Phytochemical analysis and hepatoprotective activity of Raphanus sativus var. sativus in Sprague-Dawley rats

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Abstract

Purpose: To determine the phenolic and flavonoid contents of R. sativus rhizome ethanol extract and the hepatoprotective effect of the extract in rats.

Methods: Folin–Ciocalteau and aluminum chloride colorimetric tests were used to determine the contents of phenols and flavonoids in the R. sativus extract. Male Sprague-Dawley rats induced with CCl4 to develop hepatotoxicity were treated orally with R. sativus extract for 4 weeks. The antioxidant and anti-inflammatory effects of the extract on the liver were determined by evaluating the concentration of oxidative analytes, serum liver enzymes and lipids, and hepatic histopathology and cytochrome P450 2E1 expression.

Results: R. sativus extract significantly (p < 0.05) reduced the hepatotoxic effect of CCl4 via its antioxidant activities and protection of liver tissues from oxidative damage.

Conclusion: The hepatoprotective effects of R. sativus rhizome ethanol extract are attributed to its high phenolic and flavonoid contents.

Keywords: R. savitus rhizome, Phenols, Flavonoid contents, antioxidant, Hepatoprotective

INTRODUCTION

Liver diseases are become more of a health concern, accounting for approximately 2 million deaths worldwide [1]. Current drugs in use for the treatment of hepatic toxicity are associated with intolerable side effects. As alternatives, compounds from plants are being investigated to determine their usefulness in the treatment of liver diseases [2].

Among plants with potential hepatoprotective effects is the Raphanus sativus var. Sativus. The plant, commonly known as radish and belonging to the root vegetable family, Cruciferae, is originally from Europe and Asia [3]. The rhizome of the plant reportedly has other medical...
properties including prevention from development of kidney stones [3], antioxidant [4], gastroprotective [5], hypoglycemic [6], antimutagenic, hypotensive, immunoprotective [7], anticancer [8], cardiovascular protection, and antimicrobial [9] activities. The leaves and roots of *R. sativus* contain many chemical constituents, including, alkaloids, nitrogenous compounds, coumarins, enzymes, and phenols [7].

Polyphenols such as phenolics and flavonoids are known to have various healing properties including antioxidant, free radical scavenging, antioxidant enzyme secretion enhancement, and body defence towards free oxygen species activities [8]. However, the effect of *R. sativus*, with its high phenolic content, on hepatotoxicity is not known. Hepatotoxicity can be induced with carbon tetrachloride (CCL4), through formation of free radicals, trichloromethyl and trichloromethyl peroxyl that induces lipid peroxidation [9]. Thus, the aim of this study is to ascertain the hepatoprotective potential of *R. sativus* on CCl4-induced hepatotoxicity in the rat model.

**EXPERIMENTAL**

**Plant extract preparation**

The *R. sativus* plant was procured, identified and authenticated by Department of Field Crop, College of Agriculture, University of Sulaimani. Approximately, 3 kg of freshly harvested radish rhizome were thoroughly washed to remove contaminants, and without peeling, cubed to 1 cm³ sizes, and placed under shade at room temperature until dry. Approximately, 250 g of the cubes were pulverized to powder and macerated with ethanol (1:10; w/v) for 5 days in a sealed vessel. The suspension was filtered using the Whatman No. 1 filter paper and the filtrate was concentrated using a rotary evaporator (Heidolph, Germany). Finally, concentrated product afforded 10.45 g of ethanolic extract, kept in an air-tight glass container and stored at 4 °C.

**Evaluation of phenolic and flavonoid compounds**

Folin–Ciocalteau [10] and the aluminum chloride (AlCl3) colorimetric [11] tests were used to determine the phenolic and flavonoid contents of the *R. sativus* rhizome, respectively. The phenolic content was determined by adding 1 mL of the extract to 1 mL of Folin-Ciocalteu reagent (10-fold dilution), mixed well and allowed to stand for 5 minutes at 25 °C. Then, 10 mL of sodium carbonate (Na2CO3; 7.5 %) solution was added to the mixture and made to a total volume of 25 mL with distilled water and allowed to stand for 1 hour. The absorbance was recorded at 25 °C and 765 nm using a spectrophotometer. The results were expressed as mg of Gallic acid equivalent (GAE; 5 – 100 μg/mL) per gram of dry extract. The flavonoid content was determined by adding 1 mL of 2% AlCl₃ in ethanol to a mixture of 10 μL of plant material and 10 μL of acetic acid, made to a final volume of 25 mL with distilled water and allowed to stand for 45 minutes. The absorbance of the mixture was determined spectrophotometrically at 25 °C and 415 nm and the result expressed as mg of rutin equivalent (RE; 5 – 25 μg/mL) per gram dry extract.

**Animal groups**

Male Sprague-Dawley rats, aged 7 – 8 weeks and weighing 150 ± 15 g, were obtained from the Department of Clinic and Internal Medicine, College of Veterinary Medicine, University of Sulaimani. The rats, given standard rat chow and water *ad libitum*, were humanely managed and acclimatized under 12 h dark/ 12 h light and 25 ± 3 °C ambient temperature for 1 week prior to commence of the experiment. The study was approved by the College of Veterinary Medicine, University of Sulaimani, Iraq (approval no: UNIVSUL/ACUC/2016-0909) and according to the Canadian guidelines for animal use and care [12].

**Induction of hepatotoxicity**

The rats were injected subcutaneously with a single dose of 1.2 mg/kg B.wt. CCl₄ (Sigma-Aldrich, USA) in 50 mM phosphate buffer solution (PBS). Experimentation commenced three days after the administration of CCl₄.

**Study design and animal treatment**

Thirty male Sprague Dawley rats were randomly allotted into 5 groups of 6 animals each. Group (A) was the normal non-treated rats and served as the negative control; group (B) rats were with induced hepatotoxicity and served as vehicle control; group (C) rats were with induced hepatotoxicity, treated with 100 mg/kg B.wt. silymarin (hepatoprotective drug, Sigma Aldrich, St Louis, MO, USA) and served as positive control; groups (D) and (E) were rats with induced hepatotoxicity and orally treated with 50 and 100 mg/Kg B.wt. *R. sativus* extract, respectively. Oral treatment was done by force-feeding rats daily for 28 consecutive days, using a ball-tipped stainless-steel needle.
Blood collection

On day 29, 2 mL blood collected via cardiac puncture under general anesthesia and centrifuged at 3000 × g at 4 °C for 10 minutes to collect serum. Then, the animals were euthanized with an overdose of intraperitoneal sodium thiopental. The liver was excised, washed in 0.02 M chilled EDTA solution, fat tissues removed and subjected to the oxidative stress biomarkers content assessment and histopathology.

Preparation of liver cellular fractions

Approximately, 1 g of liver samples were blended with 5 mL of 200 mM potassium phosphate buffer at pH 6.5 to obtain homogenates, which were used for the determination of malonaldehyde (MDA), glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), and myeloperoxidase (MPO) concentrations. The protein concentration of the homogenates was determined using the Bradford method (Bio-Rad, Hercules, CA, USA), with bovine serum albumin (BSA) (0.125 – 1.0 mg/mL) as the standard.

Free radical and lipid peroxidation

The MDA content of the homogenates was estimated by adding 0.5 mL of thiobarbituric acid reactive substance (TBARS) to each sample, heated at 100 °C for 1 hour, ice-cooled before centrifugation at 5000 × g for 15 minutes. The absorbance of the supernatants was estimated spectrophotometrically at 37 °C and 535 nm using 1,1,3,3-tetraethoxypropane as the standard. The results were expressed as nmol/mg protein.

The SOD was determined by mixing 1 mL of homogenate with 500 µL 200 mM Tris HCl–EDTA buffer solution at pH 8.5 and allowing for equilibration for 1 minute before incubating at 25 °C for 18 minutes. The reaction was stopped by adding 0.1 mL 1N HCl to the mixture centrifuging at 15000 × g. The absorbance of the supernatant was read spectrophotometrically at 37 °C and 405 nm. The amount of SOD that suppressed the auto-oxidation of pyrogallol by 50% was expressed as U/mg protein [13].

The concentration of GSH was determined by mixing 1 mL of homogenate with 1 mL 12.5% trichloroacetic acid and allowed to equilibrate for 8 minutes before centrifuging for 10 minutes at 1000 × g. The supernatant collected was mixed with 2 mL 0.4 M TRIS buffer at pH 8.9) and 1 mL 0.01 M dinitro-thiobenzoic acid (DTNB). The absorbance of the supernatant was read spectrophotometrically at 25 °C and 420 nm. The GSH concentration expressed as nmol/mg of tissue was determined from GSH standard curved prepared.

About 1 mL of homogenate was centrifuged at 15000 × g to collect supernatant. The CAT was determined by mixing 1 mL of supernatant with 1 mL of solution containing 200 mM Tris HCl–EDTA buffer solution of pH 8.5 and 0.5 mL 20 mM H2O2 of pH 7.0. Then, the absorbance was read spectrophotometrically at 37 °C and 240 nm and the results expressed as µmol/mg protein [14].

Anti-inflammatory evaluation

The hepatic MPO activity was determined by adding 1 mL homogenate to 1 mL 80 mM potassium phosphate buffer (pH 5.4) containing 0.5% hexadecyl-trimethyl-ammonium bromide (HTAB). The mixture was centrifuged at 15000 × g for 15 minutes at 4 °C. The absorbance of the supernatant was determined at at 37 °C and 620 nm and the concentration of MPO was expressed as absorbance/mg of protein using 0.0625 mg/mL albumin as the standard [15].

Lipid profile and serum biochemical analysis

Serum total cholesterol (TC), triglyceride (TG), low density lipoprotein (LDL), high density lipoprotein (HDL), very low density lipoprotein (VLDL), urea, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), total protein (TP), alkaline phosphatase (ALP), and total bile acids (TBA) were determined using commercial kits (Roche) and the Hitachi Chemistry Analyzer (Hitachi High-Technologies Corporation, Japan).

Histopathology analysis of liver

Fresh liver tissues were trimmed, placed in plastic cassettes and fixed in 10% formalin. After 48 hours, tissues were processed, infiltrated in paraffin, sectioned to a thickness of 5 mm, deparaffinized and rehydrated. Finally, the sections were stained with hematoxylin and eosin, viewed under light microscopy (Leica, Japan) and the histological changes in the liver sections recorded.

Western blotting

The expression of liver cytochrome P450 E1 (CYP2E1) was determined by mixing 0.5 mL of liver homogenate with 1 mL radioimmunoprecipitation assay (RIPA) buffer...
and 10 µL protease inhibitor cocktail (Pierce, Thermo Fisher Scientific, USA) to lyse the cells. The mixture was centrifuged at 15000 × g to obtain supernatant. The protein concentration of the supernatant was determined using the Bradford method (Bio-Rad, Hercules, CA, USA), with bovine serum albumin (BSA) (0.125 – 1.0 mg/mL) as the standard. The SDS PAGE (Bio-Rad, USA) was used to separate proteins in the supernatant. The separated protein transferred by blotting to the nitrocellulose membrane (PALL, USA) and blocked with 5% skimmed milk (Biobasic, USA) for 24 hours, washed with TBST (pH 7.6), and flooded with CYP2E1 (Abcam, USA) for 1 hour at 4°C. GAPDH was used as the housekeeping protein. The membrane was rinsed and flooded with horse radish peroxidase substrate for 10 minutes. Lastly, the membrane was visualised with the Chemidoc imager (UVP, USA) and the Vision Work LS Analysis software (UVP, USA) was used to analyse the result.

Statistical analysis

The data were subjected to statistical analysis using one-way analysis of variance (ANOVA) followed by Tukey multiple comparison tests (SPSS version 23.0, Chicago, USA) and the results expressed as mean ± SD (n = 3). Probability values of p < 0.05 were considered statistically significant.

RESULTS

Phytochemical profile

The of *R. sativus* ethanol extract contained 145.91 ± 0.18 mg GAE/g phenolic and 21.95 ± 1.2 mg RE/g flavonoid compounds.

Lipid peroxidation

The study showed that oral supplementation of rats with *R. sativus* extract and silymarin treatment decreased the GSH and MDA content (p < 0.05) and increased the SOD and CAT activities (p < 0.05) in liver tissues, in comparison with the vehicle group. The *R. sativus* extract at various doses also decreased liver MPO activity significantly (p < 0.05) in comparison with the positive control (Table 1).

Lipid profile

Rats of the vehicle group showed significant (p < 0.05) higher serum TC, TG, LDL, and VLDL levels and lower (p < 0.05) HDL level than the silymarin and *R. sativus* extract treatment groups (Table 2). The high LDL/HDL ratio in the vehicle group and to lesser extent in treated groups may point to incipient atherosclerotic complications. At the same time, the high TG level in the vehicle group is related to the fatty degeneration of the liver.

Biochemical profile

*R. sativus* significantly (p < 0.05) prevented the CCl4 evoked raising of serum AST, ALT, ALP, LDH, and urea while significantly (p < 0.05) restored the levels of serum TBA, TP and creatinine in dosed rats. The silymarin group also displayed significant (p < 0.05) protective covering toward CCl4-induced liver impairment through the previous-mentioned biomarkers (Table 3).

Histopathological features

The liver tissues of the control negative group rats demonstrated normal morphology with intact hepatic cells and a central vein. In opposite to this, the vehicle group showed marked hepatic injury including massive fatty changes, necrosis, degeneration, infiltration of lymphocytes, cellular hypertrophy, and the loss of cellular boundaries. On the other hand, changes improved greatly in silymarin treated rats, which displayed almost as of normal liver appearance. Additionally, treatment with *R. sativus* markedly attenuated CCl4-induced liver damage and showed normal lobular outline with a slight fatty change, necrosis, and inflammatory cells (Figure 1).

![Figure 1](https://example.com)
Table 1: Effect of *R. sativus* aqueous extract on liver homogenate lipid peroxidation and anti-inflammatory parameters of normal and CCl₄-induced hepatotoxic rats.

| Treatment            | MDA (nmol/mg) | GSH (nmol/mg) | SOD (U/mg) | CAT (U/mg) | MPO (abs/mg) |
|----------------------|---------------|---------------|-------------|-------------|--------------|
| Normal control       | 2.25 ± 1.11   | 12.7 ± 0.85   | 12.5 ± 0.75 | 28.04 ± 0.43 | 0.10 ± 0.05  |
| Vehicle control      | 4.4 ± 0.52*   | 19.9 ± 0.44*  | 6.6 ± 0.15* | 15.31 ± 0.67* | 0.17 ± 0.04* |
| Positive control     | 2.85 ± 0.55** | 13.05 ± 0.65**| 10.9 ± 0.28**| 22.45 ± 0.55** | 0.121 ± 0.05** |
| *R. sativus* extract |               |               |             |             |              |
| 50 mg/kg B.wt.       | 3.3 ± 0.42**  | 14.1 ± 0.75** | 9.4 ± 0.45**| 17.9 ± 0.61** | 0.13 ± 0.02** |
| 100 mg/kg B.wt.      | 3.0 ± 0.03**  | 13.0 ± 0.25** | 10.3 ± 0.77**| 20.75 ± 0.23** | 0.125 ± 0.11** |

Values are expressed as mean ± SD; N = 6. Normal control – Non-treated; vehicle control - carbon tetrachloride (CCl₄)-induced hepatotoxicity without; Positive control - CCl₄-induced hepatotoxicity with 100 mg/kg B.wt. silymarin treatment; *R. sativus* extract - CCl₄-induced hepatotoxicity with extract treatment. MDA – malonaldehyde; GSH – glutathione; SOD – superoxide dismutase; CAT – catalase; MPO - myeloperoxidase. *Significance level: p < 0.05, compared to normal control. **Significance level: p < 0.05, compared to vehicle control.

Table 2: Effect of *R. sativus* aqueous extract on liver homogenate lipid profile of normal and CCl₄-induced hepatotoxic rats.

| Treatment            | TC (mg/dL) | TG (mg/dL) | LDL (mg/dL) | HDL (mg/dL) | VLDL (mg/dL) |
|----------------------|------------|------------|-------------|-------------|--------------|
| Normal control       | 80.76 ± 1.23 | 112.50 ± 2.55 | 24.25 ± 1.27 | 52.61 ± 1.15 | 18.33 ± 0.48 |
| Vehicle control      | 127.5 ± 2.17* | 225.05 ± 1.72* | 101.09 ± 3.02* | 29.45 ± 1.01* | 35.54 ± 0.77* |
| Positive control     | 84.33 ± 1.06** | 130.14 ± 1.32** | 55.22 ± 4.15** | 40.04 ± 1.25** | 20.97 ± 0.49** |
| *R. sativus* extract |            |            |             |             |              |
| 50 mg/kg B.wt.       | 89.22 ± 3.02** | 145.09 ± 3.03** | 64.36 ± 2.30** | 43.18 ± 0.77** | 23.45 ± 0.52** |
| 100 mg/kg B.wt.      | 83.43 ± 1.12** | 127.23 ± 3.10** | 57.55 ± 2.01** | 48.10 ± 1.24** | 25.31 ± 0.65** |

Values are expressed as mean ± SD; N = 6. Normal control – Non-treated; Vehicle control - Carbon tetrachloride (CCl₄)-induced hepatotoxicity without treatment; Positive control - CCl₄-induced hepatotoxicity with 100 mg/kg B.wt. silymarin treatment; *R. sativus* extract - CCl₄-induced hepatotoxicity with extract treatment. TC - Total cholesterol; TG - Triglyceride; LDL - Low density lipoprotein; HDL - High density lipoprotein; VLDL - Very low-density lipoprotein (VLDL). *Significance level: p < 0.05, compared to normal control. **Significance level: p < 0.05, compared to vehicle control.

Table 3: Effect of *R. sativus* aqueous extract on serum biochemical parameters of normal and CCl₄-induced hepatotoxic rats.

| Treatment            | Urea (mmol/L) | Creatinine (µm/L) | LDH (U/L) | AST (U/L) | ALT (U/L) | ALP (U/L) | TBA (mg/dL) | TP (mg/dL) |
|----------------------|---------------|-------------------|-----------|-----------|-----------|-----------|-------------|------------|
| Normal control       | 8.45 ± 2.0    | 66.25 ± 13        | 455 ± 13  | 81.40 ± 2.10 | 49.01 ± 2.30 | 155.60 ± 2.50 | 0.27 ± 0.16 | 7.05 ± 1.20 |
| Vehicle control      | 0.30 ± 2.0    | 20 ± 13           | 583 ± 13  | 295.40 ± 2.50 | 158.03 ± 2.50 | 345.30 ± 2.50 | 0.80 ± 0.16 | 4.35 ± 1.20 |
| Positive control     | 0.50* ± 2.0   | 14.9* ± 19        | 3.00* ± 2.10 | 0.59* ± 2.50 | 2.10* ± 2.40 | 2.30* ± 2.00 | 3.00* ± 2.30 | 6.55 ± 1.20 |
| *R. sativus* extract | 0.34** ± 2.0  | 2.75** ± 5.45**   | 2.10** ± 3.30** | 1.10** ± 3.20** | 3.30** ± 3.20** | 2.11** ± 3.00** | 3.00** ± 3.00** | 6.55 ± 1.20 |
| 50 mg/kg B.wt.       | 9.55 ± 2.0    | 54.95 ± 13        | 495 ± 13  | 155.37 ± 2.50 | 111.65 ± 2.50 | 269.97 ± 2.50 | 0.71 ± 0.16 | 5.39 ± 1.20 |
| 100 mg/kg B.wt.      | 0.37** ± 2.0  | 1.92** ± 16**     | 1.50** ± 3.12** | 1.45** ± 3.45** | 2.09 ± 3.20** | 2.20** ± 3.33** | 3.00** ± 3.00** | 6.55 ± 1.20 |

Values are expressed as mean ± SD; N = 6. Normal control – Non-treated; Vehicle control - Carbon tetrachloride (CCl₄)-induced hepatotoxicity without treatment; Positive control - CCl₄-induced hepatotoxicity with 100 mg/kg B.wt. silymarin treatment; *R. sativus* extract - CCl₄-induced hepatotoxicity with extract treatment. LDH – Lactate dehydrogenase; AST – Asparate aminotransferase; ALT – Alanine aminotransferase; ALP – Alkaline phosphatase; TBA – Total bile acids; TP – Total protein. *Significance level: p < 0.05, compared to normal control. **Significance level: p < 0.05, compared to vehicle control.
Protein expressions

The non-treated rats showed lowest expression of liver cytochrome P450 2E1 among treatment groups. The highest hepatic cytochrome P450 2E1 expression was in the vehicle group of rats. The positive control and R. sativus -treated rats showed significantly (p < 0.05) lower hepatic cytochrome P450 2E1 expressions than the vehicle group (Figure 2).

DISCUSSION

The study showed that the R. sativus extract has high phenolic and flavonoid compound contents. This finding, with some variation, is similar to that reported by others on various species of radish rhizomes such as in R. sativus L., R. sativus L. cultivars Cherry Belle and Valentine [16, 17]. The variations in phenolic and flavonoid contents of R. sativus among these studies are attributed to the differences in species, chemodiversity, breeding condition, ontogenetic status, stage of maturation, degradation, and post-harvest handling [17]. Phenolic and flavonoid compounds have antioxidant properties that exert actions either directly through interactions with toxic oxygen species or indirectly by increasing the activities of tissue antioxidant enzymes [18].

Several extracts from natural plants such as Maytenus robusta Reiss [19], Phyllanthus amarus [20], Fagonia schweinfurthii Hadidi [21], and Herpetospermum caudigerum [22] were shown to protect the liver from oxidant damage induced with CCl4. In our study, we showed that administration of R. sativus significantly decreased the activities of the oxidants, MDA, and GSH, while increasing the activities of antioxidant enzymes, SOD and CAT in CCl4-induced hepatotoxicity in rats. The results suggest that the R. sativus extract is a potent compound for the protection of the liver from oxidative damage.

Treatment of rats with CCl4 had caused liver damage as evident by significant increases in concentration of serum leakage of liver enzymes, ALT, AST, and LDH. However, R. sativus prevented the CCl4 induced ascension of serum AST, ALT, ALP, LDH, and urea in treated rats. This suggests that the R. sativus extract is hepatoprotective.

Histopathologically, CCl4 caused fatty degeneration and infiltration of inflammatory cells in the rats. Even after treatment with silymarin, a known hepatoprotective drug, the liver of CCl4-treated rats still showed some hepatocellular degeneration. R. sativus extract is a good hepatoprotective agent because this compound had reduced the histopathological changes in the rat liver tissues induced by CCl4. In fact, in rats, R. sativus extract had dose-dependently reduced the CCl4-induced hepatotoxicity to minimal fatty changes with infiltration of only a few inflammatory cells, suggesting that it is even a better hepatoprotective compound than silymarin.

Lipids are primarily produced by the liver and intestinal enterocytes. Among the causes of hyperlipidemia is cholestatic Liver disease. Liver disease can cause hypercholesterolaemia, from failure of the organ to metabolize circulating cholesterol. In this study, CCl4 injection increased serum TC, TG, LDL, and VLDL but not HDL in rats. However, treatment with R. sativus extract caused decrease in serum TG, LDL, VLDL, and increase in HDL and HDL: LDL. This finding is similar to that report by other researchers [23]. The extract does not only protect the liver from oxidant damage but also has the potential to reduce risk of development of cardiovascular disorders in patients with hepatic diseases.

Cytochrome P450-2E1 or CYP2E1 is a membrane-bound protein found in high concentration in the liver. CYP2E1 catalyzes transformation of organic molecules, fatty acids, ketones, glycerol, drugs such as salicylic acid, halothane, and isoniazid, CCl4, chloroform, and environmental contaminants such as benzene and acrylamide. Increased expression of hepatic tissue CYP2E1 is associated with oxidative liver damage and hepatotoxicity [24, 25]. In our study, the highest expression of hepatic tissue CYP2E1 was in the rats with CCl4 hepatocytotoxicity. Treatment with R. sativus extract had dose-dependently decreased expression of this protein in the rat liver.

CONCLUSION

The findings of this study show that R. sativus extract lowers the hepatotoxic effect of oxidant...
compounds produced by the action of CCl₄. The antioxidant and hepatoprotective effects of *R. sativus* extract is suggested is here attributed to its phenolic and flavonoid contents. The antioxidant effect of *R. sativus* extract is primarily due to its capacity to suppress lipid peroxidation, stimulate tissue free radical scavenging and enzymatic antioxidant activities. These properties protect the liver from the tissue-damaging actions of reactive oxygen species (ROS) and subsequent inflammatory response.

**DECLARATIONS**

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**Conflict of interest**

No conflict of interest is associated with this work.

**Contribution of authors**

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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