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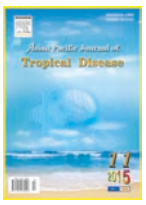

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# Evaluation of *in vitro* antioxidant and anti-inflammatory activities of *Ximenia americana* extracts

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ABSTRACT

**Objective:** To evaluate *in vitro* antioxidant and anti-inflammatory activity of *Ximenia americana* extracts.
**Methods:** Herbal extraction was done by Soxhlet extraction method with increasing polarity of solvents *viz.*, chloroform, ethyl acetate, methanol, ethanol and water. Phytochemical analysis was done using different biochemical tests. Antioxidant potential of plant extracts were analyzed by ferric ion reducing antioxidant power, phosphomolybdenum and 2,2-diphenyl-1-picrylhydrazyl, and anti-inflammatory activity by using protein denaturation *in vitro* bioassay. Total phenolic content of each extract was also determined to assess their corresponding effect on antioxidant capacity of plant.
**Results:** Phytochemical analysis showed that each solvent extract contained broad spectrum of secondary metabolites, phenolic compounds, flavonoids, tannins and glycosides, whereas compared to other solvent extracts, chloroform extract showed negative result for phenolic compounds whereas aqueous extract exhibited the highest phenolic content and the significant antioxidant capacity based on the test performed. Out of all extracts, methanol extract showed high anti-inflammatory activity.
**Conclusions:** The present study revealed that different solvent extracts of *Ximenia americana* leaves contain broad spectrum of bioactive compounds. Results confirm that aqueous extract exhibited high antioxidant activity and methanol extract exhibited high anti-inflammatory activity. Further study requires purification, characterization and structural elucidation of phenolic compounds in both extracts that may help in the development of new phytopharmaceuticals.

## 1. Introduction

Reactive oxygen species (ROS) are an integral part of normal physiological processes, continuously formed as a consequence of aerobic metabolism in eukaryotic cells. ROS at low-to-moderate concentrations play an important role in cell physiology, such as regulation of cell growth, cellular signal transduction pathways, and defense against pathogens[1,2]. In addition to their biological importance, overproduction of these extremely reactive and unstable oxygen species is considered to be the main contributor to various metabolic and cellular disturbances. Oxidative stress has been suggested to play a major role in the pathogenesis of many degenerative diseases in humans[3], *i.e.* inflammatory, cancer, diabetes, aging, cardiovascular diseases; tumor growth and Alzheimer's disease are contributed by increased cell oxidation[4-7]. In modern medicine, maintaining the balance between antioxidant defense system and ROS formation is believed to be a critical concept for healthy biological systems[8]. However in recently published data there is use of synthetic antioxidants like hydroxyl toluene, butylated hydroxyanisole, tert-butylhydroquinone and propyl gallate are widely used in the food industry to prevent oxidative deterioration[9]. However, it has been established that synthetic antioxidants appear to have carcinogenic and tumor-promoting action[10]. Therefore, it is of great importance to find new sources of safe and inexpensive antioxidants of natural origin in order to use them in food and pharmaceutical formulations. Several studies have showed that increased dietary intake of natural phenolic antioxidants correlate with decreased coronary heart disease[11]. Natural products based drugs have used against various diseases since time immemorial. Plant derived natural products hold great promise for the discovery and development of new drugs. Studies have been conducted to identify antioxidants

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from natural sources which are suitable alternatives to synthetic antioxidants[12,13]. Polyphenolic compounds or phytochemicals present in plants are important components of the human diet; Phytochemicals can be used to regulate oxidation and stress-related chronic diseases such as diabetes and cardiovascular diseases[14]. The protective effects of phenolic compounds and flavonoids are directly related to their ability to scavenge free radicals[15] by undergoing oxidation, producing toxic compounds, which elicit inhibitory effects on pathogenic microorganisms[16]. In order to protect the human body from free radicals, a number of studies have been carried out on various plants, vegetables and fruits which are a rich source of antioxidants, such as vitamin A, vitamin C, polyphenolic compounds and flavonoids[17]. Inflammation is a very common symptom of many chronic diseases and it is normal protective response to tissue injury caused by chemical or microbial agents[18]. It is well known fact that denaturation of tissue proteins lead to inflammatory and arthritic diseases[19]. Inflammation plays an important role in various diseases, such as rheumatoid arthritis and asthma. During an inflammatory response, mediator such as cytokines, interleukin-1, tumour necrosis factor and interferons are released[20,21]. Since ancient times, in various cultures worldwide, inflammatory disorders and related diseases have been treated with plants or plant derived formulations. An anti-inflammatory activity of several plant extracts and isolated compounds has already been scientifically demonstrated[22,23].

In the present study, *Ximenia americana* (*X. americana*) belonging to Olacaceae family was selected. It is used in the treatment for a wide variety of ailments by many rural communities in Asia commonly known as “wild olive”. It is extensively used as herbal remedy in treatment of malaria, leproutic ulcer, skin infections[24], as antibacterial activity, in fever, tuberculosis, stiffness, tooth decay and wounds[25]. Many works have reported the use of roots in the treatment of leprosy, syphilis, dysentery, and wounds. The stem bark has been reported to have anti-trypanosomal activity and used in treating headaches and mumps[26]. *X. americana* species, where systematic study is still not satisfactory, specially, is relative to specific biological activity of their chemical constituents. However this plant has not been studied for anti-inflammatory activity and there are very few available literatures on antioxidant activity of this plant. Therefore, the present study was aimed to evaluate *in vitro* antioxidant and anti-inflammatory activities of *X. americana*.

## 2. Materials and methods

### 2.1. Plant collection

*X. americana* leaves were collected from the campus of Karnatak University, Dharwad, India in the month of June 2014. The leaves were identified and authenticated by Dr. Kotresha K, Department of Botany, Karnataka Science College, Dharwad, Karnatak. A voucher specimen was deposited in the Department of Botany, Karnataka Science College, Dharwad, Karnataka. Fresh plant material was washed under running tap water, air dried and then homogenized to fine powder. The powder was stored in airtight containers at -20 °C for further use.

### 2.2. Crude extraction

About 100 g dried leaves were coarsely powdered and subjected to successive extraction by Soxhlet extractor. The extraction was done with different solvents in their increasing order of polarity such as chloroform, ethyl acetate, methanol, ethanol and distilled water. Each time the plant material was dried and later extracted with other solvents. All the extracts were concentrated by rotary vacuum evaporator and evaporated to dryness.

### 2.3. Phytochemical analysis

The crude powder of *X. americana* was qualitatively tested for different phytochemical constituents namely alkaloids, flavonoids, glycosides, phenols, lignins, saponins, sterols, tannins, anthraquinone and reducing sugar by following the standard procedure of Deepti *et al.*[27].

### 2.4. Estimation of total phenolic content

The total phenolic content of the *X. americana* leaf extract was estimated by using Folin-Ciocalteu method of Singleton *et al.* with slight modification[28]. Gallic acid was used as the reference standard. A volume of 0.5 mL of plant extract was mixed with 2 mL of the Folin-Ciocalteu reagent (10 fold) and was neutralized with 4 mL of sodium carbonate solution (8% w/v). The reaction mixture was incubated at room temperature for 30 min for color development. The absorbance of the resulting color was measured at 765 nm using UV-vis spectrophotometer. The total phenolic contents were estimated from the linear equation of standard curve prepared with gallic acid. The content of total phenolic compounds expressed as mg/g gallic acid equivalent (GAE).

### 2.5. Determination of antioxidant activity by using in vitro methods

#### 2.5.1. Ferric ion reducing antioxidant power (FRAP) assay

Ferric ions reducing power was measured according to the method of Oyaizu with a slightest modification[29]. Methanol and aqueous extract of *X. americana* in different concentrations ranging from 100 μL to 500 μL were mixed with 2.5 mL of 20 mmol/L phosphate buffer and 2.5 mL (1% w/v) potassium ferricyanide, and then the mixture was incubated at 50 °C for 30 min. Afterwards, 2.5 mL of (10% w/v) trichloroacetic acid and 0.5 mL of (0.1% w/v) ferric chloride were added to the mixture, which was kept aside for 10 min. Finally, the absorbance was measured at 700 nm. Ascorbic acid was used as positive reference standard.

#### 2.5.2. Phosphomolybdenum (PM) assay

Total antioxidant activity was estimated by PM assay using standard procedure of Prieto *et al.*[30]. Methanol and aqueous extract of *X. americana* in different concentration ranging from 100 μL to 500 μL were added to each test tube individually containing 3 mL of distilled water and 1 mL of molybdate reagent solution. These tubes were kept incubated at 95 °C for 90 min. After incubation, these tubes were normalized to room temperature for 20–30 min

and the absorbance of the reaction mixture was measured at 695 nm. Ascorbic acid was used as reference standard.

### 2.5.3. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging ability assay

Radical scavenging activities of methanol and aqueous extracts of *X. americana* were determined using the DPPH radical as a reagent, according to the methods of Rice-Evans *et al*.[31]. About 100 μL of a DPPH radical solution in ethanol (60 μmol/L) was mixed with 100 μL of sample solution in ethanol (different concentrations, w/v). The mixture was incubated for 30 min in the dark at room temperature and the absorbance was measured at 517 nm. Ascorbic acid was used as a standard. The DPPH scavenging activity of each sample was calculated using the following equation:

$$\text{DPPH scavenging activity (\%)} = \frac{A_c - A_t}{A_c} \times 100$$

where $A_c$ is the absorbance of the control reaction (100 μL of ethanol with 100 μL of the DPPH solution), and $A_t$ is the absorbance of the test sample. The experiment was done in triplicate. The $IC_{50}$ value is the concentration of sample required to inhibit 50% of the DPPH free radical. Lower absorbance of the reaction mixture indicated higher free radical activity.

### 2.5.4. Evaluation of in vitro anti-inflammatory activity

Anti-inflammatory activity of methanol and aqueous extract of *X. americana* was evaluated by protein denaturation method as described by Padmanabhan *et al*. with slight modifications[32]. Diclofenac sodium, a powerful non steroidal anti-inflammatory drug was used as a standard drug. The reaction mixture consisting of 2 mL of different concentrations of *X. americana* methanol and aqueous extract (100–500 μg/mL) or standard diclofenac sodium (100 and 200 μg/mL) and 2.8 mL of phosphate buffered saline (pH 6.4) was mixed with 2 mL of egg albumin (from fresh hen's egg) and incubated at (27 ± 1) ℃ for 15 min. Denaturation was induced by keeping the reaction mixture at 70 ℃ in a water bath for 10 min. After cooling, the absorbance was measured at 660 nm by using double distilled water as blank. Each experiment was done in triplicate and the average was taken. The percentage inhibition of protein denaturation was calculated by using the following formula:

$$\text{Inhibition (\%)} = \frac{A_t - A_c}{A_c} \times 100$$

where $A_t$ is absorbance of test sample, and $A_c$ is absorbance of control.

### 2.6. Statistical analysis

The results were expressed as mean ± SD. One-way ANOVA was used to analyze the variation and level of statistical significance between groups. $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Phytochemical analysis

In the present study, the phytochemical analysis was performed with chloroform, ethyl acetate, methanol, ethanol and aqueous extracts of leaves of *X. americana*. Out of five different extracts, presence of alkaloids and lignins were detected in chloroform extract; phenols, flavonoids and tannins were detected in other four solvent extracts except chloroform extract; glycosides were detected in chloroform, ethyl acetate and aqueous extracts; saponins were detected in methanol, aqueous and chloroform extracts; sterols were detected in chloroform and ethyl acetate extracts; oil and fats were present in all solvent extracts (Table 1). The present work identifies the variation in the phytoconstituents in each extract of the plant which is known to have beneficial importance in therapeutic application. Many drugs containing tannins are used in the medicine as healing agent in inflammation, burns, and piles. Apart from this, they are known to have antiviral, anti-inflammatory and antioxidant properties[33-35]. We observed presence tannins in four out of five extracts. Presence of hydroxyl group in phenol possesses a different chemical and biological activity that makes it as useful drug. Some of its biological activities include antiviral, antibacterial, anti-inflammatory, antioxidant and anticancer[36]. From these results it can be considered that selected extracts of *X. americana* can be used in broad spectrum of biomedical application.

### 3.2. Total yield of crude extract

Phytochemical analysis showed that *X. americana* leaves were rich in plant secondary metabolites. The total yield of crude extracts from *X. americana* leaves by using the solvents, *viz.* chloroform, ethyl acetate, methanol, ethanol and aqueous were 4.0%, 5.0%, 3.7%, 4.0% and 8.0% w/w respectively with reference to the air dried plant material.

**Table 1**

Phytochemical constituents of leaves extracts of *X. americana*.

| Chemical constituent | | Chloroform | Ethyl acetate | Methanol | Ethanol | Aqueous |
|---|---|---|---|---|---|---|
| Alkaloids | Iodine | - | - | - | - | - |
| | Waganer's | + | - | - | - | - |
| | Dragendroff's | + | - | - | - | - |
| Flavonoids | Pew's test | - | + | ++ | + | + |
| | Shinoda test | + | + | ++ | ++ | ++ |
| | NaOH test | - | ++ | ++ | ++ | ++ |
| Glycosides | K-K test | + | ++ | - | + | ++ |
| | Glycoside test | ++ | ++ | - | - | ++ |
| | Conc. $H_2SO_4$ test | ++ | ++ | - | - | ++ |
| | Molish Test | ++ | ++ | - | - | + |
| Phenols | Ellagic acid test | - | ++ | ++ | ++ | ++ |
| | Phenols test | - | ++ | ++ | ++ | ++ |
| Lignin | Lignin test | + | - | - | - | - |
| | Lobat test | ++ | - | - | - | - |
| Saponins | Foam test | ++ | - | + | ++ | - |
| | Haemolysis test | + | - | ++ | + | + |
| Sterols | L-B test | + | + | - | - | - |
| | Salkowsk test | + | ++ | - | - | - |
| Tannins | Gelatin test | - | + | + | + | + |
| | Lead acetate test | - | ++ | ++ | ++ | ++ |
| Anthraquinone | Bomtrager's test | - | ++ | - | - | ++ |
| Phlobatanin | | - | - | - | - | - |
| Reducing sugar | | - | - | - | - | - |
| Volatile oil | | - | - | - | - | - |
| Oils and fats | Filter paper test | ++ | + | ++ | ++ | + |
| | Saponification test | + | + | + | + | ++ |

-: Absent; +: Moderate; ++: High presence.

### 3.3. Total phenol content

In the present study, total phenolic content of different extracts of leaves of *X. americana* was determined by the Folin-Ciocalteu reagent[28] and expressed as GAE per gram of plant extracts. Aqueous extract exhibited the highest amount of phenolic content among the extracts, *i.e.* (91.40 ± 0.14) mg/g GAE followed by (90.40 ± 0.07) mg/g GAE in methanol extract (88.30 ± 0.14) mg/g GAE in ethanol extract and (83.70 ± 0.14) mg/g GAE in ethyl acetate extracts. Variation was observed in total phenolic content in each extracts.

### 3.4 FRAP assay

In the present study, methanol and aqueous extracts were subjected to FRAP assay along with standard gallic acid. In the results obtained, aqueous extract showed higher activity than the methanol extract (Figure 1) which was comparable to standard ascorbic acid.

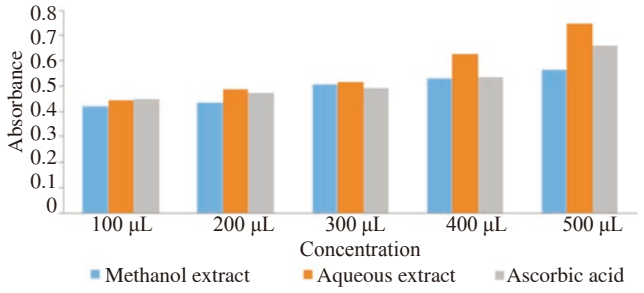


**Figure 1.** FRAP assay for extracts of *X. americana*.

### 3.5. PM assay

In the present study, methanol and aqueous extracts were subjected to PM assay along with standard ascorbic acid. Aqueous extract showed the highest activity among methanol extract and standard ascorbic acid (Figure 2).

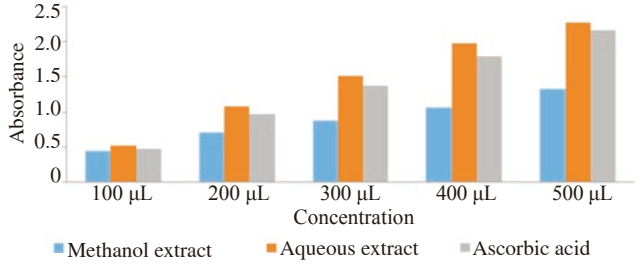


**Figure 2.** PM assay for leaf extract of *X. americana*.

### 3.6. DPPH assay

In the present study, the different concentrations of aqueous and methanol extract of leaves of *X. americana* were subjected to DPPH free radical scavenging assay. The antioxidant capacity of the extract was compared with ascorbic acid as standard antioxidant. Aqueous extract exhibited higher antioxidant activity than the methanol extract (Table 2).

**Table 2**

Percentage inhibition of DPPH free radical of *X. americana* extracts. %.

| Concentration | Inhibition | | |
|---|---|---|---|
| | Methanol extract | Aqueous extract | Standard |
| 10 µg | 63.48 ± 0.28 | 68.04 ± 0.35 | 69.06 ± 0.30 |
| 20 µg | 64.94 ± 0.28 | 70.29 ± 0.34 | 76.16 ± 0.29 |
| 30 µg | 66.51 ± 0.40 | 76.12 ± 0.35 | 78.44 ± 0.23 |
| 40 µg | 70.32 ± 0.51 | 78.07 ± 0.26 | 80.69 ± 0.23 |
| 50 µg | 73.96 ± 0.23 | 80.39 ± 0.34 | 83.87 ± 0.46 |

Values are mean ± SD, $n = 3$. Results were analyzed using One-way ANOVA.

### 3.7. In vitro anti-inflammatory assay

In the present study, known concentrations of aqueous and methanol extract of *X. americana* were subjected for anti-inflammatory activity on protein denaturation. The *in vitro* anti-inflammatory activity of the extract was comparable to the diclofenac sodium, a reference drug[32]. A significant difference in the inhibition of thermally induced protein denaturation was observed in case of aqueous and methanol extract when compared with standard drug at concentration of 100 µg/mL. Inhibition activity of methanol extract was comparable with diclofenac sodium whereas aqueous extract showed less inhibitory activity (Table 3).

**Table 3**

*In vitro* anti-inflammatory effect of *X. americana* extracts. %.

| Treatment | Concentration | Inhibition |
|---|---|---|
| Methanol extract | 100 µg/mL | 77.51 ± 2.25 |
| Aqueous extract | 100 µg/mL | 28.48 ± 2.11 |
| Diclofenac sodium | 100 µg/mL | 96.73 ± 1.09 |

Values are mean ± SD, $n = 3$. Results were analyzed using One-way ANOVA.

## 4. Discussion

In the present study, the phytochemical analysis helped in the detection of the secondary metabolites in the selected extracts of the plant. Phytoconstituents were evaluated by different quantitative biochemical tests. Natural antioxidants play an important role in the enhancement of antioxidant capacity of the blood plasma and help in the prevention of many diseases including cancer and diabetes[37]. Especially phenols and polyphenols are the main secondary metabolites present in a plant which acts as antioxidant or free radical scavengers[38]. Phenols are also known to have several biological activities such as anti-inflammatory, anti-tumor and antioxidant activities[39,40]. Natural antioxidants mainly come from plants in the form of phenolic compounds (flavonoids, phenolic acids and alcohols, stilbenes, tocopherols, tocotrienols), ascorbic acid and carotenoids[41]. The presence of phenols, flavonoids and tannins was observed in all solvent extracts except chloroform extract, whereas the presence of oils and fats was common in all solvent extracts. The study also reveals that the detected phytochemicals in leaves of *X. americana* are pharmaceutically important. These secondary metabolites are considered as natural source of antioxidant, antimicrobial and anti-inflammatory agents which have been shown to reduce the risk and progression of many diseases such as cancer and diabetes[42,43]. Especially phenolic compounds are high potent radical terminators by donating hydrogen atom to radical and inhibit lipid oxidation. Polyphenolic compounds present in plant

are known to have several biological activities like antitumor, anti-inflammatory, antioxidant and anticancer[44,45]. In the present work, aqueous extract of leaves of *X. americana* was found to be rich in total phenolic content (91.04 mg/g GAE).

Antioxidants are compounds that protect cells against the damaging effect of reactive oxygen species[28]. Recently natural antioxidants are in high demand because of their potenitial in health promotion and disease prevention. It is well known that antioxidant properties of the plant extracts cannot be evaluated by one single method due to the complex nature of phytochemicals. In the present work, three methods were used to evaluate total antioxidant capacity of aqueous and methanol extracts (FRAP, PM and DPPH).

FRAP assay is used to measure the reducing potential of the extracts. It depends on reduction reaction where Fe (III) gets converted and reduced to form Fe (II) and it forms the color complex of 510 nm absorption maxima by the action of polyphenols in the extracts. This method is used to determine reducing the capacity of extracts as well as standard. Absorption is directly proportional to reducing potential. Higher absorbance indicates the high reducing capacity of the antioxidants[29]. In the present work, aqueous extract shows higher antioxidant activity than the methanol extract (Figure 1) which was comparable to the standard ascorbic acid.

PM assay is calorimetric quantitative method which measures the reduction of Phosphate-Mo (VI) to Phosphate-Mo (V) by the sample and subsequent formation of a bluish green colored Phosphate-Mo (V) complex at acidic pH. It helps to investigate the reduction rate among antioxidant and molybdenum ligand. The PM method is routinely applied in the laboratory to evaluate the total antioxidant capacity of plant extracts. Absorbance is directly proportional to the antioxidant activity and gives reducing potential of plant extracts[30]. In the present study aqueous extract exhibited higher absorbance than the methanol extract (0.521 ± 0.003) (Figure 2).

DPPH free radical scavenging assay is an easy and widely used method for testing *in vitro* antioxidant activity of natural compounds or plant extracts[27]. In the present study, antioxidant activity of *X. americana* was evaluated using aqueous and methanol extract of plant and was compared with standard ascorbic acid. Results obtained showed that standard antioxidant had higher scavenging activity at all tested concentrations than the extracts. Among the extracts, aqueous extract exhibited higher activity than the methanol extract (68.04% ± 0.35%) (Table 2).

Significant difference was observed among antioxidant activities of evaluated extracts and aqueous extract came out as a superior total antioxidant capacity, with significant higher results in all performed assays.

Inflammation is common symptom among many chronic diseases. In the present market non-steroidal anti-inflammatory drugs are commonly used for the treatment of inflammatory diseases, but these are associated with many side effects like gastric irritation, ulcer, *etc*. [46]. Drugs from natural origin are promising and high in demand in management of inflammation conditions due to their fewer side effects and cost effective in nature. Hence in the present study simple and viable protein denaturation bioassay method was used to evaluate the anti-inflammatory activity of the plant extracts. Aqueous and methanol extract of *X. americana* leaves were subjected to anti-inflammatory assay with diclofenac sodium as reference drug. In comparison to the reference drug, methanol extract showed higher inhibitory activity (77.51% ± 2.25%) whereas the aqueous extract exhibited moderate inhibitory activity (Table 3).

The phytochemical analysis of different extracts of *X. americana* leaves showed the presence of broad spectrum of secondary metabolites which includes phenols, flavonoids, tannins, saponins, oils and fats. The above mentioned phytochemicals are known to have many biological activities such as antimicrobial, anti-inflammatory, anticancer and antioxidant[44,45]. The present study indicated that aqueous extract has higher total phenolic content than the other extracts. In the results obtained from different *in vitro* antioxidant assays, there is significant difference in antioxidant activity of extracts evaluated. Aqueous extract of leaves of *X. americana* has shown high significant antioxidant activity in all assays comparing with methanol extract. Whereas in case of anti-inflammatory activity, methanol extract shows higher activity than the aqueous extract, but aqueous extract showed moderate anti-inflammatory activity. Over viewing of the present study, it clearly shows that aqueous extract and methanol extract exhibited high potential antioxidant and anti-inflammatory activity, respectively. The results of our study suggest that *X. americana* leaves can be used as natural antioxidants to prevent the incidence and progression of many diseases. However, further detailed study is needed to isolate and purification of constituents from the plant for this antioxidant and anti-inflammatory activity.

## Conflict of interest statement

We declare that we have no conflict of interest.

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