Revista Brasileira de Farmacognosia 25 (2015) 29–34

Revista Brasileira de Farmacognosia
BRAZILIAN JOURNAL OF PHARMACOGNOSY
www.sbfgnosia.org.br/revista

Original Article

Combined administration of silymarin and vitamin C stalls acetaminophen-mediated hepatic oxidative insults in Wistar rats

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ARTICLE INFO

Article history:
Received 14 August 2014
Accepted 28 November 2014
Available online 11 February 2015

Keywords:
Antioxidants
Hepatoprotective
Reactive metabolite
Therapeutic
Lipid peroxidation

ABSTRACT

Oxidative insult by free radicals has been implicated in drug-induced hepatic damage and this has resulted in frequent episodes of liver disorders. Therapeutic efficacy of antioxidants may provide a possible solution to this menace. This study was carried out to investigate the effect of combined administration of silymarin and vitamin C in rescuing acetaminophen-induced hepatotoxicity in rats. Hepatotoxic rats were orally administered with silymarin and vitamin C at 100 and 200 mg/kg body weight, respectively. At the end of the experiment, liver function indices, antioxidant parameters and histological analysis were evaluated. We observed that the significantly increased ($p < 0.05$) activities of alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, as well as levels of thiobarbituric acid reactive substances and serum total bilirubin, were markedly reduced following co-administration of silymarin and vitamin C. The compounds also effectively reversed the reduced activities of superoxide dismutase, catalase, glutathione peroxidase, glutathione *S*-transferase and total protein concentration in the hepatotoxic rats. These findings are indicative of hepatoprotective and antioxidant attributes of the two compounds which are also supported by the histological analysis. The available evidences in this study suggest that the complementary effects of silymarin and vitamin C proved to be capable of ameliorating acetaminophen-mediated hepatic oxidative damage and the probable mechanism is *via* antioxidative action.

Introduction

Liver, the key organ in maintenance of homeostasis as well as metabolism and excretion, has an overwhelming task of detoxifying xenobiotics and chemotherapeutic agents (Ademuyiwa et al., 1994). Its role in transforming and clearing chemicals renders it susceptible to damage from these agents. Generally, this usually goes unnoticed because of the considerable capacity of hepatocytes to regenerate. However, overstressed liver compromises its detoxification role which may expose it to a variety of diseases and disorders (Amic et al., 2003). Liver injury caused by toxic chemicals and certain drugs has been recognized as a toxicological problem and linked to the occurrence of oxidative stress (Wolf et al., 1997). Free radicals' generation, arising from oxidative stress, is a common mechanism underlying hepatotoxicity caused by deleterious effect of drugs and toxicants. Oxidative stress has also been implicated in the pathogenesis of cellular damage caused by a number of toxic agents including arsenic, diclofenac, carbon tetrachloride, rifampicin and acetaminophen (Deavall et al., 2012).

Acetaminophen (paracetamol) is a widely used antipyretic and analgesic which produces acute liver damage if overdoses are ingested. The drug is mainly metabolized in the liver to excretable glucuronide and sulphate conjugates (Jollow et al., 1974; Wong et al., 1981). Hepatotoxicity caused by paracetamol ingestion has been attributed to the formation of a highly reactive metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI), by the action of hepatic cytochrome P-450 (Savides and Oehne, 1983). NAPQI is initially detoxified by conjugation with reduced glutathione (GSH) to form mercapturic acid (Moore et al., 1985). However, when the rate of NAPQI formation exceeds the rate of detoxification by GSH, it oxidizes tissue macromolecules such as lipids or sulfylhydride (SH) group of protein and alters the homeostasis of calcium after depleting GSH.

Hepatotoxicity is one of very common ailments resulting into serious debilities ranging from severe metabolic disorders to mortality (Patel et al., 2008). A good number of antioxidants have been

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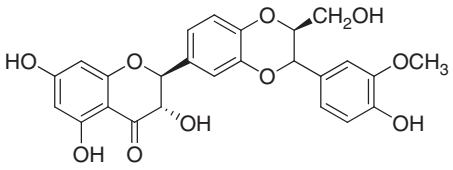
http://dx.doi.org/10.1016/j.bjp.2014.11.012

exploited in the management of liver disorders. These agents play an important role in scavenging free radicals thereby providing protection against infections and degenerative diseases (Subramaniam et al., 2000). Silymarin and vitamin C have received considerable attention over the years due to their diverse antioxidant and hepatoprotective activities (McDowell, 1989; Sies et al., 1992; Burtis and Ashwood, 1994; Shaker et al., 2010; Tong et al., 2011).

Silymarin (**1**) is a flavonoid extracted from the seed of *Silybum marianum* (L.) Gaertn. (milk thistle plant) which is a member of the aster family (Asteraceae). Silybin (silibinin), silychristin and silydinin have been identified as its other active principles (Rui, 1991). *S. marianum* is one of the oldest and most thoroughly researched plants in the treatment of liver diseases (Morazzoni and Bombardelli, 1995). Seeds of the plant have been used for more than 2000 years to treat a range of liver and gallbladder disorders including hepatitis, cirrhosis and icterus (Morazzoni and Bombardelli, 1995). It is also commonly used as in herbal therapy especially for treating liver diseases partly due to its antioxidant activity (Tong et al., 2011). In drug- and chemically-induced oxidative stress, silymarin has been reported as the primary therapeutic modality of choice (Ferenci et al., 1989; Blázovics and Fehér, 2001; Féher and Lengvel, 2012).



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Vitamin C is a six-carbon compound structurally related to glucose. It consists of two inter-convertible compounds: L-ascorbic acid, which is a strong reducing agent, and its oxidized derivative, L-dehydroascorbic acid (Elias and Oputiri, 2013). Vitamin C is found in citrus, soft fruits and leafy green vegetables while kidney and liver are good animal-derived sources of the compound (Stangeland et al., 2008). Vitamin C is hydrophilic and exerts its antioxidant action by inhibiting lipid peroxidation and oxidative cell damage (Xavier et al., 2007). Pathogenic dysfunction of tissues owing to cell death *via* apoptosis is one of the important outcomes of oxidative stress that could be ameliorated by vitamin C (Santos et al., 2009). Ergul et al. (2010) have also reported the effect of vitamin C on oxidative liver injury induced by isoniazid in rats. They found that isoniazid-mediated hepatic onslaught was associated with oxidative stress and treatment with vitamin C ameliorated liver damage appreciably.

Accordingly, since the attention of today's clinical practice is focused on antioxidant consumption against drug-induced oxidative stress, the present study examined the combined effects of silymarin and vitamin C on acetaminophen-mediated hepatic oxidative insult in rats.

Materials and methods

Chemicals and reagents

Silymarin was procured from Sigma-Aldrich Chemicals Company (St. Louis, Mo, USA). Paracetamol and vitamin C were products of Emzor Pharmaceuticals, Lagos, Nigeria. Assay kits were purchased from Randox Laboratories limited, United Kingdom and Sigma-Aldrich Chemicals Company (St. Louis, Mo, USA). Distilled water was obtained from Biochemistry Laboratory, Kwara State University, Malete, Ilorin, Nigeria. Other chemicals and reagents were of analytical grade.

Experimental animals

Wistar strain albino rats with a mean weight of 180.00 ± 2.33 g were obtained from the Animal House of Al-Hikmah University, Ilorin, Nigeria. The animals were kept in clean metabolic cages placed in a well-ventilated room with optimum condition (temperature: 23 ± 1 °C, photoperiod: 12 h natural light and 12 h dark, relative humidity: 45–50%). They were acclimatized to animal house conditions for ten days and were allowed free access to food and water *ad libitum*. The research was carried out following approval from the Ethical committee on the use of Laboratory Animals of Al-Hikmah University, Ilorin, Nigeria. A clearance number HUI/ECULA/014/04/002 was assigned and issued for the research.

Induction of liver damage

Hepatotoxicity (liver damage) was induced in rats according to the procedure described by Kanchana and Mohamed (2011). Briefly, the animals were orally administered with 400 mg/kg body weight (b.w.) of acetaminophen once daily for seven days. Feed and water were made available to the animals *ad libitum* throughout the induction period.

Animal grouping and treatments

Thirty albino rats were randomized into five groups of six rats each. Group 1 animals served as normal control and were given distilled water. Group 2 comprised animals induced with liver damage but not treated. Animals in groups 3–5 were hepatotoxic rats administered with therapeutic doses of silymarin (200 mg/kg b.w.), vitamin C (200 mg/kg b.w.) and both compounds co-administered (100 mg/kg b.w. each) respectively (Mongi et al., 2011; János and Gabriella, 2012; Sabzevarizadeh and Najafzadeh, 2012; Santhrani et al., 2012). All administrations were done once daily for seven days using oral intubator with *ad libitum* provision of food and water throughout the experimental period.

Preparation of serum and excision of liver

Twenty-four hours after the last treatment, the rats were humanely sacrificed by diethyl ether anaesthetization. Blood was collected by cardiac puncture into centrifuge tubes and allowed to stay for 20 min before centrifuging at 3000 × *g* for 15 min using a bench centrifuge (Beckman and Hirsch, Burlington, IO, USA). Serum was carefully aspirated and used for liver function tests. The liver was excised, cleaned of fat and sliced into two portions. A portion of the liver was homogenized in Tris–HCl buffer (0.05 mol/l Tris–HCl and 1.15% KCl, pH 7.4) for antioxidant analyses, while the other portion was fixed in saline formaldehyde solution for histological examination.

Liver function indices, antioxidant analyses and histopathological examination

Adopting the procedure described by Reitman and Frankel (1957), serum activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined. Total bilirubin and protein concentrations were determined using the methods of Jendrassik and Grof (1938) and Lowry et al. (1951) respectively, while the activity of alkaline phosphatase (ALP) was assayed according to the method of Rec (1972). Activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione transferase (GST) were assayed using the methods of Marklund and Marklund (1974), Sinha (1972), Thabrew et al. (1987) and Rotruck et al. (1973) respectively. Following the procedure described by Devasagayam and Tarachand (1987), level of

lipid peroxidation measured in terms of thiobarbituric acid reactive substances (TBARS) was determined in the liver homogenate. Histopathological examination of the liver was carried out using the method of Bancroft and Stevens (1990).

Statistical analysis

All data were subjected to one-way analysis of variance (ANOVA) using SPSS software package for windows (Version 16) and expressed as mean (X) ± standard error of mean (SEM) ($n = 6$). Significant difference between the treatment means was determined at 5% confidence level using Duncan's Multiple Range Test.

Results

Liver function tests

Table 1 shows the effects of silymarin and vitamin C on the activities of serum ALP, ALT, AST as well as concentrations of total bilirubin and total protein in the experimental rats. Oral administration of 400 mg/kg b.w. of acetaminophen for seven days caused a significant ($p < 0.05$) increase in the activities of these enzymes and level of total bilirubin as well as significant reduction ($p < 0.05$) in the concentration of total protein when compared with the normal control. The elevated activities of the assayed enzymes and total bilirubin concentration induced by acetaminophen were significantly attenuated ($p < 0.05$) following treatment with either silymarin/vitamin C or both. The reduced concentration of total protein was also significantly increased ($p < 0.05$) after treatment with either silymarin/vitamin C or both compounds. The effects were however more pronounced in the rats wholly treated with vitamin C and those co-administered with both silymarin and vitamin C.

Antioxidant analyses

The effects of silymarin and vitamin C on the antioxidant status of rat liver are presented in Table 2. TBARS was significantly increased ($p < 0.05$) in the hepatotoxic rats. Separate and combined treatments with silymarin and vitamin C significantly reduced ($p < 0.05$) the level of TBARS comparable to normal. A significant reduction ($p < 0.05$) was also observed in the activities of SOD, CAT, GPx and GST in liver of hepatotoxic rats when compared with normal control. Co-treatment with silymarin and vitamin C resulted in significant increase ($p < 0.05$) in the activities of these enzymes which was comparable to normal.

Histopathological analysis

The liver micrographs of the rats are presented in Fig. 1. The liver of the control rats showed normal architecture with well-preserved cords of hepatocytes, well-demarcated sinusoids and no area of infiltration by inflammatory cells (Fig. 1A). This is in contrast to the features observed in the liver of hepatotoxic rats. There were drastic alterations in liver architecture ranging from extensive fatty change, distended hepatocytes, vacuolated cytoplasm, compressed sinusoids, fatty degeneration area of necrosis, to infiltration by inflammatory cells (Fig. 1B). However, the liver micrographs of rats treated with silymarin/vitamin C or both showed distinct and essentially normal cords of hepatocyte with non-prominent fatty change (Fig. 1C–E).

Discussion

Drug-induced liver disorders occurred frequently and can be life threatening. Oxidative stress occasioned by highly reactive intermediates (free radicals) has been linked to acetaminophen-mediated hepatotoxicity in rats (Balamurugan, 2007). The catastrophic free radical events such as lipid peroxidation, protein oxidation and DNA oxidation are rarely the cause of cell death in realistic *in vivo* condition. This is because the antioxidant defense arsenal in liver cells is capable of detoxifying free radicals and repair damage resulting from highly reactive metabolites (Jaeschke et al., 2003). However, when the antioxidant defense system is overwhelmed, free radicals may inflict direct oxidative damage to cellular macromolecules, leading to cell death (Sabiu et al., 2014). Timely intervention with exogenous antioxidants augments the cellular defense system to prevent these ill effects on cellular macromolecules. Studies have reported the antioxidants and cytoprotective activities of silymarin and vitamin C (Crocenzi et al., 2003; Santos et al., 2009; Mor and Ozmen, 2010). This study, thus, demonstrates the antioxidant and hepatoprotective potentials of silymarin and vitamin C in acetaminophen-mediated hepatic oxidative damage in rats.

Increased activities of serum AST, ALT and ALP are indicative of cellular leakage and loss of functional integrity of liver cell membrane. The extent of drug-induced hepatotoxicity is assessed by the release of these intracellular enzymes *via* the hepatocyte membrane into circulation (Sabiu et al., 2014). Specifically, increased activity of AST is indicative of liver damage due to viral hepatitis, cardiac infarction and muscle injury. ALT is more specific to the liver and thus a better parameter for assessing liver injury (Willianson et al., 1996) while serum ALP activity gives a clue to the functionality of the hepatocytes. Elevated activity of serum ALP may be attributed to increased synthesis in the face of increasing biliary pressure (Gini and Muraleedhara, 2010). In the present study, elevated activities of AST, ALT and ALP in acetaminophen-treated rats

Table 1
Effects of silymarin and vitamin C on liver function indices of acetaminophen-induced hepatotoxic rats ($n = 6$, $X \pm$ SEM).

Treatments	AST (U/L)	ALT (U/L)	ALP (U/L)	Total bilirubin (mg/dl)	Total protein (g/dl)
Control	85.36 ± 4.51[a]	47.15 ± 2.35[a]	75.23 ± 5.31[a]	0.92 ± 0.06[a]	9.32 ± 0.32[a]
Acetaminophen induced	147.81 ± 9.20[b]	160.28 ± 4.52[b]	126.21 ± 3.36[b]	1.98 ± 0.09[b]	5.46 ± 0.06[b]
Acetaminophen induced + silymarin	110.17 ± 5.32[c]	63.65 ± 2.25[c]	90.32 ± 2.63[c]	0.70 ± 0.04[c]	7.75 ± 0.32[c]
Acetaminophen induced + vitamin C	100.36 ± 4.63[a]	60.87 ± 3.19[a]	70.25 ± 9.61[a]	0.80 ± 0.05[a]	8.99 ± 0.15[a]
Acetaminophen induced + silymarin + vitamin C	90.04 ± 4.68[a]	55.39 ± 2.86[a]	73.61. ± 2.92[a]	0.88 ± 0.02[a]	9.25 ± 0.35[a]

Values with different superscripts along the same column for each parameter are significantly different ($p < 0.05$).

Table 2
Effects of silymarin and vitamin C on antioxidant status of acetaminophen-induced hepatotoxic rats ($n = 6$, $X \pm$ SEM).

Treatments	TBARS (mM/100 g tissue)	SOD (U/mg protein)	CAT (U/mg protein)	GPX (U/mg protein)	GST (U/mg protein)
Control	14.64 ± 1.75[a]	7.25 ± 0.90[a]	3.98 ± 0.35[a]	18.56 ± 0.31[a]	1.02 ± 0.05[a]
Acetaminophen induced	18.80 ± 1.55[b]	5.65 ± 0.93[b]	2.18 ± 0.25[b]	16.42 ± 0.79[b]	0.59 ± 0.03[b]
Acetaminophen induced + silymarin	15.30 ± 1.75[a]	6.69 ± 0.52[a]	2.99 ± 0.23[a]	17.48 ± 0.30[a]	0.82 ± 0.02[a]
Acetaminophen induced + vitamin C	14.01 ± 1.63[a]	6.99 ± 0.32[a]	3.55 ± 0.97[a]	18.27 ± 0.08[a]	0.92 ± 0.02[a]
Acetaminophen induced + silymarin + vitamin C	14.52 ± 1.09[a]	7.20 ± 0.59[a]	3.81 ± 0.05[a]	18.48 ± 0.21[a]	0.99 ± 0.02[a]

Values with different superscripts along the same column for each parameter are significantly different ($p < 0.05$).
TBARS, thiobarbituric acid reactive substances; SOD, superoxide dismutase; CAT, catalase; GPX, glutathione peroxidase; GST, glutathione *S*-transferase.
AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase.

may be an indication of liver damage and cell necrosis resulting from formation of NAPQI in excess of GSH detoxification capacity. This agrees with earlier reports by Balamurugan (2007), Gini and Muraleedhara (2010), and Kanchana and Mohamed (2011). These authors opined that overdose of acetaminophen could be toxic to the hepatocytes. Conversely, the significant reduction in enzyme activities of rats treated with silymarin and vitamin C suggests that both compounds were able to ameliorate the deleterious effects of acetaminophen. The effects were more pronounced in the vitamin C-treated rats as well as those co-treated with both silymarin and vitamin C.

The serum levels of total protein and bilirubin may indicate the state of the liver and the type of damage (Sabiu et al., 2014). Hypoproteinemia is a feature of liver damage which may be attributed to a decrease in protein synthesis (Oloyede and Sunmonu, 2009). In this study, hypoproteinemia was observed in the acetaminophen-treated rats and may be a consequence of impaired hepato-cellular functions. In addition, the observed hyperbilirubinemia may be due to excessive heme destruction and blockage of biliary tract in acetaminophen-treated rats. This obstruction might have resulted to mass inhibition of conjugation reaction and release of unconjugated bilirubin from damaged and dead hepatocytes (Wolf et al., 1997). This agrees with the earlier reports by Ajiboye et al. (2010), Kanchana and Mohamed (2011), and Chinnasamy et al. (2011) where acetaminophen was reported to have caused alteration in serum concentrations of total bilirubin and total protein. Co-treatments with silymarin and vitamin C, however, reduced the level of bilirubin and increased protein concentration suggesting that they offered considerable level of hepatoprotection at the tested regimen.

Oxidative insult to a cell induces peroxidation of membrane-bound lipids whose toxic products cause damage of macromolecules. In the present study, the increased concentration of TBARS in the liver of hepatotoxic rats is suggestive of facilitated lipid peroxidation leading to tissue damage and failure of body's antioxidant defense mechanisms to prevent formation of excessive free radicals. It has been reported that acetaminophen caused significant increase in hepatic lipid peroxidation due to free radical injury in necrotic livers of rats (Gini and Muraleedhara, 2010). Reactive oxygen species in conjunction with NAPQI are required to initiate lipid peroxidation which has been opined as an important initiation event in the toxicity mechanism of acetaminophen (Thabrew et al., 1987). The significantly reduced concentration of TBARS in the liver of silymarin and vitamin C-treated rats indicates their possible antiperoxidative attribute and thus antioxidative potential.

The body is endowed with an effective mechanism to counter the ravaging effect of free radical-induced damage. This is attained *via* endogenous antioxidant enzymes, such as SOD, CAT, GPX and

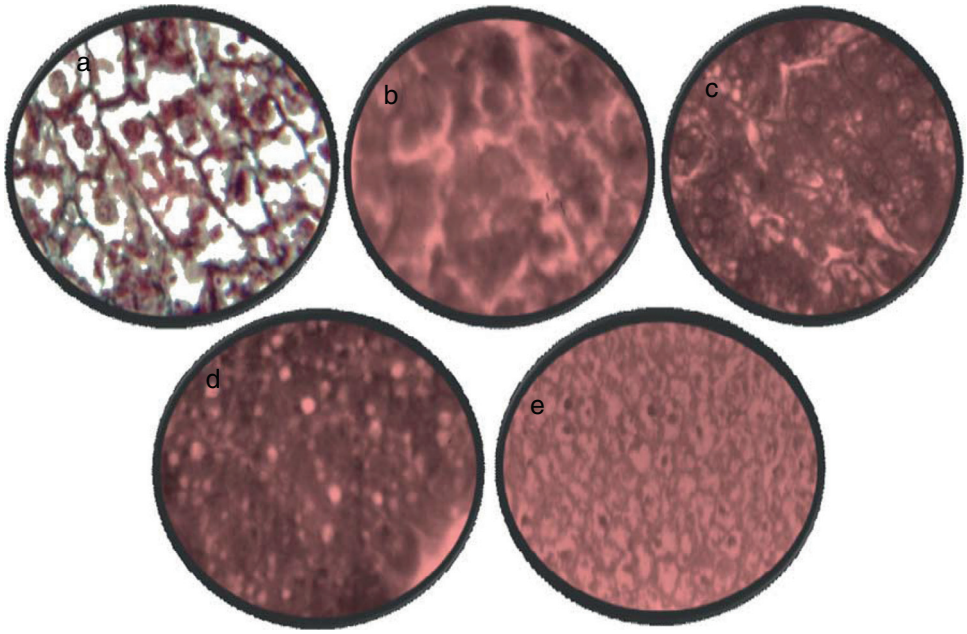

Fig. 1. Light micrograph (400×, Haematoxylin + eosin stained) of the liver of (A) normal control rat, (B) untreated acetaminophen-induced hepatotoxic rat, (C) silymarin-treated acetaminophen-induced hepatotoxic rat, (D) vitamin C-treated acetaminophen-induced hepatotoxic rat and (E) silymarin + vitamin C-treated acetaminophen-induced hepatotoxic rat.

GST. An imbalance between free radicals production and antioxidant defense system results in oxidative stress which further deregulates cellular functions leading to various pathological conditions. In the present study, the reduced activity of antioxidant enzymes in acetaminophen-treated rats is an obvious reflection of excessive formation of free radicals resulting in tissue damage. However, the significant increase in their activities following treatment with silymarin and vitamin C is an indication of antioxidant effect.

In the present study, the protection offered by silymarin against acetaminophen-induced hepatotoxicity may be generally linked to its beneficial attributes as revealed by Valenzuela and Garride (1994). These include its ability to scavenge free radicals, increase cellular GSH content and regulate membrane permeability. The authors also reported that silymarin has the capacity to regulate nuclear expression by means of a steroid-like effect.

Similarly, the ability of vitamin C to trap free radicals, protect biomembranes from peroxide damage and effectively scavenge reactive oxygen species as reported by Sminorff and Wheeler (2000) may be suggestive of its effect exhibited in the treatment groups. Bendich (1990) has also reported vitamin C as an excellent electron donor to free radicals which subsequently quench their deleterious activity on cellular macromolecules, thus playing a role in antioxidant mechanism. In addition, the 200 mg/kg b.w. of vitamin C exploited in this study may be sufficient enough to contribute to its therapeutic potential exhibited in the treated rats. This agrees with the report of Mongi et al. (2011) where vitamin C was reported to have offered protection against biochemical toxicity induced by deltamethrin in male Wistar rats. This, perhaps, could also be a tenable fact for the rats wholly administered with vitamin C and those co-administered with silymarin for having pronounced hepatoprotective and antioxidant potentials compared to rats treated with only silymarin. This submission is in conformity with the report of Sabzevarizadeh and Najafzadeh (2012) that vitamin C modulates myoglobinuric hepatic failure better than silymarin by binding to various harmful substances in rats.

Another important consideration in assessing the efficacy of potential therapeutic agents against hepatic injury is their effect on histology. The effects are manifestations of inflammatory insult on the liver and often complement enzyme analysis (Adesokan and Akanji, 2007). The apparently annulled degenerative threats posed by acetaminophen on the architectural features of hepatocytes in the rats wholly treated with vitamin C and in combination with silymarin suggest that the two compounds conferred a reasonable level of integrity on the liver. In fact, architectural organization of some of the hepatocytes was almost completely restored to normal. The effects noticed were in consonance with the results of biochemical analysis obtained and in agreement with the finding of Balamurugan (2007), where recovery towards normalization of serum enzymes and liver histological architecture caused by acetaminophen were attributed to antioxidant agents.

Conclusively, the restoration of degenerative insults inflicted by 400 mg/kg b.w. acetaminophen by co-administration with silymarin and vitamin C is an indication of their inherent hepatoprotective and antioxidant attributes in rats. Though, the effects were prominently exhibited by vitamin C, their complementary efficacy is formidable and thus recommended against hepatic oxidative damage.

Authors' contributions

SS and SOT designed the study and were involved in the manuscript preparation. AOT evaluated the compounds in acetaminophen-treated rats. SS, AOE and SOT performed biochemical estimations and histopathology. All authors read and approved the final manuscript.

Conflict of interest

All authors have nothing to declare.

Acknowledgements

The authors are immensely thankful to the Biochemistry Units of Kwara State University, Malete, Ilorin, Nigeria and Al-Hikmah University, Ilorin, Nigeria for providing ample support and access to research facilities.

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