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Original Research Article

HPLC-MS Analysis and evaluation of Antioxidant and Anti-Inflammatory Potential of *Cinnamomum cassia* Extract

Fatima Z. Labbaci[1]*, Hamza Belkhodja[2], Fatima Z. Elkadi[3], Ahmed Megharbi[4], Khadidja Belhouala[5]

[1] *Laboratory of Clinical and Metabolic Nutrition. Faculty of Nature and Life Sciences, University of Oran 1 Ahmed Ben Bella, Algeria.*
[2] *Laboratory of Bioconversion, Microbiology Engineering and Health Safety, University of Mustapha Stambouli, Mascara, 29000, Algeria.*
[3] *Department of Biology, University of Djilali Liabes, Sidi Bel Abbes, 22000, Algeria*
[4] *Faculty of science and technology, University of Relizane, Bourmadia, Relizane, 48000, Algeria.*
[5] *Laboratory Research on Biological Systems and Geomatics, University of Mustapha Stambouli, Mascara, 29000, Algeria*

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ABSTRACT

In the current decade, growing interest has been in using herbs and spices as antioxidants and anti-inflammatory agents. This study aims to investigate the chemical composition and evaluation of the antioxidant effect of *Cinnamomum cassia* extract. First, the chemical composition of *C. cassia* methanolic extract was determined using the HPLC-MS method. Then, the antioxidant potential was evaluated using the DPPH scavenging and total antioxidant capacity (TAC) methods. For the anti-inflammatory potential, three methods were performed: Membrane stabilization assay and inhibition of protein denaturation. five compounds were identified in the methanolic extract: Quercetin-O-hexoside, Quercetin-O-pentoside, Kaempferol-O-coumaroyl, Diosmin and Cinnamic acid. The methanolic extract exhibited the highest scavenging activity recording activity of 93.08 ± 0.05% at 1000 µg/ mL concentrations compared to the aqueous extract (77.03 ± 0.07%). The methanolic extract also presented the highest value of TAC (208.85 mg AAE/g) compared to the aqueous extract (199.56 ± 0.47 mg AAE/g). Methanolic extract presented the highest percentage for HRBC protection (87.27 ± 0.09%) at concentrations of 1000 µg/ mL. For the protein denaturation method, the aqueous extract showed an inhibition percentage of 32.86 ± 4.66% against methanolic extract (24.56 ± 4.11%). *Cinnamomum cassia* manifested an important free radical scavenging activity and anti-inflammatory potential. Therefore, it can be considered an alternative treatment against oxidative stress and biomolecules damages.

Keywords: *Cinnamomum cassia*, Oxidative stress, Inflammation, HPLC-MS

Introduction

Recently, a new concept has invaded the biological and medical sciences, "oxidative stress", where the cell no longer controls the excessive presence of toxic oxygen radicals.[1] The overproduction of the latter beyond the antioxidant capacities of biological systems gives rise to oxidative stress, which is implicated in the appearance of several diseases such as arteriosclerosis, cancer, inflammatory diseases, ischemia and ageing. Inflammation is usually a beneficial process: its purpose is to eliminate the pathogen and repair tissue damage. However, synthetic anti-inflammatory or antioxidant chemical substances are always accompanied by undesirable side effects, while phytochemicals are beneficial and without side effects.[2]
Utilizing various medicinal plant parts (such as roots, leaves, seeds, bark, fruits, or flowers) offers a solution. Medicinal and aromatic plants and even cultivated or spontaneous spice plants have exciting biological properties which find application in various fields, including medicine, pharmacy, cosmetology and agriculture.[3] Plant extracts from the *Cinnamomum cassia* species, used in Chinese medicine, are among the available natural resources.

*Corresponding author. E mail: Labbaci.fatimazohra@yahoo.fr
Tel: 00213657088763

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Cinnamomum cassia is a spice that comes from the bark of the cinnamon tree from the Lauraceae family. The species was commonly used in pharmacies, agrifoods, cosmetics, perfumery, biscuits, or culinary arts.[4] Several chemical investigations on this species have led to the isolation and identification of a large number of biologically active compounds such as eugenol, cinnamic aldehyde, cinnamyl acetate, O-methoxy-cinnamaldehyde, etc., possessing various pharmacological properties, namely anti-inflammatory, antimicrobial, anti-carcinogenic, antioxidant, tonic and hyperemic.[5,6]
After determining their chemical composition using HPLC analysis, the current study aims to assess the antioxidant and anti-inflammatory properties of aqueous and methanolic extracts of *Cinnamomum cassia* bark.

Materials and Methods

Plant

The plant *Cinnamomum cassia* was supplied by a herbalist in the Mascara region (Algeria) in March 2022 under the industry name "Calinuts". It was obtained from the province of Pleiku in Vietnam (13° 59′ north, 108° 00′ east). It was washed, dried at room temperature and finely ground into a fine powder using an electric mill (SM-450, MRC instruments, UK).

Preparation of polyphenolic extracts

Aqueous extraction was carried out by maceration, 10 g of the plant was mixed with 100 mL of distilled water. The mixture was filtered after magnetic stirring for 24 hours at room temperature. Then, the aqueous extract was dried. Methanolic extraction was carried out by decoction. A mixture of 10 g of plant and 100 mL of methanol (98%) was left in magnetic stirring at 40 °C for 24 hours. The extract was then filtered,

and the solvent was removed by evaporation using a rotary evaporator (Stuart RE300, Keison, UK). The quote was dried in an oven at 45°C and stored at four °C until use.[7]

HPLC analysis
Identification of *C. cassia* bioactive components was conducted on a Shimadzu-system (prominence I. LC-2030C 3D) equipped with a UVVIS Diode Array Detection (DAD) and a LCQ advantage max ion trap mass spectrometer. The separation was performed on ascentis express C18 column (15 cm x 4.6 mm). The flow rate was 0.8 mL/min and the injection volume was 5 µl. PDA wavelength range was 190–400 nm and the chromatograms were extracted at 280 nm (time constant: 0.025 s; sample frequency: 40 Hz). MS acquisition was performed using an ESI interface.

DPPH Free radical scavenging assay
The antioxidant activity of *C. cassia* has been determined according to the method of Mahendran et al.[8] It utilized DPPH as a relatively free radical that absorbed light with a visible wavelength of 517 nm. The DPPH solution was prepared by solubilizing 2.4 mg of DPPH in 100 mL of absolute methanol. 25 µL of extract at different concentrations were added to 975 µl of DPPH. A standard antioxidant (ascorbic acid) was also prepared under the same conditions to be a positive control. The negative control consisted of DPPH and methanol. The mixture was left in the dark for 30 minutes. The assay was performed using a spectrophotometer (Shimadzu UV-1280, Japan) at a wavelength of 517 nm. The percentage of antiradical activity was estimated according to equation (1).

Anti-radical activity [%] = [(A1-A2) / A1] x 100 (1)
A1: Absorbance of the negative control
A2: Absorbance in the presence of the extract

Total Antioxidant Capacity
The Total Antioxidant Capacity (TAC) (Phosphomolybdate test) was evaluated by the method of Prieto et al.[9] The approach was based on the reduction of molybdenum Mo (VI) presented in the form of molybdate MoO_4^{2-} to molybdenum Mo (V) MoO^{2+} ions in the presence of the sample to form a green to yellowish complex of phosphate/Mo (V) at acid pH. A volume of 0.3 mL extract was mixed with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Then the mixtures were incubated at 95°C for 90 min. After cooling, the absorbance of the solutions was measured at 695 nm using a spectrophotometer (Shimadzu UV-1280, Japan) against the blank, which contained 3 mL of the reagent solution and 0.3 ml of the methanol and incubated (POL-EKO-APARATURA, ProfiLab, Germany) as the sample. Total antioxidant capacity was expressed in milligram ascorbic acid equivalent per gram (mg AAE/g).

Membrane stabilization assay
The human red blood cell (HRBC) membrane stabilization method has been used to study vitro anti-inflammatory activity.[10] The blood was collected from a healthy human volunteer and mixed with an equal volume of Alsever solution (2 % dextrose, 0.8 % sodium citrate, 0.5 % citric acid and 0.42 % NaCl). All the blood samples were stored at 4 °C for 24 h before use. It was centrifuged (Benchtop Centrifuge, Hermle, Germany) at 2500 rpm for 5 min and the supernatant was removed. The cell suspension was washed with sterile saline solution (0.9 %w/v NaCl) and centrifuged at 2500 rpm for 5 min. This was repeated thrice until the supernatant was clear and colorless, and the packed cell volume was measured. The cellular component was reconstituted to a 40 % suspension (v/v) with phosphate-buffered saline (10 mM, pH 7.4) and was used in the assays. The extract was prepared (62,5 to 1000 µg/mL) using distilled water, and to each concentration, 1 mL of phosphate buffer, 2 mL hyposaline and 0.5 mL of HRBC suspension were added. It was incubated at 37 °C for 30 min and centrifuged at 3000 rpm for 20 min. The hemoglobin content of the supernatant solution was estimated using a spectrophotometer (Shimadzu UV-1280, Japan) at 560 nm. Ascorbic acid was used as the reference standard, and control was prepared by omitting the extracts. The percentage inhibition of hemolysis or membrane stabilization was calculated using the formula (2).
% Inhibition of hemolysis = 100 × {OD1 – OD2/OD1} (2)
OD1 = Optical density of hypotonic-buffered saline solution alone.
OD2 = Optical density of test sample in a hypotonic solution.

Inhibition of thermal denaturation of proteins (BSA)
In-vitro anti-inflammatory activity was performed using bovine serum albumin denaturation (BSA) method.[11] The reaction mixture (0.5 ml) consisted of 0.45 mL bovine serum albumin (5% aqueous solution) and 0.05 mL of extractor ibuprofen (125 to 1000 µg/mL). pH was adjusted at 6.3 using a small amount of 1 N HCl. The samples were incubated at 37°C for 20 minutes and then heated at 57°C for 30 minutes. After cooling the models, 2.5 mL phosphate buffer saline (pH 6.3) was added to each tube. Absorbance was measured using a spectrophotometer (Shimadzu UV-1280, Japan) at 660 nm; for the control test, 0.05 ml distilled water was used instead of extract, while the product control test lacked bovine serum albumin. The percentage inhibition of protein denaturation was calculated using the formula (3).
Protection (%) = 100- (OD sample/OD control) X 100 (3)

Statistical analysis
Effects between variables were tested using multi-way ANOVA. Conditions of use (normality, homoscedasticity and sample independence) have been verified. In addition, Statistical analysis was performed using R software (3.6.3).

Results and Discussion

HPLC analysis
The chromatogram was presented in Figure 1. A total of 5 compounds were identified in the methanolic extract of *C. cassia*: Quercetin-O-hexoside, Quercetin-O-pentoxide, Kaempferol-O-coumaroyl, Diosmin and Cinnamic acid. Mainly, Kaempferol-O-coumaroyl (a derivative of coumarin) appeared after 33 minutes with a very high content (21.9%) compared to the other compounds, followed by Quercetin-O-pentoxide (14.64%), which occurred after 32.36 minutes, while standard cinnamic acid, which was known to be present in *C. cassia*, was eluted at 36.16 minutes with a percentage of 0.56% as shown in Table 1.
These results agreed with those of Hussain *et al.*[12] where the HPLC chromatogram showed the presence of quercetin, chlorogenic acid, cinnamaldehyde and kaempferol in cinnamon extract. Results showed that kaempferol was present in the highest concentration while cinnamaldehyde was presented in the lowest concentrations. According to the findings of Rahayu *et al.*[13] LC-MS study of a *C. cassia* extract, kaempferol, procyanidin dimer, procyanidin trimer, and one terpenoid, linalool, were all discovered in high concentrations. Derivatives of kaempferol and procyanidin were frequently utilized as antioxidant and anti-ageing cosmetic ingredients.[14,15] According to Akkawi *et al.*[16] coumarin and cinnamic acid were phenolic components in the *C. cassia* extract's phenolic profile. According to Wang *et al.*[17] the LC-UV chromatograms of samples showed that cinnamaldehyde was a dominating component in the species *C. verum*, *C. burmannii*, *C. loureiroi*, and *C. cassia*.
The results of the developed UPLC-UV/MS method for quantitative and qualitative analysis of coumarin derivates showed that indeed cinnamon contained only traces of coumarin (Woehrlin *et al.*[18] Coumarin derivatives have been used in several countries for the treatment of edemas, renal cell carcinoma, and other tumors. [19] The most significant cinnamon components responsible for many of the biological actions associated with cinnamon were found in the bark of numerous cinnamon species using chromatographic methods. Among these were cinnamic acid and a few lesser-presented substances, including kaempferol, coumarin, and quercetin.[20, 21] Previous studies have shown that cinnamic acid affects the in vitro growth of *Plasmodium falciparum* and the permeability of the membrane of malaria-infected erythrocytes.[22] The administration of quercetin to animals undergoing acute stress suppressed activation of the hypothalamic-pituitary-adrenal (HPA) axis, which was a significant component of the stress response and played a major role in the aetiology of stress-related pathologies.[23]

Table 1: Characterization of the methanolic extract of *C. cassia*

RT	Lambda max	Compound	Area	% area
26.394	197/211/668/271/692	Quercetin-O-hexoside	3743534	3.403
32.863	288/299/197/639/792	Quercetin-O-pentoside	16109318	14.645
33.006	288/224/197/668/636	Kaempferol-O-coumaroyl	24090122	21.900
34.952	677/276/525/381/464	Diosmin	1102383	1.002
36.167	676/212/787/551/334	Cinnamic acid	616606	0.561

Table 2: IC_{50} and TAC values of *C. cassia* and Ascorbic acid

	IC_{50} (mg/ml)	TAC (mg AAE/g)
Methanolic	0.038	208,85
Aqueous	0.06	199.56 ± 0.47
Ascorbic acid	0.035	

DPPH Free radical scavenging activity

In the DPPH scavenging assay, the methanolic extract exhibited the highest scavenging activity, recording activity of 93.08 ± 0,05% at concentrations of 1000 µg/ mL when compared against aqueous extract (77.03 ± 0.07%). It was noted that ascorbic acid presented the highest effect compared to both extracts. The inhibition percentage appeared proportional to sample concentrations (Figure 2). Free radical scavenging activities of the various extracts were presented as IC_{50} and TAC values in Table 2. For the test of total antioxidant capacity, methanolic extract also gave the highest value of TAC (208.85mg AAE/g) compared to aqueous extract (199.56 ± 0,47 mg AAE/g).

There were significant differences in antioxidant activity among the different extraction solvents. This suggested, in an indirect manner, that the solvent type, which affected the concentration of total phenolic and flavonoids responsible for the antioxidant potential, played a significant role in the antioxidant activity of the various extracts.[24] In the study of Singh *et al*.[25] methanolic, ethanolic, and acetone extracts of *C. cassia* were tested for DPPH free radical scavenging. The ethanolic extract exhibited the highest scavenging activity when compared to other extracts, recording activity of 88.26% ± 0,09% at concentrations of 1000 µg/ml, and the results were comparable with the same concentration of ascorbic acid (87,66 ± 0,52%). The methanolic and acetone extracts had the 63.08 ± 0.16% and 70.79 ± 0.20% scavenging activity at a concentration of 1000 µg/mL, respectively. DPPH findings followed the results of Abeysekera *et al*.[26] who reported that the methanolic extract showed more free radical scavenging activity (60.49 ± 0.48%). Prakash *et al*.[27] said that the methanolic extract of Cinnamomum bark effectively scavenged reactive oxygenic species, including superoxide anions, hydroxyl radicals, and other free radicals, under in vitro conditions.

While the results of Eweys *et al*.[28] indicated that the DPPH radical scavenging activity was recorded as 67.63% for lyophilized cinnamon extract. Yang *et al*.[24] demonstrated that the antioxidant activity of the organic extract of *C. cassia* presented an IC50 value of 72 µg/ml. Other researchers also reported that the extract of Indonesian cinnamon bark possessed antioxidant potential with IC_{50} values ranging from 75.48 µg/mL to 136.88 µg/mL.[29] According to Yang *et al*.[30] Cinnamomum bark, in addition to cinnamaldehyde, benzaldehyde, and eugenol, was composed of a relatively high rate of phenolic compounds, which played an antioxidant role and acted as a potent hydrogen donor. Phenolic substances and flavonoids were effective electron donors due to the hydroxyl groups that directly contributed to free radical scavenging and antioxidant activity.[31, 32] Varalakshmi *et al*.[33] mentioned that Cinnamomum bark was a potential source of natural antioxidants and could be used in all preparations to fight against free radicals, mediators of oxidative damage. According to Şimşek *et al*.[34] Malondialdehyde (MDA) levels in rats' liver, heart, and kidneys under heat stress considerably decreased when cinnamon extracts were added to their diet. Moselhy and Junbi[35] explained this by activating antioxidant mechanisms (superoxide dismutase) in cells and inhibiting the lipid peroxidation reaction chain.

Membrane stabilization assay

The anti-hemolytic effect of *C. cassia* extracts was evaluated against hypotonic hemolysis to study the stabilization of the red blood cell membrane against osmotic stress. For comparative purposes, ascorbic acid was used. Figure 3 presented the protection percentage of the extracts of *C. cassia* against hypotonic hemolysis.

Results showed that the proportion of membrane stabilization depended on extract concentration. Methanolic extract presented the highest percentage for HRBC protection (87.27 ± 0,09%) at 1000 µg/ mL concentrations compared to aqueous extract (64.41 ± 0,05%). It was noted that ascorbic acid presented the highest effect (98.71± 0,05%) compared to both extracts. From the results of Shalihah *et al*.[36] the membrane stabilization activity test showed that cinnamon extract had an IC_{50} of 84.45 ± 3.55 µg/mL, which was higher than the IC_{50} value of diclofenac sodium (standard) (64.505 ± 1.272µg/mL). According to the results of Suransh *et al*.[37] the cinnamon extract demonstrated a significant anti-inflammatory activity at all the doses tested compared to the control, and the percentage membrane stabilization shows an increase with the increase in the concentration of the extract. The extract of cinnamon bark contained some constituents such as flavonoids, saponins, triterpenoids, steroids, and tannins. Tannins and saponin compounds stabilized membranes by binding to cations.[38] It was demonstrated that free radical inhibitors could stabilize erythrocyte membranes from hypotonic solutions.[39] The erythrocyte membrane was analogous to the lysosomal membrane, and its stabilization implied that the extract might as well stabilize the lysosomal membrane. Stabilizing the lysosomal membrane was important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophils such as bactericidal enzymes and proteases, which caused further tissue inflammation and damage upon extracellular release.[40]

Inhibition of thermal denaturation of proteins (BSA)

When the temperature is raised, it can increase the kinetic energy and cause the molecules that make up the protein o move or vibrate very quickly, damaging the molecule. The protein denaturation process takes place permanently and does not change. A denatured protein will decrease its liquid solubility to settle.[41] Figure 4 represented the percentage inhibition of BSA denaturation by the extracts of *C. cassia* compared to ibuprofen. At 1000 µg/ ml concentrations, aqueous extract registered an inhibition percentage of 32.86 ± 4.66% against methanolic extract (24.56 ± 4.11%). At the same time, ibuprofen showed the highest rate with a value of 91.59 ± 0.04%.

From the results of Shalihah *et al*.[36] the cinnamon extract has protein denaturation inhibitory activity, which can be seen from the IC_{50} value of 57.412±0.718µg/mL. This value is still higher than the IC50 of diclofenac sodium (14.655±1.131µg/ml). Results of Suransh *et al*.[37] showed that the percentage of inhibition of cinnamon extract was increased with the increase in extract concentration. The percentage inhibition was 32.81 at 100 µg/ml; 49.88 at 200 µg/ml;63.13 at 400 µg/ml; 76.73 at 600 µg/ml and 85.71 at 800 µg/ml.

Many studies have been conducted on the effectiveness of extracts or compounds derived from *C. cassia* in inhibiting inflammation effects.[42,43] Research by Rao and Gan on *Cinnamomum cassia* showed an inhibitory effect on nitric oxide production by inhibiting the activation of factor NF-κB.[44] This was in line with other research showing that cinnamon's active ingredients were crucial in preventing induced inflammation.[45] According to Budiastuti *et al*.[46], the extract of Cinnamomum bark has been shown to have an acute anti-inflammatory

effect in experimental animal rats. Flavonoids were known to have anti-inflammatory activity with the mechanism of action inhibiting prostaglandin formation through the cyclooxygenase (COX) and lipoxygenase-5 (LOX-5) pathways.[47] Flavonoid and saponins compounds are anti-inflammatory because they inhibit inflammatory mediators and free radicals.[48]

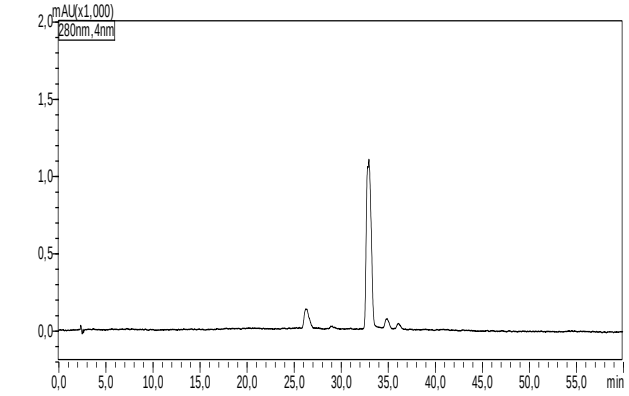

Figure 1: HPLC-ESI/MS base peak chromatogram of *C. Cassia* methanolic extract.

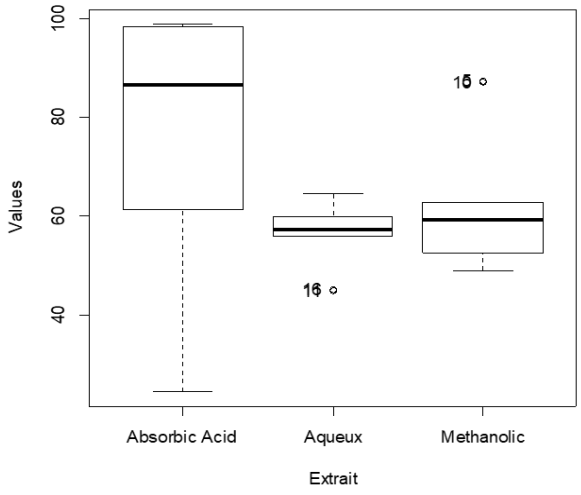

Figure 2: Inhibition percentage of *C. cassia* and Ascorbic acid.

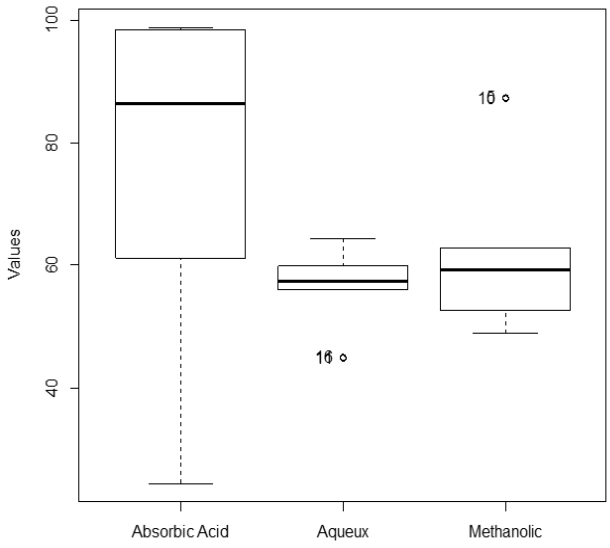

Figure 3: Membrane stabilization percentage of *C. cassia* extract.

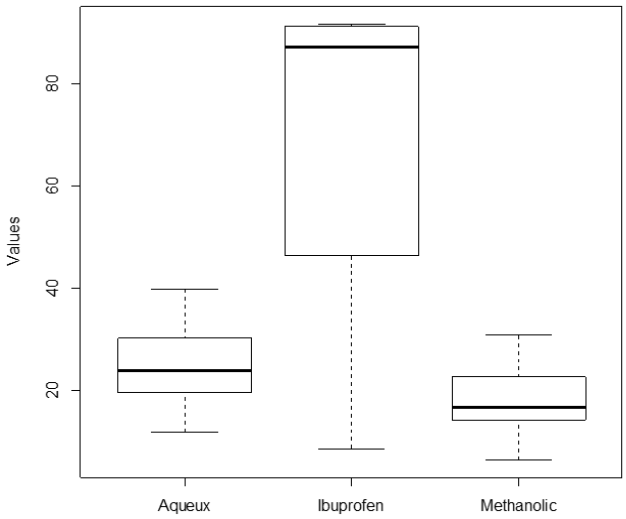

Figure 4: Inhibition percentage of *C. cassia* extract.

Conclusion

Cinnamomum cassia manifested an interessant free radical scavenging activity and anti-inflammatory potential. Therefore, it can be considered an alternative treatment against oxidant stress and biomolecule damage.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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