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Original Article

# ANTIOXIDANT AND HEPATOPROTECTIVE POTENTIALS OF *STEMONOCOLEUS MICRANTHUS* HARMS (FABACEAE) STEM BARK EXTRACT

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**ABSTRACT**

**Objective:** This study evaluated the antioxidant and hepatoprotective activities of the methanol-dichloromethane (1:1) extract of *Stemonocoleus micranthus* Harms (Fabaceae) stem bark (SME).

**Methods:** *In vitro* ferric reducing power, hydrogen peroxide and α, α-diphenyl–β–dipicryl–hydrazyl (DPPH) free radical scavenging assays, were used to determine the antioxidant activity of SME (25, 50, 100, 200 and 400 µg/ml). Also the effects of SME (100, 200 and 400 mg/kg) on liver enzymes alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) in carbon tetrachloride ($CCl_4$)-induced hepatic oxidative damage were studied in rats.

**Results:** The results showed that SME (25-400 µg/ml) significantly ($P$<0.01) reduced iron III ($Fe^{3+}$) to iron II ($Fe^{2+}$) with 400 µg/ml eliciting 135.4% reducing power. The SME demonstrated significant ($P$<0.01) hydrogen peroxide scavenging with 400 µg/ml eliciting 20.37% activity, comparable to ascorbic acid (20.32%). The SME (25-400 µg/ml) also elicited 77-81% DPPH free radical scavenging, lower than ascorbic acid (25-400 µg/ml) with 83-88% activity. The *in vivo* study showed that SME protected the rats from liver damage as shown by the reduction of liver enzymes in serum. The SME (400 mg/kg) elicited 7.7, 33.8 and 7.2% inhibition of ALP, ALT and AST respectively. The acute toxicity test revealed that SME has high margin of safety, with oral lethal dose ($LD_{50}$)>5 g/kg. Phytochemical analyses on the extract revealed the presence of alkaloids, carbohydrates, flavonoids, glycosides, proteins, reducing sugars, saponins, resins, steroids, tannins, terpenoids, fats, and oils.

**Conclusion:** The findings suggest that the methanol-dichloromethane extract of *S. micranthus* stem bark possess antioxidant and hepatoprotective effects.

**Keywords:** *Stemonocoleus micranthus,* Antioxidant, Hepatoprotective, Albino rats, Ascorbic acid



## INTRODUCTION

All biological systems contain redox elements that function in physiological regulation and maintenance of homeostasis [1]. The activity of these redox elements are organized and coordinated through circuits reliant on common control nodes. A disruption of the function of these redox circuits and the consequent imbalance between antioxidant and pro-oxidant mechanisms leads to excessive oxidative metabolism, ultimately resulting in oxidative stress.

Oxidative stress is characterized by the accumulation of oxidants, including reactive oxygen species (ROS) (e. g., oxygen ions, free radicals, and peroxide), which provoke cell damage. Factors such as infection, poor diet, and exposure to pollutants, toxins, alcohol, drugs and radiation [1] have been implicated in the development of oxidative stress.

It has been established that oxidative damage caused by ROS leads to deoxyribonucleic acid (DNA) lesions, loss of enzyme functions, changes in cell integrity and functions, and eventual necrotic cell death or apoptosis [1]. Generation of free radicals and their oxidative damage to proteins, DNA and other biomolecules are known to play a definite role in the pathology of a wide variety of ailments including heart disease, pain, inflammation, cancer, diabetes, Alzheimer's disease, hepatic damage, glaucoma, among others. Antioxidants fight free radicals and protect against various diseases [2]. Antioxidant mechanisms include inhibition of generation of ROS and scavenging activity against ROS, reducing power, metal chelation, activity as antioxidant enzymes and inhibition of oxidative enzymes [3].

Phytoconstituents in plants have been shown to possess antioxidant properties and there is a great need for a continued search for such plants for the benefit of man. One of such plants with putative antioxidant activity is *Stemonocoleus micranthus* Harms (Fabaceae). *S. micranthus* is a large forest tree with leaflets similar to those of *Detarium species,* but with fewer numbers of lateral veins. Its morphology has been described [4]. The name *S. micranthus* came from the Greek word "Stamen stealth" referring to the filament of the stamens which are united at the base being the only one species in the genus [5]. It seems to be most closely related to Augouardia, which is abundant in Gabon [4]. The stem bark decoction is a traditional remedy for contaminated wounds, pains, rheumatoid arthritis and joint pains, to treat infertility in women and enhance conception [4, 5]. However, non-medicinal uses of the wood include in light construction and other wood works [5]. Although the description of the plant is well documented, information on its pharmacological and phytochemical properties is sparse. The analgesic, narrow spectrum antibacterial, central nervous system (CNS) depressant and local anesthetic properties of the stem bark methanol extract have been demonstrated [6]. Since the stem bark extract of *S. micranthus* is used in ethnomedicine to manage rheumatoid arthritis, a disease triggered by oxidative damage, the study sought to evaluate its antioxidant effect. Furthermore, we evaluated the hepatoprotective potentials of the extract, as studies have shown the involvement of oxidative stress in the pathogenesis of liver damage [7-9].

## MATERIALS AND METHODS

### Animals

Adult Swiss albino rats (102-160 g) of both genders were obtained from the Laboratory Animal Facility of the Department of Pharmacology & Toxicology, University of Nigeria, Nsukka. The animals were kept in steel cages within the Facility and allowed free access to water and standard livestock pellets. All animals used in the experiments were handled in compliance with the National

Institute of Health Guidelines for Care and Use of Laboratory Animals (Pub No. 85–23, revised 1985) and with prior permission from the National Health Research Ethics Committee (NHREC) of the University of Nigeria, Nsukka, with protocol ethical clearance number NHREC/05/01/2013B.

**Chemicals, reagents and solvents**

All chemicals used were of analytical grade and include: methanol (Sigma-Aldrich, Germany), dichloromethane (Sigma-Aldrich, Germany), distilled water, chloroform, normal saline, hydrogen peroxide, $H_2O_2$ (40%), potassium dihydrogen phosphate ($KH_2PO_4$), sodium hydroxide (NaOH), potassium ferricyanide ($K_3[Fe(CN)_6]$), trichloroacetic acid ($C(Cl_3)COOH$; TCA), ferric chloride ($FeCl_3$), ascorbic acid, α,α-diphenyl-β-dipicryl-hydrazyl (DPPH) radical, buffer tablets, carbon tetrachloride ($CCl_4$), liquid paraffin.

**Equipment/Instruments**

The equipment/instruments used include: electrical animal weighing balance (B. Bran Scientific & Instruments Co., England), analytical weighing balance, digital pH meter, centrifuge tubes, incubator, centrifuge (B. Bran Scientific & Instruments Co., England), test tube racks, UV-Visible spectrophotometer (Easy-Way Medical England 752W, England), milling machine (Lab mill, serial No. 4745, Christy and Norris Ltd., England), soxhlet apparatus, and rotary evaporator (B. Bran Scientific & Instruments Co., England).

**Collection and preparation of plant material**

Fresh stem barks of *S. micranthus* were collected from a forest in Orba, Nsukka in May. The identity of the plant was established and authenticated by Mr A. Ozioko of the International Centre for Ethnomedicine Drug Development (InterCEDD), Nsukka, Nigeria. The stem bark was carefully separated from the woody part, cut into small pieces, sun-dried, pulverized and stored in an airtight container before extraction.

**Extraction of plant material**

About 2.37 kg of the powdered plant material was extracted with about 10 liters of a 1:1 mixture of methanol-dichloromethane by continuous extraction in a soxhlet extractor. The filtrate was concentrated in a rotary evaporator to obtain *S. micranthus* stem bark extract (106.28 g; 4.5% w/w).

**Preliminary phytochemical analysis**

The SME was subjected to phytochemical analysis using standard procedures [10, 11].

**Acute toxicity test**

The acute toxicity and lethality ($LD_{50}$) of SME in mice was estimated using previously described method [12]. The study was carried out in two stages.

In stage one, mice (n = 3) received oral administration of 10, 100, or 1000 mg/kg of SME (suspended in distilled water) and were observed for 24 h for signs of toxicity and death. At the end of 24 h, no death was recorded. Consequently, a fresh batch of mice (n = 1) received 1600, 2900, and 5000 mg/kg respectively of SME in the second stage of the test and were observed for 24 h for deaths.

***In vitro* antioxidant tests**

**Assay of ferric reducing power**

The reducing power was determined as previously described [13]. Briefly, 1.0 ml SME of different concentrations (25, 50, 100, 200 and 400 µg/ml) were each mixed with 2.5 ml of 0.2M phosphate buffer (pH 6.6). To each of the mixtures, 2.5 ml of potassium ferricyanide (30 mM) was added and incubated at 50 °C for 20 min. Thereafter, 2.5 ml of 0.6 M trichloroacetic acid (TCA) was added to the reaction mixture and centrifuged for 10 min at 3000 rpm. The upper layer of each solution (2.5 ml) was decanted and mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (6 mM). The change in colour was observed and the absorbance of each mixture was measured at 700 nm using a UV-Visible spectrophotometer. The same treatment was used for the different concentrations (25, 50, 100, 200 and 400 µg/ml) of the reference standard, and the control. Ascorbic acid was used as the reference standard [14], while distilled water served as the control. Tests were performed in triplicates. A higher absorbance of the reaction mixture indicated greater reducing power; the reducing power (%) was calculated using the relation:

$$\text{Reducing power (\%)} = \frac{\text{A1} - \text{A0}}{\text{A0}} \times 100$$

Where A0 = the absorbance of control

A1 = the absorbance of test.

**Hydrogen peroxide radical scavenging assay**

The ability of SME to scavenge hydrogen peroxide was determined as previously described [15] with minor modifications. A solution of hydrogen peroxide (43 mM) was prepared in 0.2 M phosphate buffer (pH 7.4). In the reaction mixture, 3.4 ml each of different concentrations of SME and ascorbic acid (25, 50, 100, 200 and 400 µg/ml) was added to 0.6 ml of 43 mM $H_2O_2$ solution. Distilled water was used as a control. The absorbance at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide, using a UV-Visible spectrophotometer. Tests were performed in triplicates. A lower absorbance reading of the reaction mixture indicated greater scavenging ability. Scavenging activity (%) was calculated using the relation:

$$\text{Scavenging activity (\%)} = \frac{\text{A0} - \text{A1}}{\text{A0}} \times 100$$

Where: A0 = the absorbance of control

A1 = the absorbance of test.

**DPPH radical scavenging assay**

The DPPH radical scavenging activity was measured using previously described methods [16, 17] with some modifications. The reaction mixture (3.0 ml) consisting of 1.0 ml of DPPH in methanol (100 µM), 1.0 ml of methanol and 1.0 ml each of the different concentrations (25, 50, 100, 200 and 400 µg/ml) of SME or standard was prepared. The reaction mixtures were incubated for 10 min in a dark cupboard, and their absorbances were measured at 517 nm using a UV-Visible Spectrophotometer. The same treatment was given to the control that consisted of 1.0 ml of 100 µM DPPH solution in methanol+2.0 ml of methanol, and the absorbance was determined at 517 nm. Tests were performed in triplicates. A lower absorbance reading of the reaction mixture indicated greater scavenging ability. The scavenging activity (%) was calculated using the formula:

$$\text{Scavenging}(\%) = \frac{\text{A0} - \text{A1}}{\text{AO}} \times 100$$

Where: A0 = absorbance of control

A1 = absorbance of test.

***In vivo* antioxidant test**

**Carbon tetrachloride-induced biologic oxidation**

The carbon tetrachloride-induced liver damage model was used as described by [18]. After seven days of acclimatization, 30 albino rats of both genders were randomly grouped (n = 5). On the first day of treatment, the animals in the test groups (II to VI) were given single oral doses of SME (100, 200 or 400 mg/kg) respectively, while control animals received ascorbic acid and distilled water respectively. About 24 h later, oral treatment commenced for the next 8 d after challenging the animals with intraperitoneal (i. p) administration of carbon tetrachloride ($CCl_4$) in liquid paraffin (1:2) 1.0 ml/kg as follows:

Group I: Naive control (0.9% normal saline; 1 ml/kg/day, i. p).

Group II: $CCl_4$+Distilled water (2 ml/kg/day).

Group III: $CCl_4$+SME (100 mg/kg/day).

Group IV: $CCl_4$+SME (200 mg/kg/day).

Group V: $CCl_4$+SME (400 mg/kg/day).

Group VI: $CCl_4$+ascorbic acid (25 mg/kg/day).

Groups II-VI was challenged with $CCl_4$ on alternate days for 1 w.

After 24 h of the last treatment, blood was withdrawn from the retro-orbital plexus of each rat and allowed to clot for more than an hour. The serum was separated by centrifugation at 3000 g at room temperature, and used for biochemical estimation of the liver enzymes–alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) [19, 20].

**Statistical analysis**

Data obtained were analyzed using One-Way ANOVA in Graph Pad Prism 5.03 and further subjected to Dunnett's post-hoc test. Differences between means of treated and control groups were accepted significant at confidence intervals of 99% and 99.9% for the *in vitro* studies, and 95% for the *in vivo* study.

**RESULTS**

**Phytochemical constituents of SME**

The phytochemical analysis showed that SME tested positive to alkaloids, glycosides, flavonoids, reducing sugars, carbohydrates, steroids, terpenoids, saponins, tannins, proteins, fats and oils (table 1).

**Acute toxicity**

The SME caused no death in the first 24 h in mice that received 10, 100 and 1000 mg/kg. Also, no death occurred at the end of the next 24 h with the second set of mice that received 1600, 2900 and 5000 mg/kg. Therefore, the oral $LD_{50}$ of SME in mice was estimated to be>5 g/kg. There were no observed signs of toxicity during and after administration of the SME to the animals.

**Ferric reducing power of SME**

The SME had significant ($P$<0.01) higher absorbance values compared to control (data not shown). The SME and ascorbic acid elicited concentration-related and significant ($P$<0.01) ferric reducing power relative to control as shown by the higher percentage values (table 2).

The SME exhibited electron donating capacity and reduced $Fe^{3+}$to $Fe^{2+}$with 25 and 50 µg/ml having lower reducing power than the same concentrations of the standard. However, at higher concentrations, the extract (100, 200 and 400 µg/ml) had higher reducing power than similar concentrations of the standard (table 2).

**Table 1: Phytochemical constituents of methanol-dichloromethane extract of *S. micranthus* stem bark**

| Phytochemical constituent | Relative presence |
|---|---|
| Alkaloids | + |
| Carbohydrates | ++++ |
| Fats and Oils | ++ |
| Flavonoids | ++++ |
| Glycosides | ++++ |
| Proteins | +++ |
| Reducing Sugars | +++ |
| Saponins | ++ |
| Resins | – |
| Steroids | ++ |
| Tannins | ++++ |
| Terpenoids | + |

–= not present; += present in small concentration; ++= present in moderate concentration; +++= present in high concentration; ++++= abundantly present; SME = *S. micranthus* stem bark extract.

**Effect of SME on hydrogen peroxide radical scavenging activity**

The SME had significant ($P$<0.01) lower absorbance values compared to control (data not shown). The SME and ascorbic acid elicited concentration related significant ($P$<0.01) and moderate $H_2O_2$ radical scavenging activity (table 3). Also, the activity of SME was comparable to that of ascorbic acid.

**Table 2: Reducing power (%) of methanol-dichloromethane extract of *S. micranthus* stem bark**

| | Reducing power (%) | |
|---|---|---|
| Dose (µg/ml) | Ascorbic acid | SME |
| 25 | 19.71±0.04 | 9.98±0.24 |
| 50 | 26.80±0.45 | 36.61±0.00 |
| 100 | 39.70±0.10 | 66.73±0.06 |
| 200 | 61.17±0.20 | 121.80±0.36 |
| 400 | 104.34±0.04 | 135.53±1.24 |

n = 3; SME = *S. micranthus* stem bark extract.

**Effect of SME on DPPH free radical scavenging activity**

The SME had significant ($P$<0.001) lower absorbance values compared to control (data not shown). The SME and ascorbic acid elicited significant ($P$<0.001) inhibition of DPPH free radical compared to control. However, the SME (100 µg/ml) elicited highest free radical scavenging activity as shown by 80.59% inhibition of DPPH free radical (table 4).

**Effects of SME on $CCl_4$-induced biologic oxidation**

The SME–treated rats had reduced levels of liver enzymes; however, only ALP and ALT for SME 100 and 400 mg/kg respectively were significantly ($P$<0.05) reduced (table 5, fig. 1). In addition, there were observed a substantive non-dose related decrease in the values of ALP, ALT and AST in the SME treated groups. Furthermore, SME 400 mg/kg treated rats had the lowest levels of ALT and AST.

**Table 3: Hydrogen peroxide radical scavenging activity (%) of methanol-dichloromethane extract of *S. micranthus* stem bark**

| | Scavenging activity (%) | |
|---|---|---|
| Dose (µg/ml) | Ascorbic acid | SME |
| 25 | 2.00±0.06 | 6.83±0.06 |
| 50 | 2.87±0.28 | 15.63±0.06 |
| 100 | 12.79±0.00 | 17.83±0.06 |
| 200 | 19.00±0.08 | 18.66±0.11 |
| 400 | 20.32±0.00 | 20.37±0.10 |

n = 3; SME = *S. micranthus* stem bark extract.

**Table 4: DPPH radical inhibiting (%) activity of methanol-dichloromethane extract of *S. micranthus* stem bark**

| | Scavenging activity (%) | |
|---|---|---|
| **Dose (µg/ml)** | **Ascorbic acid** | **SME** |
| 25 | 82.59±0.00 | 78.08±0.05 |
| 50 | 86.71±0.05 | 80.13±0.05 |
| 100 | 87.69±0.05 | 80.59±0.05 |
| 200 | 87.83±0.05 | 80.45±0.17 |
| 400 | 87.97±0.12 | 77.06±0.09 |

n = 3; SME = *S. micranthus* stem bark extract.

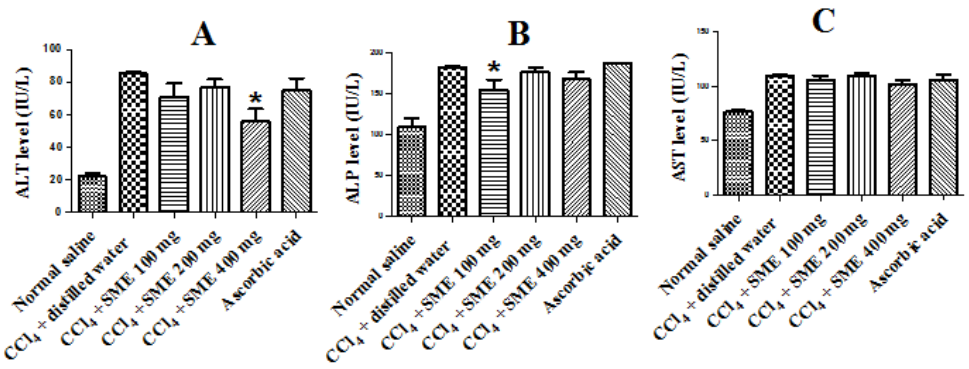


**Fig. 1: Effect of methanol-dichloromethane extract of *S. micranthus* stem bark on serum liver enzymes of $CCl_4$ hepatotoxic rats. n=5; **P*<0.05 compared to negative control using One-Way ANOVA (Dunnett's Post-Hoc test); A = alanine transaminase (ALT), B = alkaline phosphatase (ALP), C = aspartate transaminase (AST); SME = *S. micranthus* stem bark extract**

**Table 5: Hepatoprotective effect (%) of methanol-dichloromethane extract of S. micranthus stems bark on $CCl_4$ hepatotoxic rats**

| Group | Treatment | Dose (mg/kg) | ALP (IU/l) (%) | ALT (IU/l) (%) | AST (IU/l) (%) |
|---|---|---|---|---|---|
| I | Normal saline | 1 ml/kg | - | - | - |
| II | $CCl_4$+distilled water | 2 ml/kg | 0 | 0 | 0 |
| III | $CCl_4$+SME | 100 | 14.9* | 16.4 | 3.2 |
| IV | | 200 | 2.8 | 9.1 | -0.002 |
| V | | 400 | 7.7 | 33.8* | 7.2 |
| VI | $CCl_4$+ascorbic acid | 25 | -0.02 | 11.8 | 3.5 |

n = 5;**P*<0.05 compared to control (group II), using One-Way ANOVA, Dunnett's Post-Hoc test; values represent decrease in serum liver enzymes (%) relative to control (group II); SME = *S. micranthus* stem bark extract; A = alanine transaminase (ALT), B = alkaline phosphatase (ALP), C = aspartate transaminase (AST).

## DISCUSSION

Assessment of the antioxidant potentials of *S. micranthus* stem bark extract demonstrated that it elicited ferric reducing, $H_2O_2$ and DPPH radical scavenging, and hepatoprotective activities.

The SME exhibited significant (*P*<0.01) and concentration-dependent ferric reducing power which was greater than that of ascorbic acid, especially at higher doses, even though the reverse was the case at lower doses. The reducing power of a compound is associated with electron donating capacity [21, 22]. It may serve as a significant indicator of its potential antioxidant activity [23], as reductants reduce $Fe^{3+}$/ferricyanide complex to the $Fe^{2+}$form. Comparison of the reducing power of SME with that of a hydro-methanol extract of *Phoenix dactylifera* fruits with established antioxidant activity [2] showed that SME had a higher reducing power, and thus, greater antioxidant activity.

The SME exhibited moderate $H_2O_2$ radical scavenging activity, which was greater than that of the reference standard. Hydrogen peroxide is a non-radical form of ROS that is formed in living organisms by superoxide dismutase. The $H_2O_2$ is not by itself very active, but it can cross biological membranes and generate hydroxyl radicals, which are toxic to cells and can damage a number of biomolecules [24]. The ability of extract to scavenge and remove $H_2O_2$ indicates antioxidant activity and may partly account for the beneficial therapeutic effects of the plant, as removal of $H_2O_2$ is very important for the protection of living organisms from oxidative damage.

The DPPH scavenging assay demonstrated significant (*P*<0.001) and remarkable antioxidant activity of SME. The DPPH scavenging assay is a widely accepted method used to evaluate antioxidant activity in a relatively short time compared to other methods. The DPPH is a stable nitrogen-centred free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule [3]. The SME (25-400 µg/ml) exhibited marked DPPH radical inhibition (77–81%) as shown by a decrease in DPPH absorbance thus demonstrating a high DPPH scavenging activity. The decrease in absorbance of DPPH caused by antioxidants is due to the reaction between the antioxidant molecules and radical, which results in the scavenging of the radical by hydrogen donation [25]. Therefore, the effect of SME on DPPH scavenging is likely due to the hydrogen donating ability of the plant extract, and suggests antioxidant activity.

The *in vivo* antioxidant study was undertaken to evaluate the ability of SME to protect against $CCl_4$-induced liver damage in rats. Carbon tetrachloride is reported to produce free radicals, which affect the cellular permeability of hepatocytes leading to elevated levels of ALP, ALT and AST in the serum [26]. The serum levels of AST, ALT and ALP were taken as markers for oxidative stress induced by $CCl_4$. The animals treated with SME had reduced levels of liver enzymes implying that the generated free radicals were mopped up by the plant extract. The use of SME protected the liver from damage by $CCl_4$ as is evident by improved biochemical markers of liver damage in all extract-treated groups. Results suggest the ability of the extract to protect the liver from free radical-induced oxidative damage

hence, facilitating normal repair process and regeneration of diseased liver [25, 26].

Earlier investigations have demonstrated that flavonoids, triterpenes and tannins hinder free radicals formation, and hence are antioxidants [27]. The highly abundant flavonoids, tannins, saponins and terpenoids in SME may account for the observed inhibition of free radical formation and antioxidant effect, and suggest potential inhibition of free radicals mediated oxidative damage [26]. Flavonoids, tannins, terpenoids and saponins are collectively called phenolic compounds. Phenolic compounds have been demonstrated to possess scavenging ability attributable to their hydroxyl groups [25]. Oral administration of the extract (10-5000 mg/kg) to mice did not cause death, suggesting a high level of safety.

## CONCLUSION

In conclusion, the results of the study indicate that *S. micranthus* stem bark extract possess antioxidant activity and protected rats against carbon tetrachloride induced liver damage. Studies are on-going to determine the mechanism of its antioxidant activity.

## ACKNOWLEDGEMENT

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## CONFLICT OF INTERESTS

Declared none

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