.* [5] have demonstrated that coagulated tofu containing some meaningful phenolic acids and polyphenols, which could serve as protective agents in the body system.

These results were further confirmed by *in vivo* hepatoprotective study as presented in Table 1. It reveals that rats intoxicated with acetaminophen demonstrated a high level of serum marker enzymes (AST, ALT, ALP, and LDH). These elevations of serum marker enzymes might be due to hepatocellular injuries. This finding is in line with the report of M.T. Olaleye *et al.* [2] that increased in serum levels of marker enzymes could indicate cellular membrane damage and their subsequent leakages into the blood circulation. Increased activities of these enzymes could also signify acute hepatocellular injuries (drug-induced necrosis and acute viral hepatitis leakage. [24] These results are supported by the previous studies that the elevations of serum marker enzymes indicate liver injuries. [2, 4-5, 25-26] On the contrary, the hepatoprotective effects of tofu flavonoid extracts were demonstrated in rats treated with ACE and tofu flavonoid extracts, simultaneously, as indicated by a significant reduction (p<0.05) in

serum activities of marker enzymes (Table 1). This indicates the ability of tofu flavonoid extracts to protect the liver cells from oxidative injury due to intoxicated rats with acetaminophen. The serum level reduction of marker enzymes could also be associated with the presence of protective phytochemical compounds such as flavonoids in the tofu flavonoid extracts, as earlier suggested in this study. Kuppan Nithianantham *et al*. [22] have suggested that flavonoid extracts might have some functional mechanisms in protecting the structural integrity of hepatic cells and prevent leaking of enzymes into the blood stream. Previously, parallel findings have been reported. [5, 14, 27] Interestingly, raised levels, but no significant differences (p<0.05) in the activities of serum marker enzymes were observed in rats intubated with tofu flavonoid extracts only compared to normal control (Table 1). This might signify the potent antioxidant activities of tofu flavonoid extracts. Yakubu *et al*. [5] and Anderson and Theron [28] have demonstrated that soy products contained high antioxidant properties that scavenge the free radicals generated in the extracellular cells. Yakubu *et al*. [5] and Alfa *et al*. [12] have reported that vegetables and tofu respectively, are rich in phenols (flavonoid). Flavonoids are potent antioxidant agents compared to vitamin C and E, reportedly used to neutralize free radical activities. [12]

The *in vivo* protective activities of tofu flavonoid extracts by evaluating the levels of serum chemistry (glucose, total protein, albumin, and total cholesterol) were conducted (Table 2). High raised significantly (p<0.05), in the serum glucose and total cholesterol were observed in rats intoxicated with ACE. The rise in serum chemistry might be an indication of hepatotoxicity generated by ACE intubated. However, the reduction of serum sugar and total cholesterol as an indication of hepatoprotection against oxidative stress in animals treated with ACE and tofu flavonoid extracts could be associated with the potent antioxidant activity of soy product flavonoid extracts, which is a neutralizer of the extracellular free radicals. Oboh [29] has demonstrated that the antihypercholesterolemic effect of soy flavonoid might cause the reduction of the plasma concentrations of LDL as well as the ratio of plasma LDL to HDL. Flavonoids can protect membrane lipids from oxidation, and major sources of flavonoids are vegetables, fruits, and soybeans. [2] Contrarily, serum total protein and albumin reduction in rats intoxicated with ACE signify the production of oxidative stress caused by ACE. However, the raised in total protein and albumin in animals topped with ACE and tofu flavonoid extracts, simultaneously, could explain the protective mechanisms of tofu flavonoid extracts. This result is in line with that reported by Yakubu *et al*. [5] that elevated total protein and albumin were observed in rats treated with various coagulated tofu and acetaminophen, simultaneously. The elevation in total protein and albumin might be due to high effectiveness and stabilized endoplasmic reticulum leading to protein synthesis, which is an evidence of hepatoprotection. [30] Acetaminophen administered alone may adversely affect the protein metabolism, possibly by preventing the protein synthesis in the liver. Probably, supplementing rats intoxicated by acetaminophen with flavonoid extracts could stimulate protein and albumin synthesis in the liver. The stimulation has been advanced as a contributory hepatic cell protective mechanism that can enhance cell regeneration. [31] The protective activities of tofu flavonoid extracts as demonstrated by no significant difference of serum chemistry levels in rats treated with flavonoid extracts justify their potent antioxidant activities. Kuppan Nithianantham *et al.* [20] and Yakubu *et al.* [5] have reported the presence of antioxidant activities, total phenolic acids, and polyphenols in soybeans and its products. This finding is supported by high total flavonoids contents of coagulated tofu, as earlier reported in this study. It was also established in this study that ACE intoxication prolongs the prothrombin time (PT) in rat serum. However, in rats supplemented with tofu flavonoid extracts, the PT prolongation was inhibited. The decrease of PT in rats topped with ACE and tofu flavonoid extracts could be associated with a potential hepatocellular generative capacity by tofu flavonoid extracts. PT, an index of hepatocellular synthetic activity is a vital diagnostic method in hepatic cell injuries. Worsening PT prolongation in acute hepatic necrosis may be associated with high risk of liver injury. [24, 32]

The hepatic protective activity of tofu flavonoid extracts against acetaminophen-induced hepatocellular injuries in rats were further evaluated by histopathological observation of liver sections (Figure 4). It was clearly established that advert effects, like necrosis and inflammatory across the hepatic cells, were observed in the liver sections of animals treated with ACE. These damages observed on the liver architecture might be associated with the production of oxidative stress caused by ACE-intoxication. Acetaminophen intoxication after 14 days of experiment causes hepatic necrosis, fatty infiltration, lymphocytic and neutrophil infiltration in rats. [2, 22] Obek *et al*. [21] have demonstrated that ACE-intoxication may result in free radical generation and glutathione depletion. Glutathione is a potent neutralizer of the free radical activities, which also causes oxidation of glutathione to glutathione disulfide that lead to the diminished glutathione stores in the liver cells. [5, 22] The glutathione preservative mechanism signifies a protection against ACE-induced hepatic toxicity. [2] The ability of tofu flavonoid extracts to accelerate the recovering of hepatic cells in rats treated with the ACE and tofu flavonoid extracts showed the protective activities of the tofu extracts against ACE intoxications, and thus prevented the leakage of the marker enzymes into blood circulation as earlier reported in this study.

These findings from the histological study confirmed the hepatic protective effects of tofu flavonoid extracts against ACE-intoxication. This result correlates with the reports of M.T. Olaleye *et al.* [2] and Kuppan Nithianantham *et al.* [22] that flavonoid extracts enhanced improvement of the hepatic cell injuries caused by ACE-intoxication.

Interestingly, intact hepatic cell architectures were observed in rats topped with tofu flavonoid extracts as compared to the liver cell sections of the normal control. This observation could be attributed to the potent antioxidant activities of tofu flavonoid extracts. It was reported that the potent antioxidant activities exhibited in coagulated tofu, [5] flavonoid extracts [2] could be attributed to the presence of protective phytochemicals such as phenolics and polyphenols. [33]. Polyphenols (flavonoids) are potent free radical scavengers in the body system. [2] Kuppan Nithianantham *et al.* [22] reported that soy foods and vegetables are richer in polyphenols (flavonoids) that have potent antioxidant capacity than vitamin C and E, reportedly used in preventing the production of free radicals.

Conclusively, the present study demonstrated that all the coagulated tofus are rich in total flavonoids and their flavonoid extracts exhibited hepatoprotective activity against ACE-intoxication in rats. The liver protection ability of tofu flavonoid extracts may be associated with their free radical neutralizing and antioxidant capacities. Therefore, supplementation of tofu in our diets is highly recommended as it can be used as a functional food to prevent liver injuries.

ACKNOLEDGEMENTS

The authors acknowledged the supports of Pest Management Technology and Pre-ND Sciences Departments, Niger State College of Agriculture, Mokwa for providing facilities for the conduct of this research.

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Source of Support: Nil, Conflict of Interest: None declared.