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Inhibitory Activity of Methanolic Extracts of Six (06) Medicinal Plants on the Growth of *Escherichia coli* Resistant to Imipenem by Production of Carbapenemase Isolated at the Tengandogo Hospital in Ouagadougou

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Abstract

The fight against bacterial infections is essentially based on the use of antibiotics. Unfortunately, bacteria have developed resistance mechanisms against antibiotics, particularly those of the β-lactam family. Thus, this study aims to evaluate the inhibitory activity of six medicinal plants resulting from an ethnobotanical investigation of these formidable bacteria. Plant methanol extracts were obtained using Soxhlet. The photometric method was used to measure the absorbances of bacterial (*Escherichia coli*) suspensions and for the phytochemical assay of plant extracts. *Terminalia laxiflora* (*T. laxiflora*) Engl. & Diels proves to be the most effective plant with an optical density (OD) of 0.322 against 1.133 of the positive control and 0.901 of the antibiotic. The total phenolic content of the methanolic extract of *T. laxiflora* Engl. & Diels was 68.45 ± 0.11 mg EAG/100 mg extract. The total flavonoids were 2.71 ± 0.09 mg EQ/100mg of extract. The antioxidant content of the extract (820.46 ± 0.17 µmol EAA/g of extract) is higher than the contents of quercetin (646.85 µmol EAA/g of extract) and trolox (765.99 µmol EAA/g of extract). As for the FRAP method, the extract gave a content (4190.93 ± 1.79 µmol EAA/g of extract) which exceeds that of quercetin (5991.29 ± 1.33 µmol EAA/g of extract). At the level of

the ABTS method, the extract still gave a value (11935.45 ±3.18 µmol EAA/g of extract) higher than trolox (8137.61 ± 4.04 µmol EAA/g of extract).

Keywords

Medicinal Plants, *Escherichia coli*, Carbapenemase, Tengandogo Hospital

1. Introduction

The fight against bacterial infections is essentially based on the use of antibiotics [1]. Unfortunately, bacteria have developed sophisticated resistance mechanisms to fight against the lethal effects of antibiotics, including those of the β-lactam family [2]. The misuse of antibiotics or their inappropriate use is mainly responsible for the emergence of antibiotic resistance [3]. The development of each new compound follows its resistance [2]. Resistance to β-lactams and more particularly the emergence of β-lactamases increasingly described throughout the world constitutes a real public health problem. Indeed, carbapenems, which are very often the last active molecules in the therapeutic arsenal available to fight multiresistant bacteria, are also affected [4]. A study showed that *Escherichia coli* (*E. coli*) had the highest distribution frequency of isolated ESBL-producing pathogens according to species and with a prevalence of 65.88% [5]. Another study revealed that *E. coli* had the highest prevalence of resistance among the strains studied [6] [7].

Today, the resistance of bacteria to antibiotics, in particular carbapenems, has become one of the most important problems in anti-infective therapies in the world and in the pharmaceutical industry, especially since it appears after the development of each medication.

According to a WHO report, the antibiotic resistance crisis is estimated at 300 million deaths by the year 2050 if nothing is done, with a loss of up to 100 billion dollars (64 billion sterling) for the global economy.

In Burkina Faso, investigative works have carried out carbapenemases in several samples such as from diarrhea and from chickens consumed [7] [8].

Efforts are being made in the search for new antibiotics, but the problem remains [1]. This makes essential the search for medicinal plants that inhibit carbapenemase produced by pathogenic bacteria.

Medicinal plants offer a variety of metabolites with microbial disease control potential [9]. Moreover, one of the strategies to fight against bacterial resistance is the search for enzyme inhibitors [10].

One study revealed the activity of Terminalia glaucescens, mangifera indica and Mitracarpus villosus on carbapenem-resistant enterobacteria [11].

Extracts of medicinal plants such as *Combretum micranthum* and *Boswellia dalzielii* were used in combination with β-lactam antibiotics. These extracts were effective against strains resistant to β-lactam antibiotics (cephalosporins), and the work was patented [12].

Thus, do the plants used by traditional health practitioners have properties that inhibit the carbapenemase produced by *E. coli*?

It is to answer this question that six (06) plants from an ethnobotanical survey were studied for *E. coli* isolated from patients hospitalized at the Tengandogo hospital in Ouagadougou.

The general objective related to this study was to search for medicinal plants with carbapenemase inhibiting properties.

2. Material and Methods

2.1. Study Framework

The samples were collected in the Classified Forest of Dindéresso in Bobo-Dioulasso.

The extraction work and phytochemical assays were carried out at the Laboratory of project N˚BKF5021 within UNB. The biological activities of the extracts were carried out at the Pietro Aninigoni Biomolecular Research Center (CERBA) in Ouagadougou.

2.2. Methodology

2.2.1. Plant Selection

The interview took place from September 4 to 30, 2020, using a semi-structured questionnaire. Traditional practitioners were interviewed in the local language (Dioula and Mooré); and the survey focused on the following main points: socio-demographic characteristics.

knowledge of infectious diseases such as diarrheal diseases, urinary tract infections, pneumonia, meningitis and difficult-to-transmit wounds; medicinal plant parts used; and pharmacological characteristics.

After the survey, the sheets were grouped by pathology [13]. The plants have been arranged with their citation according to the use in ethno-therapy for diseases. The frequency of citations was calculated according to the following formula: $F = Nc/Nt \times 100$.

With F: Frequency of citation, Nc: Number of citations for the plant considered, Nt: Total number of citations for all the plants.

The collection of plants was made taking into account the frequency of citation and their availability in the collection area.

2.2.2. Processing of Plant Samples

1) Grinding plant samples

After a minimum of 14 days of drying, the plants were pulverized using an aluminium mortar and pestle to obtain powdered samples. Each pulverized sample is sieved and then placed in freezer bags and then properly labelled. These samples are kept for later use.

2) Extraction

The extraction was done by a Soxhlet type device. Indeed 15 g of plant powder

was weighed and then put in the cartridge. A volume of 200 mL of methanol was measured and placed in a 250 mL flask. Then the temperature was set at 65˚C for an operation of at least 4 hours (not exceeding 06 hours). After this operation the extract is removed and put in a Petri dish under ventilation to allow evaporation of the solvent.

3) Extraction

One hundred (100) mg of the extract was weighed and added to a volume of 1 mL of 99% DMSO. The mixture was homogenized to obtain a concentration of 100 mg/mL. This previous solution was supplemented with 1% DMSO until reaching 10 mL. The final solution had a concentration of 10,000 µg/mL.

2.2.3. Bacterial Strains

The bacterial strains were pathogenic strains and from previous studies. They come from samples taken from patients hospitalized at the Tengandogo hospital in Ouagadougou and are resistant to imipenem. They were stored in LB medium with glycerol at −80˚C. The awakening of the strain was done by suspending the bacterial strains in LB medium without glycerol and incubated for 24 h at 37˚C.

After awakening, 10 µL of each bacterial strain was placed in 5 mL of Luria Bertani (LB) medium and homogenized by vortexing. Each strain was then inoculated onto Miller Hinton (MH) solid medium. Antibiograms were then performed using 30 µL/mL imipenem discs and incubated again for 24 h at 37˚C. Inhibition diameters were measured to verify strain resistance. A colony isolated from one of the resistant Petri dishes was selected and suspended in 5 mL LB for further testing.

2.2.4. Preparation of Culture Media

MH medium was prepared by dissolving 38 g of MH powder in 1 L of distilled water. LB medium was prepared by putting 25 g of LB powder in 1 L of distilled water. The pH of the media was adjusted to 7. The medium and all the material to be used during the manipulations were sterilized in an autoclave at 121˚C for 15 minutes. This medium was poured into the Petri dishes which were used for the antibiogram test. The antibiogram was performed on solid medium using the disc diffusion method following the recommendations of the antibiogram committee of the French Society of Microbiology (SFM). The manipulations were carried out under sterile conditions.

2.2.5. Inhibitory Activity of Plant Extracts

The plant extracts were tested on the strain in order to determine which plant has the best activity on the carbapenemases, enzymes produced by the bacteria.

From the formula $C_iV_i = C_fV_f$, the volumes of plant extracts (10,000 µg/mL), antibiotic (50 µg/mL), and LB medium were adjusted to a final volume of 5 mL. The quantity of bacterial suspensions taken from each was 20 µL.

Inoculations on liquid medium were carried out according to the following conditions:

- T1: Negative control = liquid LB alone

- T2: Positive control = LB + strain
- T3: LB + strain + 50 µg/mL of imipenem alone
- T4: LB + strain + 50 µg/mL of plant extract alone
- T5: LB + strain + 50 µg/mL of imipenem + 50 µg/mL of plant extract

Before and after 4 hours of incubation at 37˚C with stirring, 300 µl of each solution was taken and placed in reading plates for reading the ODs at 600 nm according to a plate plan. Readings were performed in triplicate. GEN5 1.07 software was used for absorbance readings.

2.2.6. Phytochemical Analysis

After biological testing of all plant extracts, *T. laxiflora* Engl. & Diels proves to be the most effective and was selected for the phytochemical assays.

1) Assay of total polyphenols

The quantification of the total polyphenols of the extract was carried out using the Folin Ciocalteu reagent [14].

Procedure: after 100th dilution of a stock solution of 10 mg/mL of each extract with distilled water, 125 µL of each extract at 0.1 mg/mL were mixed with 625 µL of the Folin Ciocalteu reagent at 0.2 N. This mixture was incubated for 5 minutes, then 500 µL of an aqueous solution of sodium carbonate (75 $mg{\cdot}mL^{-1}$) was added. After 2 h of incubation, the absorbances were measured at 760 nm against a blank consisting of water using a spectrophotometer. The standard calibration curve was drawn using gallic acid (0 - 200 mg/L).

The average of the readings was used and the results were expressed in mg of gallic acid equivalents (GAE)/100 mg of extract.

2) Assay of total flavonoids

The total flavonoids of each extract were assayed using the colorimetric method [15].

Procedure: the same dilution as before was prepared with methanol. Thus, 625 µL of each extract (0.1 mg/mL) was mixed with 625 µL of aluminum trichloride (AlCl3) in 625 µL of methanol (2%). The absorbances read at 415 nm were taken after 10 min against a blank consisting of 625 µL of extracts and 625 µL of methanol without $AlCl_3$. Quercetin (0 - 50 mg/L) was used as a reference compound to produce the calibration curve. The average of three readings was used and the results were expressed as mg quercetin equivalent (EQ)/100 mg extract [16].

3) Determination of antiradical and antioxidant content

ABTS radical discoloration test

The ABTS radical bleaching test by the extracts has been evaluated [16].

Procedure: a methanolic dilution to 100th of the extracts (10 mg/mL) was used. Thus, 10 µL of each diluted extract (0.1 $mg{\cdot}mL^{-1}$) were reacted with 990 µL of $ABTS^{+}$ solution and then incubated in the dark. Absorbance was taken 15 min after initial mixing at 734 nm. Ascorbic acid (0 - 10 µg/mL) was used as a standard. Trolox and quercetin standards were taken as reference radical scavengers. The free radical scavenging capacity was expressed in µmol of ascorbic acid equivalent (EAA)/g of extract.

DPPH radical scavenging test

The DPPH radical scavenging capacity of the extracts was evaluated [17].

Procedure: The methanol dilution previously described was used. 375 µL of each extract was mixed with 750 µL of the DPPH solution (0.02 mg/mL of methanol) for the test and with only 750 µL of methanol for the blank. After incubation for 15 min at ambient laboratory temperature, the absorbance was read against the blank at 517 nm. The test was carried out in triplicate. The standard calibration curve was drawn using ascorbic acid (0 - 10 µg/mL). Trolox and quercetin standards were taken as reference scavengers. The mean radical scavenging activity was expressed in µmol of ascorbic acid equivalent (EAA)/g of extract.

Iron (III) reduction activity to Iron (II) FRAP

The FRAP test of extracts was performed to assess the ability of extracts to reduce Iron (III) to Iron (II) [17].

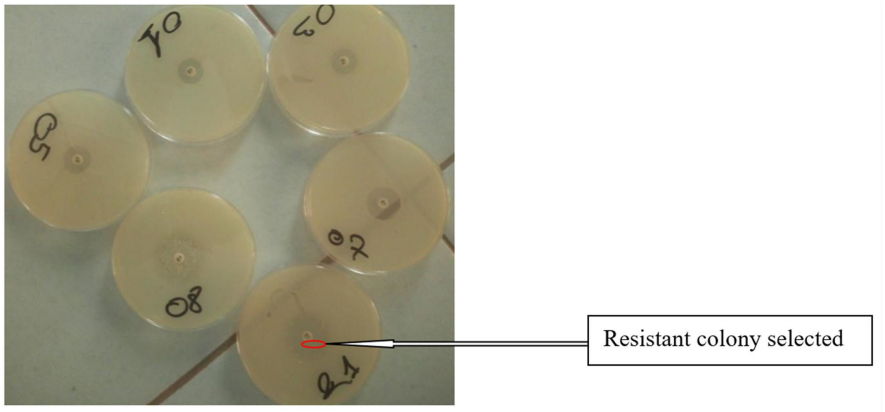
Procedure: The previous dilution with distilled water was used. Thus, 0.5 mL of each extract (100 µg·mL^{-1}) was mixed with 1.25 mL of phosphate buffer (0.2 M, pH 6.6) and 1.25 mL of aqueous hexacyanoferrate solution potassium [$K_3Fe(CN)_6$] (1%). After 30 min of incubation at 50˚C, 1.25 mL of trichloroacetic acid (10%) was added and the mixture was centrifuged at 3000 rpm for 10 min. Then, 0.625 mL of the supernatant was mixed with 0.625 mL of distilled water and 0.125 mL of a freshly prepared $FeCl_3$ solution (0.1%). Absorbances were read at 700 nm. Trolox and quercetin standards were taken as reference ferric reducers. The determination of the reducing activity of iron (III) was carried out in triplets and expressed in µmol of equivalent ascorbic acid (EAA)/g of extract.

2.3. Data Processing

Microsoft Excel 2013 spreadsheet was used for data entry and processing.

3. Results

3.1. Bacterial Strain Selection



Picture 1. strain susceptibility test to imipenem discs.

A strain of *E. coli* from a visceral sample pus and resistant to imipenem was se-

lected (Picture 1).

3.2. Inhibition Test

Among the extracts tested, 06 extracts showed their ability to inhibit carbapenemases produced by bacteria. The optical densities of the samples contained in tube number 5 (T_5) decreased compared to those of the positive controls (strain alone). In particular, the OD of T5 with T. laxiflora Engl. & Diels (the most active extract) was 0.322 against 1.133 of the positive control and 0.901 with the antibiotic alone.

Figure 1 shows the graphical representation of the different ODs measured. The six (06) extracts presented attempt to reduce the optical density of the samples contained in tube number 5 (antibiotic + plant extract) compared to the positive control (LB + strain). However, the extract that reduced the optical density the most was the extract of *T. laxiflora* Engl. & Diels (Combretaceae). The extract therefore has the best inhibitory activity.

LB = Luria Bertani nutrient medium, ATB = antibiotic, ECH32 = *T. laxiflora* Engl. & Diels, ECH47 = Bark of the trunk of *Pteleopsis suberosa* Engl. & Diels, ECH17 = Root of *Dicrostachys cinerea* (L.) Wight & Arn., ECH37 = Trunk bark of *Ficus ingens* (Miq.) Miq., ECH46 = Trunk bark of *Sclerocarya birrea* (A. Rich.) Hochst., ECH43 = Bark of the trunk of *Diospyros mespiliformis* Hochst. ex A.DC., OD = Optical Densities.

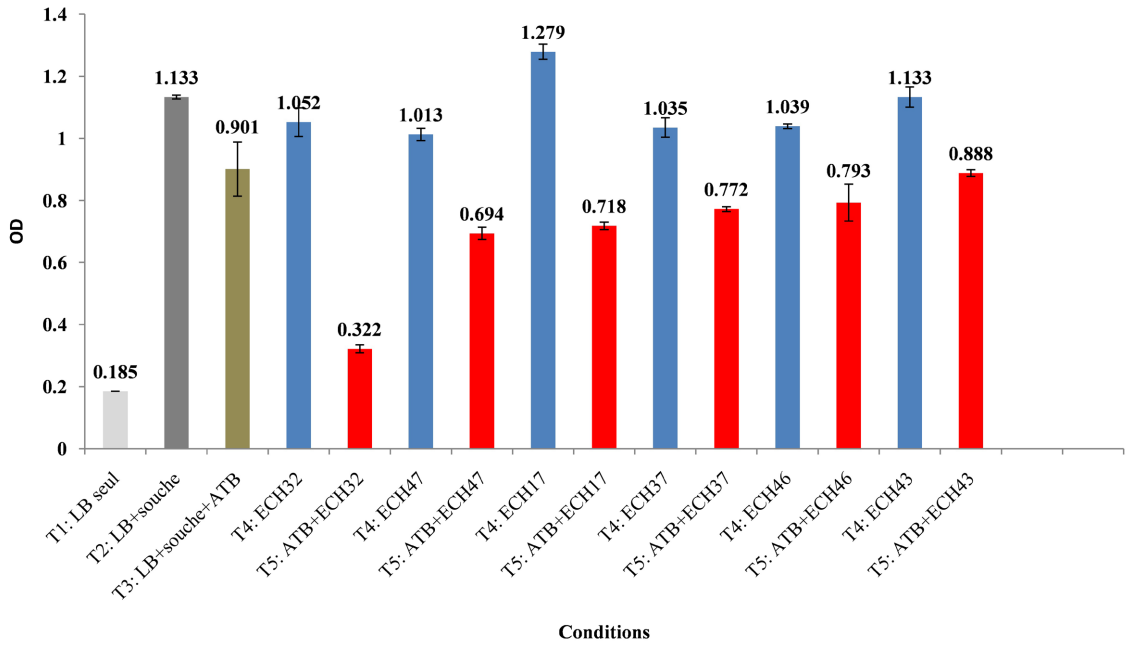


Figure 1. Optical densities of different study conditions.

3.3. Content of Total Polyphenols and Flavonoids

The total phenolic content of the methanolic extract of *T. laxiflora* Engl. & Diels was 68.45 ± 0.11 mg EAG/100 mg extract. That of the total flavonoids were 2.71

± 0.09 mg EQ/100mg of extract (Table 1).

Table 1. Content of total phenolics and flavonoids.

Extract	Total polyphenols (mg EAG/100 mg extract)	Total flavonoids (mg EQ/100mg extract)
T. laxiflora Engl. & Diels	68.45 ± 0.11	2.71 ± 0.09

3.4. Antioxidant Activities

The figure below (Figure 2) compares the antioxidant content of *T. laxiflora* and that of the standards. By the DPPH method, the antioxidant content of the extract (820.46 ± 0.17 µmol EAA/g of extract) is higher than the content of quercetin (646.85 µmol EAA/g of extract) and trolox (765.99 µmol EAA/g of extract). As for the FRAP method, the extract gave a content (4190.93 ± 1.79 µmol EAA/g of extract) which exceeds that of quercetin (2211.24 ± 0.64 µmol EAA/g of extract). At the level of the ABTS method, the extract still gave a value (11935.45 ± 3.18 µmol EAA/g of extract) higher than trolox (8137.61 ± 4.04 µmol EAA/g of extract).

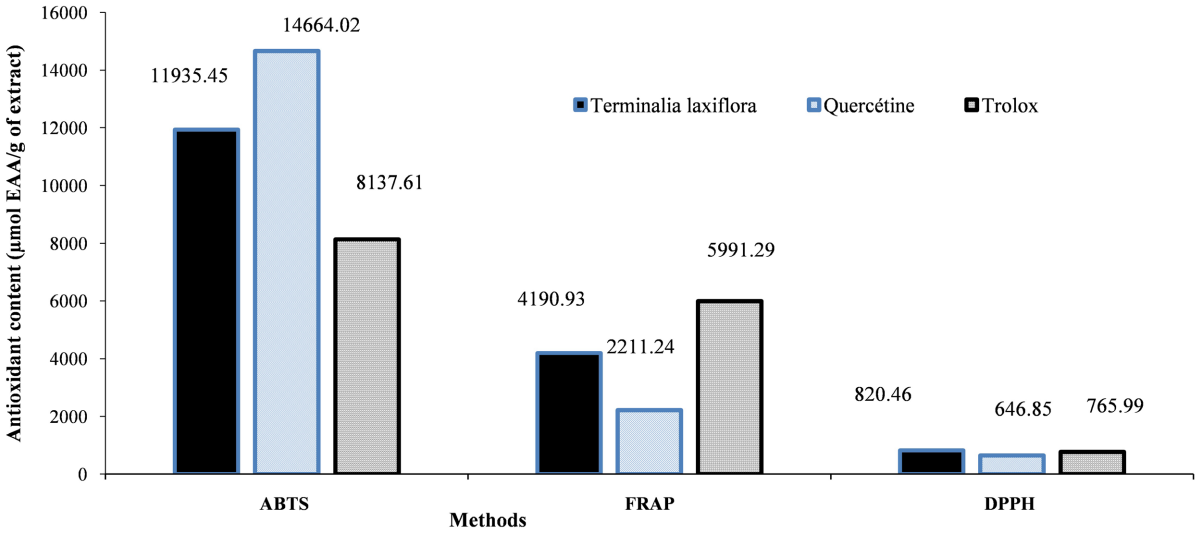


Figure 2. comparison between the antioxidant content of the extract and that of the standards.

4. Discussion

More and more *E. coli* is becoming resistant to imipenem. Its resistance to imipenem has been shown in Togo with a percentage of 0.6% [18]. The excessive use of antibiotics or their inappropriate use could explain the resistance of *E. coli* to carbapenems. This same idea is shared by some authors [3]. This species of bacteria is even more represented in pathologies caused by ESBL-producing bacteria [5].

In view of the optical densities measured, each of the six (06) extracts had an effect on the resistant strain of *E. coli* from a visceral sample pus by lowering the optical density of the bacterial suspension. The optical density of the solution increases linearly with the number of bacteria and therefore a decrease reflects the

drop in the number of bacteria [19].

This justifies that the drop in the OD of the solution (*T. laxiflora* + IMP) in our results indicates a decrease in the number of *E. coli* resistant to imipenem because the OD is high for the extract and imipenem alone. The methanolic extract of each plant would act, in the presence of imipenem, on *E. coli* to inhibit carbapenemases in order to allow imipenem to play its bactericidal role. Among these methanolic plant extracts, the extract of *T. laxiflora* Engl. & Diels gave better activity on the bacterial strain by decreasing the OD density from 0.901 to 0.322, thus reflecting a negative effect on the *E. coli* strain. A study carried out in Nigeria showed that another species of the same family (*Terminalia glaucescens*) was also active on carbapenemase-producing bacteria [11]. Furthermore, the methanolic extract of *T. laxiflora* Engl. & Diels had shown good activity in inhibiting Glucosyltransferase (GTF), the enzyme responsible for the synthesis of polysaccharides in bacteria [20].

The dosage of the methanolic extract from the bark of *T. laxiflora* Engl. & Diels gave high values. These results could explain the ability of the extract to inhibit carbapenemases. Indeed, studies have shown that *T. laxiflora* Engl. & Diels was rich in polyphenols [21] [22].

The high antioxidant content of the methanolic extracts from the bark of *T. laxiflora* Engl. & Diels could be due to the presence of total phenolics other than flavonoids. There is a positive link between antioxidant activity and total phenolic content [23] [24]. Antioxidant potential by the DPPH, ABTS and FRAP methods is high. By the DPPH method, the values are higher than those obtained in a study with petroleum ether, Chloroform, Ethyl acetate and Aqueous extracts. According to authors, phenolics seem to be efficient donors of hydrogen to the radical DPPH [25]. These results are in agreement with other research which showed that *T. laxiflora* Engl. & Diels was rich in polyphenols with a strong reduction of the DPPH radical (IC_{50} = 4.86 µM) [26].

Therapeutically, these compounds form the basis of the active ingredients found in medicinal plants. They would also play a role in antibacterial activity [27].

The fact that these plants are not administered in controlled doses could be a limiting factor in their use. Future studies will assess the toxicity of these plants in vivo. Toxicity studies were also carried out using the brine shrimp Artemia salina standard method. The results showed that *T. laxiflora* is not toxic.

5. Conclusion

The aim of the study was to find plants with properties that inhibit carbapenemase produced by bacteria. To this end, the ethnobotanical survey revealed plants used in the treatment of diseases caused by these bacteria. Of the six (06) plant extracts that had carbapenemase inhibiting properties, the trunk bark of *T. laxiflora* Engl. & Diels had a good inhibitory effect. Moreover, the extract of the latter was rich in total phenolics and had a high content of antioxidants by the methods of ABTS, FRAP and DPPH.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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