COMBINED EFFECTS OF VANILLIC AND SYRINGIC ACIDS ON HEPATIC MARKERS, LIPID PEROXIDES AND ANTIOXIDANTS IN ACETAMINOPHEN INDUCED HEPATOTOXICITY IN WISTAR RATS: BIOCHEMICAL AND HISTOPATHOLOGICAL EVIDENCES

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Summary

The aim of this study was to compare the possible protective effects of vanillic acid (VA) and syringic acid (SA) on acetaminophen (APAP)-induced hepatotoxicity in rats. Toxicity was induced in adult male Wistar albino rats, weighing between 140-160 g, by administration of APAP 750 mg/kg of body weight i.p. Rats were treated with VA and SA (50 mg/kg body weight) by oral administration. APAP-induced rats exhibited elevation in the activities of plasma hepatic markers aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and the level of lipid peroxidative markers (thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides). The toxic effect of APAP was also assessed by means of determining the activity of enzymatic antioxidants superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and the non-enzymatic antioxidants (vitamin C, vitamin E and reduced glutathione (GSH) levels) decreased significantly in APAP rats. Increase in the levels of total cholesterol, phospholipids, triglycerides and free fatty acids in the plasma were observed in APAP-treated rats. Liver histology also showed convincing evidence regarding their protective nature against fatty changes induced during APAP intoxication. Among the two phenolic compounds, SA showed higher activity than VA. Thus, VA and SA have the ability to reverse APAP-induced hepatotoxicity rats.

Keywords: Acetaminophen, Vanillic acid, Syringic acid, Lipid peroxidation, Antioxidants

Running title: - Effects of Vanillic and Syringic Acids on Acetaminophen-induced Hepatotoxicity Rats

Introduction

Liver disease remains one of the serious health problems throughout the world. Hepatic dysfunction due to ingestion and inhalation of hepatotoxin is increasing worldwide [1]. Acetaminophen (APAP), also known as paracetamol is a commonly used analgesic and antipyretic drug. The drug is safe at therapeutic levels, but an acute APAP overdose can lead to potentially fatal hepatic and renal necrosis in humans and experimental animals [2]. It is becoming clear that reactive oxygen (ROS) and nitrogen (RNS) species take an important part in the development of hepatotoxicity caused by APAP [3]. The initial step of its toxicity is cytochrome P450 (CYP) metabolism of APAP to the reactive intermediate N-acetyl-*p*-benzoquinone imine (NAPQI) [3]. At therapeutic doses this metabolite is removed by conjugation with glutathione (GSH). However at large doses of APAP, conjugation with GSH leads to its depletion [4].

Syringic acid (SA) (4-hydroxy-3, 5-dimethoxybenzoic acid) and vanillic acid (VA) (4- hydroxyl-3-methoxy benzoic acid) are reported to possess antimicrobial [5], anti-cancer [6], anti-DNA oxidation activities [7] and hepatoprotective activity [8]. Phenolic are an interesting group of compounds in nature. Show (Fig.1) VA and SA are phenolic having a similar chemical structure, but addition methyl group of SA in C_5 and both are found in fruits, plants and medicinal plants worldwide. Hence, in the present study we continued to investigate the curative effect of VA and SA against APAP-induced liver damage in rats to estimated biochemical parameters including assessment of lipid peroxidation, GSH, liver functions test (LFTs), enzymatic and non-enzymatic variables as well as histopathology.

Materials and Methods

Chemicals

Acetaminophens (APAP), vanillic and syringic acid were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). All other chemicals used in this study were of analytical grade obtained from E. Merck and HIMEDIA, Mumbai, India.

Animals

Male albino Wistar rats, 6–7 weeks old (weighing 140–160 g) were procured from the Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College and Hospital, Annamalai University and maintained in an air-conditioned room (25 ± 2ºC) with a 12 h light/12 h dark cycle. Rats were fed on standard pellet diet (Pranav Agro Industries Ltd., Pune, Maharashtra, India) and water *ad libitum.* All the experimental studies were conducted in the Department of Biochemistry, Faculty of Science, Annamalai University, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH 1985); the experimental study was approved by the Ethical Committee of Rajah Muthiah Medical College and Hospital (Reg No.160/1999/CPCSEA, Pro. No.595), Annamalainagar, Tamil Nadu.

Experimental design

Hepatotoxicity was induced in animals by an intraperitoneal injection of acetaminophen (750 mg/kg body weight) in a freshly prepared physiological saline solution kept in warm boiling water bath and used after cooling at 37°C, as a single dose on the seventh day [9]. The animals were randomly divided into six groups of six animals each as given below. Vanillic and syringic acid were administered orally once in a day in the morning for 7 days. The compound was suspended 0.9% saline vehicle solution and fed by intubation.

Group I: Control rats received 0.9% saline only
Group II: Control + VA (50mg/kg BW)
Group III: Control + SA (50 mg/kg BW)
Group IV: APAP control (750 mg/kg BW)
Group V: APAP + VA (50 mg/kg BW)
Group VI: APAP + SA (50 mg/kg BW)

The experimental duration was 7 days. On 8th day the rats were sacrificed by cervical dislocation. Blood was collected in a dry test tube and allowed to coagulate at ambient temperature for 40 min. Serum was separated by centrifugation at 2000 rpm for 10 min. The blood, collected in a heparinized centrifuge tube was centrifuged at 2000 rpm for 10 min and the plasma was separated by aspiration. After the separation of plasma, the buffy coat, enriched in white cells, was removed and the remaining erythrocytes were washed three times with physiological saline. Liver tissue (250 mg) were sliced into pieces and homogenised in appropriate buffer in cold condition (pH 7.0) to give 20% homogenate (w/v). The homogenate was centrifuged at 1000 rpm for 10 min at 0 °C in cold centrifuge. The supernatant was separated and used for various biochemical estimations.

Biochemical estimations

The activities of serum AST, ALT and ALP were assayed by the method of Reitman and Frankel [10] and Kind and King's method [11]. Lipid peroxidation was assessed by measuring the levels of thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides in the plasma following the procedures of Niehaus and Samuelson, [12] and Jiang et al., [13]. The activities of erythrocyte SOD, CAT and GPx were assayed by the method of Kakkar, et al., [14], Sinha [15] and Rotruck [16]. The levels of non-enzymatic antioxidants such as ascorbic acid, α-tocopherol and reduced glutathione were measured by Roe and Kuether [17], Baker et al [18] and Ellman [19], respectively. Plasma and tissue total cholesterol, triglycerides, free fatty acids, and phospholipids were estimated by the methods of Allain et al [20], McGowan et al [21], Falholt et al [22], and Zilversmit and Davis [23] were estimated.

Histopathological observations

For light microscopic observations, samples from liver were fixed in Bouin's fixative and processed routinely for embedding in paraffin. Tissue sections of 5mm thickness were stained with hematoxylin and eosin (H&E) and examined under light microscope.

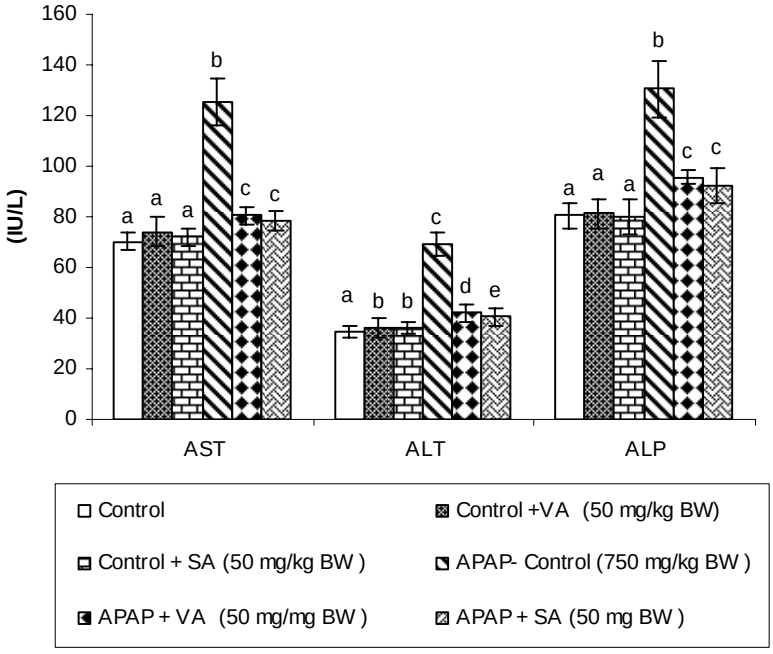
Statistical analysis

Data were analysed by one way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using a statistical software package (SPSS for Windows, V. 13.0, Chicago, USA). Results were presented as means ± S.D. P-values < 0.05 were considered as statistically significant.

Results

Fig. 1 shows the levels of serum hepatic markers in control and experimental rats. Intraperitoneal administration of APAP caused abnormal liver function in all rats. Activities of serum hepatospecific enzymes such as AST, ALT and ALP were significantly increased ($p < 0.05$) in APAP treated rats. Administration of VA and SA (50 mg/kg) with APAP significantly decreased ($p < 0.05$) the activities of serum hepatic markers when compared to normal rats.

Fig.1. Effect of VA and SA on the activities of hepatic marker enzymes in the plasma of control and APAP-treated rats



Values are given as means ± S.D. for six rats in each group. Values not sharing a common superscript differ significantly at $p < 0.05$. Duncan's Multiple Range Test (DMRT).

Table 1. Effect of VA and SA on TBARS, lipid hydroperoxides, SOD, CAT, GPx, vitamin-C, vitamin-E and GSH in the liver of control and APAP - treated rats

Parameters	Control	Control +VA (50 mg/kg BW)	Control + SA (50 mg/kg BW)	APAP- Control (750 mg/kg BW)	APAP + VA (50 mg/mg BW)	APAP + SA (50 mg BW)
TBARS(mmols/100g wet tissue)	0.74 ± 0.03^{a}	0.70 ± 0.05^{a}	0.70 ± 0.02^{a}	1.86 ± 0.07^{b}	1.04 ± 0.09^{c}	0.97 ± 0.03^{c}
Lipid hydroperoxides (mmols/100g wet tissue)	93.21 ± 5.57^{a}	89.78 ± 6.50^{a}	91.66 ± 7.02^{a}	157.67 ± 9.45^{b}	114.11 ± 6.74^{c}	110.11 ± 8.11^{c}
SOD (U*/mg protein)	7.55 ± 0.65^{ab}	7.85 ± 0.21^{ab}	8.16 ± 0.46^{b}	6.01 ± 0.31^{c}	7.18 ± 0.46^{d}	7.21 ± 0.33^{d}
CAT ($U^{\#}$/mg protein)	75.45 ± 4.51^{ab}	79.45 ± 5.17^{b}	78.64 ± 4.52^{b}	5.37 ± 2.74^{c}	68.2 ± 3.94^{d}	69.98 ± 4.58^{da}
GPx ($U^{\$}$/mg protein)	9.29 ± 0.29^{a}	9.45 ± 0.35^{ab}	9.73 ± 0.53^{b}	5.92 ± 0.34^{c}	7.92 ± 0.52^{d}	8.78 ± 0.50^{a}
Vitamin-C (mg/100mg wet tissues)	0.90 ± 0.06^{a}	$0.82 \pm 0.05b^{a}$	$0.83 \pm 0.06b^{a}$	0.30 ± 0.02^{c}	0.65 ± 0.03^{d}	0.80 ± 0.04^{d}
Vitamin-E (mg/100mg wet tissues)	6.32 ± 0.42^{a}	6.39 ± 0.31^{a}	6.41 ± 0.51^{a}	2.03 ± 0.25^{b}	5.32 ± 0.35^{c}	5.70 ± 0.36^{c}
GSH (mg/100mg wet tissues)	110.57 ± 8.44^{a}	109.68 ± 8.52^{a}	110.04 ± 7.52^{a}	6791 ± 4.30^{b}	91.51 ± 5.78^{c}	93.84 ± 5.25^{c}

U^{*} = enzyme concentration required to inhibit the chromogen produced by 50% in one minute under standard conditions
$U^{\#}$ = μmole of H_2O_2 consumed/minute.
$U^{\$}$ = μmole of GSH utilized/minute.
Values are given as means ± S.D. for six rats in each group.
Values not sharing a common superscript differ significantly at $p < 0.05$. Duncan's Multiple Range Test (DMRT).

Table 2 shows the effect of VA and SA on the lipid profile of the plasma in APAP treated rats. The levels of total cholesterol, triglycerides, phospholipids and free fatty acids significantly increased in APAP treated rats and administration of VA and SA decreased these levels.

Table 2. Effect of VA and SA on total cholesterol, triglycerides, phospholipids and free fatty acids in the plasma and liver of control and APAP - treated rats

Parameters	Control	Control + VA (50 mg/kg BW)	Control + SA (50 mg/kg BW)	APAP- Control (750 mg/kg BW)	APAP + VA (50 mg/mg BW)	APAP + SA (50 mg BW)
Total cholesterol (mg/dL)	75.80±5.88[a]	72.91±4.31[a]	72.35±4.01[a]	112.63±10.24[b]	92.12±5.21[c]	87.13±5.07[c]
Triglycerides (mg/dL)	58.12±6.08[a]	60.98±3.81[a]	59.93±3.41[a]	110.08±8.07[b]	71.43±5.67[c]	70.66±5.89[c]
Phospholipids (mg/dL)	99.74±5.07[a]	98.74±6.81[a]	96.38±6.16[a]	145.74±11.09[b]	112.28±7.11[c]	110.63±7.28[c]
Free fatty acid (mg/dL)	50.73±3.15[a]	53.73±3.38[a]	52.95±3.46[a]	105.73±8.04[b]	64.32±4.22[c]	62.50±3.41[c]

Values are given as means ± S.D. for six rats in each group.

Values not sharing a common superscript differ significantly at $p < 0.05$. Duncan's Multiple Range Test (DMRT).

Rats induced with APAP showed a considerable (P< 0.05) increase in the levels of TBARS and LOOH in the tissue (liver) compared to normal control rats. Oral treatment with VA and SA to APAP-induced rats showed considerable (P< 0.05) decrease in the levels of TBARS and LOOH in tissues compared with APAP alone induced rats (Table 1). The activities of enzymic antioxidants such as SOD, catalase, GPx, GRx, GST, GSH, vitamin C and E in the liver of normal and APAP -induced rats are shown in Table 1. APAP-induced rats exhibited a significant (P < 0.05) decrease in the activities of these enzymic and non-ezymatic antioxidants in the liver compared to normal control rats. Treatment with VA and SA to APAP -induced rats significantly increased the activities of these enzymes and non-ezymatic compared with APAP alone induced rats.

Histopathological observations

Fig.3. Histophathology of liver (A) Normal rats showing central vein surrounded by normal hepatocytes (B) Normal rats + vanillic acid showing central vein surrounded by normal hepatocytes (C) Normal rats with syringic acid showing central vein surrounded by normal (D) Liver showing cell swelling and loss of intadness with APAP (E) APAP hepatotoxic rats + vanillic acid treatment showing congested central vein and normal hepatocyte (F) APAP hepatotoxic rats with syringic acid showing small area of degenation and almost normal architecture.

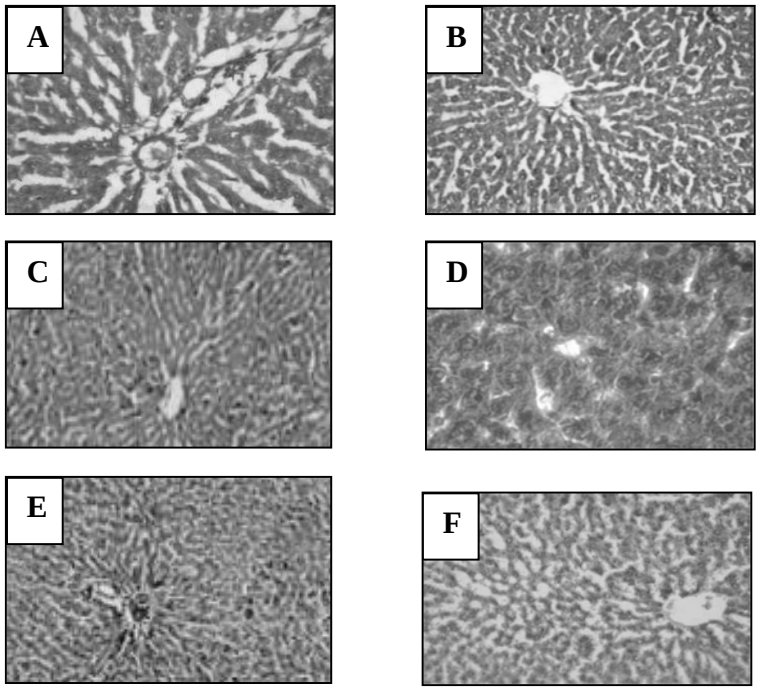


Fig. 3. Histophathology of liver (A) Normal rats showing central vein surrounded by normal hepatocytes (20X); (B) Normal rats + vanillic acid showing central vein surrounded by normal hepatocytes (20X); (C) Normal rats with syringic acid showing central vein surrounded by normal (20X); (D) Liver showing cell swelling and loss of intadness with APAP of 750 mg/kg BW (20X); (E) APAP hepatotoxic rats + vanillic acid treatment showing congested central vein and normal hepatocyte (20X); (F) APAP hepatotoxic rats with syringic acid showing small area of degenation and almost normal architecture (20X).

Discussion

Acetaminophen is a clinically important over-the-counter drug commonly used for its analgesic and antipyretic properties. At therapeutic doses, it is considered as a safe drug. However, it can cause hepatic necrosis, nephrotoxicity, extra hepatic lesions, and even death in humans and experimental animals when taken in overdoses [24]. APAP is mainly metabolized in liver to excretable glucuronide and sulphate conjugates [25]. However, hepatoxicity of APAP has been attributed to the formation of toxic metabolites when a part of APAP is activated by hepatic cytochrome P450 [26], to a highly reactive metabolite N-acetyl-*p*-benzoquinoneimine [27]. Due to liver injury, the transport function of the hepatocytes gets disturbed, resulting leakage in the plasma membrane [28], In the assessment of liver damage by acetaminophen or any other hepatotoxin, the determination of enzyme levels such as ALT and AST is largely used [29]. Necrosis or membrane damage releases the enzyme into circulation; therefore, it can be measured in serum. High levels of AST indicate liver damage, such as that due to viral hepatitis, cardiac infarction and muscle injury. ALT catalyses the conversion of alanine to pyruvate and glutamate, and is released in a similar manner. Therefore, ALT is more specific to the liver, and is thus a better parameter for detecting liver injury [30]. Serum ALP level on the other hand, is related to the function of hepatic cell. Increase in serum level of ALP is due to increased synthesis of the enzyme, in presence of increasing biliary pressure [31]. Fig.1 shows that APAP alone caused a significant elevation of serum levels of AST, ALT and ALP in the experimental animals. Administration of APAP to experimental animals increased the serum levels of AST, ALT and ALP. Our results using the model of APAP-induced hepatotoxicity in rats demonstrated that the different VA and SA caused significant inhibition of elevated serum AST, ALT and ALP levels. Effective control of alkaline phosphatase activity points towards an early improvement in the secretory mechanism of the hepatic cell. This results indicate that the hepatoprotective activity of the VA and SA probably through the correction of cellular integrity of hepatic cell and its regeneration.

The NAPQI induced covalent binding and depletion of cytosolic and mitochondrial GSH trigger the loss of cellular homeostasis leading to liver injury [32]. The APAP induced depletion of GSH was restored by syringic acid treatment, which supported the involvement of exogenous administration of antioxidants in modulation of GSH metabolism. Thus, syringic acid may play a key role against APAP intoxication by influencing the cellular GSH pool. In the present study, APAP induced hepatic injury was found due to increase in MDA, which is in agreement with the previous studies [33]. VA and SA treatment significantly inhibited MDA production, implying a reduction in LPO and cellular injury that protected the liver against APAP induced peroxidative damage. Several active constituents and phenolic compounds present in VA and SA could act synergistically and inhibited LPO as an effective chain breaking antioxidant to diminish APAP induced peroxidative damage in the test organs.

Reduction in the activity of SOD is likely to be a result of futile cycling of P450, caused by NAPQI which utilized reducing equivalent of NADPH with concomitant reduction of molecular superoxide anion radical ($O_2^{\bullet-}$), hence there will be a reduction in superoxide dismutase activity [34]. Catalase is a crucial enzyme in cellular antioxidative defense mechanisms and efficiently degrades endogenously produced hydrogen peroxide. Catalase

activity was found to be significantly decreased after a toxic paracetamol dose [35]. This would allow for the accumulation of reactive oxygen species and hydrogen peroxide, which can exacerbate the hepatocellular damage initiated by NAPQI. Low activity of GPx is one of the early consequences of a disturbance of the pro-oxidant/antioxidant balance in favour of the former [36]. GPx may play an important role in the removal of lipid hydroperoxides. The balance between these enzymes is important for the efficient removal of oxygen radicals from tissues [36]. Therefore, reduction in the activity of these enzymes may result in a number of deleterious effects due to the accumulation of superoxide radicals and H_2O_2. The administration of VA and SA resulted in the elevation of the activities of these enzymes, thus, protecting the tissues from free radical damage.

GSH plays a critical role in cellular functions, which include the maintenance of thiol status of proteins, destruction of H_2O_2, and lipid peroxides, and free radicals, detoxification of foreign compounds and biotransformation of drugs [37]. APAP metabolite, NAPQI is a strong electrophile that reacts with nucleophile such as GSH to produce non-toxic compounds, 3-S-glutathionyl APAP, and a reaction represents detoxification of the reactive metabolite. In our study acetaminophen treated rats showed significant decrease in the levels of GSH which coincides with the previous reports [38]. The decrease in glutathione is due to the formation of an acetaminophen-glutathione conjugate [39]. If the levels of GSH in a tissue lower, then that tissue can be shown to be more susceptible to injury by various chemicals that would normally be conjugated to GSH [40]. VA and SA treated rats showed significant increase in the levels of GSH indicating that the extract may limit the GSH depletion and the severity of the oxidative stress thereby protecting liver.

Vitamin functions as a free radical scavenger of O_2 radicals and successfully prevents detectable oxidative damage under all types of oxidative stress. Ascorbic acid appears to trap the peroxyl radical in the aqueous phase with a rate large enough to intercept virtually all these radicals before they can diffuse into plasma lipids [41]. The level of vitamin C was significantly depleted in APAP rats; this depletion may be due to the excessive utilization of non-enzymatic antioxidant involved in quenching the enormous free radicals produced during APAP intoxication [42]. Vitamin E, the major lipophilic antioxidant plays a vital role against oxidative stress [43]. The peroxyl radicals that escape the antioxidants in the aqueous phase diffuse into the lipids, where they initiate lipid peroxidation. The propagation is inhibited by the lipophilic chain breaking antioxidant such as α-tocopherol [44]. GSH may maintain the vitamin E levels either by direct reduction of α-tocopherol radical to vitamin E or via the reductive action of vitamin C. The level of α-tocopherol was significantly decreased in acetaminophen rats. It may be due to increased utilization in scavenging the oxyradicals generated by lipid peroxidation and/or due to decreased vitamin C and GSH levels, which can result in decreased conversion of vitamin E radical to vitamin E. VA and SA treated rats showed significant increase in vitamin C and vitamin E levels.

The plasma concentration of lipids is largely a balance of synthesis and degradation by the liver. Alterations in the balance after an over dosage of APAP cause hyperlipidemia. In our study we observed a marked increase in the levels of plasma lipid profile in APAP treated rats. Elevation in the level of cholesterol in plasma of APAP administered rats may be due to increased synthesis of cholesterol in the liver and excess cholesterol leaking out into the blood. Administration of VA and SA decreased the cholesterol level in the liver.

We observed significantly reduced levels of lipid in the plasma and liver of rats receiving APAP and VA and SA, showing the beneficial VA and SA on lipid profile against APAP toxicity. The increased fatty acid accumulation may be due to increased lipid breakdown. Our results also showed increased levels of free fatty acid in liver. Fatty acid from different sources can accumulate as triglycerides in the liver as a consequence of variety of metabolic disturbance [45]. The increase in the level of phospholipids in our study is corroborated by earlier investigation [46]. Co administration of VA and SA or silymarin may reduce the levels of free fatty acids and phospholipids thereby minimizing the toxic effect of APAP.

It has been reported that suppressive effect of syringic and vanillic acid on the ConA-induced liver injury might be due to their scavenging of ROS generated by activated NADPH oxidase in the lymphocytes. ConA induces a massive recruitment of activated T cells to the liver. Schwabe reported that ConA-induced liver injury is largely dependent on membrane bound TNF-a on the infiltrating T cells. The TNF binds to its receptor on hepatocytes to induce ROS production. Syringic acid and vanillic acid could scavenge the ROS to suppress hepatocyte death [8]. Thus, these phenolic offers a remarkable protection against APAP induced liver injury. Further, syringic acid gives a higher than vanillic acid. Syringic acid is an addition of methyl group the antioxidant potency. The addition of methoxyl group in the ortho position of the hydroxyl group enhances the radical-scavenging capacity of phenolic acids. [47].

In conclusion, our study reveals that VA and SA exhibits potent antioxidant and hepatoprotective properties in APAP-hepatotoxic rats and these properties are comparable with the control rats. Histologic studies of liver tissue also support our biochemical findings.

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