# Phytochemical Screening and Biological Activities of *Opilia amentacea* Roxb. (Opiliaceae)

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*Article*

# Phytochemical Screening and Biological Activities of *Opilia amentacea* Roxb. (Opiliaceae)

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**Abstract:** Dermatoses are essentially due to infectious or free radical aggression, immunoallergic disorders, or can be secondary to general diseases. To investigate new antimicrobial sources against dermatoses, *Opilia amentacea* crude ethanol extracts and fractions were subjected to phytochemical screening and tested for antioxidant and antimicrobial activities on Gram-positive cocci, Gram negative bacilli and fungi. Phytochemical screening revealed secondary metabolites such as sterols, triterpenes, flavonoids and tannins. Leaves and stem bark of *Opilia amentacea* exhibited the highest total phenolic contain (65,92 ± 0,93 and 65,71 ± 0,57 mg GAE/g DW respectivily) and flavonoids (38,8 ± 1,05 and 26,81 ± 0,19mg QE/g DW respectively). Dichloromethane fractions of leaves (FDFe) and stem bark (FDET) exhibited the best antioxidant activity with FRAP values of 741,15 and 662,84 mMol AAE/g and IC50 values of ABTS of 88,40 and 36,85 µg/mL respectively. These fractions were also the most active on Gram positive bacilli while Hexane leaves (FHFe) and hexane root bark (FHER) fractions exhibited the best antifungal activity against *Candida tropicalis* at MIC values of 0.23 mg/mL and 0.43 mg/mL respectively. Gram-negative bacteria were particularly resistant. This study constitutes a solid scientific basis that could justify the traditional uses of *Opilia amentacea* as an antidermatosis plant.

**Keywords:** *Opilia amentacea;* phytochemical screening; antioxidant activity; antimicrobial activity; Minimal inhibitory concentration; Minimal bactericidal concentration

## 1. Introduction

Dermatoses are a complex of diseases that manifests in the target organs, namely the skin, mucous membranes, and phaneras. Their prevalence has significantly increased in developing countries [1] because of promoting factors such as humidity, aging of the general population, increase in the number of bedridden patients and endocrine pathologies, iatrogenic (transplant) and infectious (HIV/AIDS) immunosuppression and the emergence of multi-resistant pathogens [2,3]. A study in Senegal reported that infectious dermatoses were responsible of 64.7% of dermatological emergencies in the dermatology department of the regional hospital of Thiès [4].

Dermatoses can occur in several contexts and are due either to external aggression (microorganism, free radicals), immuno-allergic disorders, or secondary to general diseases such as endocrine diseases (diabetes, thyroid pathologies), liver diseases and vitamin deficiencies [5,6].

The most common infectious dermatoses are caused by *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Pseudomonas aeruginosa,* and/or fungal strains such as *Candida albicans* and *Candida tropicalis* [1]. About one-third of the 126 cases of surgical site secondary infection are due to *S. aureus* (39. 31%), followed by *Escherichia coli* (29, 23%), and *Pseudomonas aeruginosa* (12. 95%) according to a study carried out in Niger [7].

Nowadays, modern medicine encounters various inconvenients such as antibiotic resistance, high costs of active molecules, difficulties of access to treatment of microbial diseases by the most vulnerable groups due to the remoteness or absence of health services [8]. That is why many people use medicinal plants to treat infectious diseases [9].

Moreover, plants are important for pharmacological research and drug development, not only when bioactive molecules are used directly as therapeutic agents but also as starting materials for drug synthesis or as models for pharmacologically active compounds [10].

Herbal preparations active on dermatosis should satisfy one or more of these therapeutic principles: antiseptic, anti-inflammatory, antioxidant, healing, immunostimulant and hemostatic.

Numerous studies have shown that plant extracts possess biological properties such as immune system induction [11], anti-inflammatory [12], antioxidant [13], antimicrobial [14], antimutagenic and anticancer [15]. According to the literature, secondary metabolites such as polyphenols [16], triterpenes [17], and flavonoids [14] have antimicrobial, anti-inflammatory, antioxidant, healing and fibroblast proliferation stimulation properties.

Therefore, the evaluation of the biological properties of medicinal plants claimed to possess antimicrobial properties is gaining attention nowadays.

*Opilia amentacea* (Syn. *Opilia celtidifolia*) is one of the medicinal plants of the Burkinabe pharmacopeia used in the treatment of infectious dermatoses.

The genus *Opilia* (*Opiliaceae*) includes two species, namely *amentacea* and *campestris*. *Opilia amentacea* Roxb. (*OA*) is a West African woody climber plant, a heavily- branched shrub or tree up to 10 m. *O. amentacea* grows in fringing forests and savannahs, often on anthills [18]. It is widespread from Senegal to Nigeria and dispersed over the drier parts of tropical Africa [11].

*O. amentacea* has been subjected to many investigations, such as ethnobotanical, chemical, and pharmacological studies.

Ethnopharmacological surveys on *O. amentacea* have shown that West African traditional healers are widely using this medicinal plant to cure a various ailments including malaria, dermatitis and the lack of appetite [19]. It was also known as anti-constipation, an abdominal pain killer, a remedy for intestinal worms and other diseases [20].

In Burkina Faso, *O. amentacea* (OA) is used in the country's west for malaria treatment and the country's center for skin diseases, hence the name "waagsalga," referring to skin diseases in the local Moore language [21].

Because of the frequent and widespread use of medicinal plants in primary health care, many investigations were carried out to validate the recipes of traditional medicine scientifically.

Phytochemicals screening reported the presence of saponosides, coumarins, steroids, tannins, polyphenols, flavonoids, alkaloids and active polysaccharides [20,22] in the plant. Furthermore, previous pharmacological studies revealed that a saponin fraction of methanol extracts of the stem bark and the polysaccharide fractions of the aqueous extracts of the leaves of O. *amentacea* possess interesting biological properties, including intestinal antispasmodic, uterine stimulant, hypotensive and depression of the coronary outflow.

The plant was subjected to a preliminary investigation on the antimicrobial properties of the plant ; then, the aqueous and organic extracts (96° ethanol, 80° hydro-ethanol, n-hexane) of *O. amentacea* leaves, stem and root barks were tested on American type culture collection (ATCC) reference strains and showed an antibacterial activity. The study was undertaken for more chemical and biological investigations on the plant.

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## 2. Materials and Methods

### *2.1. Plant materials*

The leaves, stem and root barks of *Opilia amentacea* Roxb were harvested in Kombissiri. The samples were identified and authenticated by a botanist from the National Center for Scientific and Technological Research (CNRST), Ouagadougou, Burkina Faso and specimen deposited in herbarium under reference number 8730. The plant materials were washed thoroughly with water and dried under shade; and later on powdered with a mechanical grinder. The powders were stored in freezer bags away from light at room temperature for further use.

### *2.2. Microbial strains*

The antimicrobial effects were investigated on seven ATCC microbial strains including two Gram-negative bacilli (*Escherichia coli ATCC 25922; Pseudomonas aeruginosa ATCC 27653*), three Gram-positive cocci (*Staphylococcus aureus ATCC 25923; Streptococcus pyogenes ATCC 19615; Streptococcus agalactiae ATCC 13813*) and two fungal strains (*Candida albicans ATCC 90028; Candida tropicalis ATCC 750)*. These microbial strains were purchased from LGC standard distributor ATCC in South Africa.

### *2.3. Culture media*

Mueller Hinton agar (MH) (Liofilchem, lot: 032720504) was used for the isolation and susceptibility testing of *S. aureus* and Gram-negative bacilli (*E. coli* and *Pseudomonas aeruginosa*). Chocolate agar (GC Medium: Liofilchem, lot: 071320502) + Isovitalex (GC+IVx) were used for isolation and antibiogram of streptococci (*S. pyogenes* and *S. agalactiae*).

Sabouraud agar serves for the isolation and susceptibility testing of fungi strains (*C. albicans and C. tropicalis*).

MH (Liofilchem, lot: 011822506), Sabouraud (Liofilchem, lot: 07280501) and Brain Heart Broth (BHI) (Liofilchem, lot: 110420503) were used for Minimum Inhibitory Concentration (MIC) determination.

### *2.4. Chemicals and Standards*

All the solvents used were of analytical quality. Ethanol, n-hexane and ethyl acetate were purchased from CARLO ERBA (Val de Reuil Cedex, France). 1-butanol and dichloromethane were purchased from Honeywell (Riedel-de Haën Chemicals Seeize GmbH, Germany). Dimethyl sulfoxide (DMSO) (≥99, 7%), vanillin (≥ 99%), sodium carbonate, ferric chloride, aluminum chloride (≥98%), 1, 3, 5-Triphenyltetrazolium Chloride (TTC), DPPH (2, 2- diphenyl-1-picrylhydrazyl), ABTS (2, 20-azino-bis (3-ethylbenzothiazoline)-6-Opisulphonic acid), and Folin-Ciocalteu were purchased from Sigma-Aldrich (Laborchemikalien GmbH, Germany). The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was purchased from EMD (Millipore Corp MW 414.3, Lot 3727633; USA). All the following standards were acquired from Sigma-Aldrich (St. Louis): Gallic acid, 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox), Catechin, Quercetin and Ascorbic acid. Water was of Milli-Q-quality.

### *2.5. Plant extracts preparation*

One hundred grams of each sample powder were macerated at room temperature (25 ± 2 °C) for 24 h with 500 mL of ethanol 96°. The mixture was agitated in enclosed flasks using a stirrer (Modal T25 Digital Ultra Turrax) during maceration and filtered using a Buchner funnel and Wattman No. 5 filter paper. The filtrate was concentrated under decreased pressure with an evaporator (Heidolph Rotacool, Allemagne Type: Laborota 4003) at 40°C. The concentrated extract was dried at 40°C in an oven and the dry residue was weighed and stored at +4ºC until use.

- *Fractionation*

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The ethanolic extract was fractionated by liquid partition using organic solvents of increasing polarity: hexane, dichloromethane, ethyl acetate, and butanol and separatory funnel. 5g of each crude ethanol extract was initially dissolved in 25 mL of distilled water and extracted with 25 mL of organic solvent (hexane, dichloromethane, ethyl acetate, and butanol successively). The experiment was performed three time for each solvent. The total organic phase was collected and concentrated at 40°C using a rotary evaporator. Finally, the aqueous fraction was frozen and lyophilized. The dried fractions were stored at 4°C.

*2.6. Phytochemical Screening*

Phytochemical screening was performed on the extracts using standard protocols and Thin Layer Chromatography (TLC).

2.6.1. Characterization reactions in tubes

Crude ethanol extracts of *Opilia amentacea* were analyzed for the presence of secondary metabolites using standard procedures as described by Ciulei (1982).

The principle is based on the ability of functional groups of these compounds to react with specific chemical reagents to give characteristic reactions.

- *Preparation of ethanolic extract solutions*

Crude ethanolic extract (1 g) was dissolved in 5 mL of ethanol 96% and 50 mL of distilled water was added. A part of the mixture at 20 mg/mL was hydrolyzed in an acid medium according to the following procedure:

- *Hydrolysis of extract solutions*

30 mL of the mixture (20 mg/mL) was mixed with 15 mL of 10% chlorhydric acid in a 100 mL ground-neck flask containing a few glass beads. The flask's content was brought to boil under reflux for 30 min. After cooling, the hydrolyzed extract was extracted using liquid/liquid partitioning with 2 x 10 mL dichloromethane in a 100 mL separator funnel. The collected organic phases were dried on anhydrous sodium sulfate, filtered on Whatman n°5 paper, and concentrated under reduced pressure in the rotary evaporator.

The concentrated organic extracts were dispatched in test tubes to characterize O-heteroside compounds such as steroidal and triterpene glycosides, flavonic, anthracenic, coumarinic derivatives and cardenolides through their genins.

The non-hydrolyzed solutions of ethanolic extract were used to screen phenolic compounds, reducing compounds, saponosides, alkaloid salts and anthocyanins.

- *Screening for Tannins*

1 mL of the ethanolic extract solution was diluted with 1 mL of distilled water in a test tube. After homogenization, two drops of a 2% ferric chloride solution were added to the content of the test tube. The appearance of a bluish-black or intense green color indicated the presence of tannins [20].

- *Screening for Flavonoids (Shibata or cyanidin reaction)*

1mL of ethanolic extract solution was placed in a test tube containing some magnesium clippings. 0.5 mL of concentrated hydrochloric acid (37%) was added. The appearance of a red or orange color after bubbling of the mixture indicated the presence of flavonols and flavanones, respectively.

- *Screening for Leucoanthocyans*

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1 mL of ethanolic extract solution was mixed with 0.5 mL of hydrochloric butanol solution (Bate Smith reagent) over a boiling bath water. The immediate appearance of a red color indicate a positive test for leucoanthocyans.

- *Screening for anthracenosides/anthraquinones (Bornträger reaction)*

1 mL of the hydrolyzed organic extract was evaporated in a water bath. The dry residue was dissolved with 0.5 mL of dichloromethane (DCM). 0.5 mL of a 25% ammonia solution was added. The mixture was shaken vigorously and allowed to stand. The formation of a cherry red color in the aqueous phase indicates the presence of anthracenosides/anthraquinones.

- *Screening for sterols and terpenoids (Liebermann Burchard Reaction)*

1 mL of the hydrolyzed organic extract was evaporated in a water bath. The residue was dissolved in 0.5 mL of dichloromethane (DCM) and 0.5 mL of acetic anhydride.

The mixture was homogenized, followed by carefully adding 1 mL of concentrated sulfuric acid. The formation at the interface of the two liquids of a reddish-brown or reddish-purple ring with a greenish or purplish supernatant, was a positive indication for the presence of steroids and terpenoids.

- *Screening for coumarins and derivatives (Feigl reaction)*

1 mL of the hydrolyzed organic extract was evaporated in a water bath. The residue was dissolved in 1 mL of hot distilled water, shaken, and dispatched into two non-fluorescent hemolysis tubes (T and E). Distilled water (0.5 mL) was added to T-tube (negative control), and 0.5 mL of a 10% ammonia solution in the E-tube (sample). The contents of both tubes (T and E) were observed under ultraviolet radiation at 254 nm and 365 nm. The intense greenish or bluish fluorescence in tube E indicates the presence of coumarins and derivatives.

- *Screening for saponosides by the foam test*

2 mL of ethanolic extract solution was mixed with 2 mL of distilled water in 16 mm diameter test tubes. The tubes were capped with parafilm and then shaken vigorously for 5 min. The development of foam column with a height of at least 1 cm and persistent for 5 min indicates the presence of saponosides.

- *Screening for cardiotonic heterosides*

1 mL of the hydrolyzed organic extract was evaporated in a water bath. The residue was dissolved in 1 mL of 50% ethanolic solution and dispatched into two non-fluorescent hemolysis tubes (T1 and T2). 0.5 mL of Baljet reagent was put into T1 and 0.5 mL of Kedde reagent was put into T2. 0.5 mL of a 1N NaOH solution was added to both tubes that were shaken and left to stand. The appearance of an orange (T1) and purplish (T2) color indicates the presence of cardiotonic heterosides.

- *Screening for reducing compounds*

To the ethanolic extract solution, diluted ½ with distilled water, was added 1 mL of Fehling's reagent (I+II). The mixture was heated over a boiling water bath. The appearance of a brick-red precipitate attests the presence of reducing compounds.

- *Screening for alkaloids*

10 mL of alcoholic extract solution was placed in a beaker and concentrated on a hot plate. The residual aqueous solution was diluted with 10 mL of alkalinized distilled water (pH = 8-9) with NaOH solution. The alkaline extract solution was extracted by liquid/liquid partitioning with 2 x 10 mL of DCM in a separatory funnel.

The organic phases were evaporated to dryness. The dry residue (alkaloid bases) was triturated with 0.5 mL of a 2% hydrochloric acid solution (alkaloid salts). The acid solution was dispatched into

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two watch glasses (V1 and V2). To the content of V1 were added two drops of Draggendorf's reagent (potassium iodobismuthite), and to V2, two drops of Mayer's reagent (potassium mercuri-ioduro). The appearance of precipitates of red or orange-yellow (V1) and whitish-yellow (V2) color attests the presence of alkaloids.

- *Thin Layer Chromatography (TLC)*

Phytochemical analysis of *Opilia amentacea* extracts (OCFe, OCET and OCER) was also performed on 20 cm × 20 cm silica gel 60 F254 TLC plates (Macherey-Nagel, Germany).

Extracts dissolved in 96°C ethanol at the concentration of 10 mg/mL were used for thin layer chromatographic analysis. The mobile phase used consisted of ethyl acetate, methanol and distilled water (77; 13; 10). The mobile phase prepared was placed in TLC migration chambers.

Sample solutions were applied on the TLC plates using the capillary microtubes. Typically, 10 µL volumes of samples were applied. The distance between deposit points was 1 cm. Distances from the bottom edge was 1.5 cm, and 1 cm from the side edges. Plates were dried after development using a hairdryer and immersed vertically in the migration chambers containing mobile phases. The migration was performed over a path of 8 cm at 0.5 cm from the superior edge. After migration, TLC plates were dried at room temperature.

The dried TLC plates were observed under white light and ultraviolet light at 254 nm and 365 nm. Secondary metabolites on the plates were revealed using 10% ferric chloride ($FeCl_3$) for tannins; vanillin sulfuric acid for steroidal and triterpenic glycosides, anisaldehyde sulfuric acid for saponosides and Neu's reagent for flavonoids groups The spraying of particular chemical reagents revealed the investigated secondary metabolite groups before observation.

2.6.2. Quantitative phytochemical assessment

- *Determination of Total Phenolics : Folin-Ciocalteu Method*

Total phenolic content (TPC) of *Opilia amentacea'* ethanol crude extracts (OCFe, OCET, OCER) was determined using the Folin–Ciocalteu spectrophotometric method according to a method previously described by Koala *et al*. [23] with slight modifications. Phosphomolybdate reagent and sodium tungstate were reduced during phenols oxidation in alkaline medium to a mixture of tungsten blue and molybdenum.

Briefly, 25 µL of of diluted sample solution (1 mg/mL) were mixed with 125 µL of diluted Folin–Ciocalteu reagent (FCR) solution (0.2 N). After 5 minutes at room-temperature incubation, 100 µL of a saturated sodium carbonate solution (7.5% in water) were added to the mixture. After 60 minutes in the dark and at 37°C the absorbance of the resulting blue hue was measured using a SHIMADZU UV-Vis spectrophotometer at 760 nm. Using the calibration curve equation $Y= 0.0516x + 0.0673$, $R^2 = 0.9936$, the phenolic content of plant extracts was calculated. The data is reported as the equivalent of milligrams of gallic acid (GAE) per gram of dry weight. Each measurement was performed in triplicate (n = 3).

- *Determination of Total Flavonoids*

The determination of total flavonoid contents (TFC) in *Opilia amentacea* extracts was performed using aluminum trichloride method and quercetin as a reference. The method evaluates all compounds reacting with aluminum chloride (AlCl3). Briefly, crude ethanolic extract was exhaustively dissolved in methanol at room temperature at 1 mg/ml and filtered. Quercetin was used as standard and a calibration curve is made from concentrations ranging from 0.001 to 0.5 mg/mL of a quercetin solution. Then, 100 µL of the sample solution was mixed with 100 µL of a 2% methanol solution of aluminum trichloride ($AlCl_3$). After 1 h room-temperature incubation, the absorbance of the supernatant was measured at 415 nm using a spectrophotometer. The TFC of the extract was obtained by relating the absorbance read to the standard curve equation $y =0.0151x + 0.0593$, $R^2=0.9992$. The total flavonoid contents were expressed as mg quercetin equivalents per gram of dry weight. All determinations were performed in triplicate (n = 3).

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*2.7. Biological properties*

2.7.1. In vitro antimicrobial assay

The *in vitro* antimicrobial activity of the fractions was assessed using the Agar diffusion method to determine the inhibition zones and the microdilution method to determine the MIC and the minimal bactericidal concentration (MBC).

- *Disc method in Agar medium*

The antimicrobial activity of the fractions on the reference strains was performed using the disk diffusion agar medium. Petri dishes containing 20 mL of appropriate agar medium were inoculated with an 18-24 h culture of bacterial strains from the nutrient broth. The inoculum was adjusted to about $10^8$ UFC/mL with sterile saline solution.

Fractions were solubilized in DMSO at 100 mg/mL. Twenty-five microliters of each sample were applied separately to sterile filter paper disks (Whatman No 1; 6 mm in diameter) and placed on the surface of the inoculated medium.

Antibiotics (ciprofloxacin 5 µg, erythromycin 15 µg, and nystatin 100 IU) were used as a positive control, and diluted DMSO as a negative control. A swab-inoculated petri dish was used as a control for bacterial growth. Inoculated Petri dishes were placed in an oven according to the incubation conditions of the inoculated strains (Table 1). All tests were performed in triplicate.

- *Microdilution method*

The microdilution method (NCCLS, 2000) used to determine the MICs was performed on 96-well microplate with samples having an inhibition zone ≥ 10 mm. Fresh colonies of each test strain were inoculated in sterile MH, sabouraud and Brain Heart Infusion (BHI) broths and incubated according to the conditions of each strain. The overnight culture was appropriately diluted in broth to obtain viable counts of approximately $10^8$ UFC/mL. Diluted solutions (0.025-25 mg/mL) of each sample were prepared from stock solution of extract. To each microplate well, 100 µL of each diluted sample was added to 100 µL of inoculum prepared from each strain tested.

DMSO 1/5 was used as a negative control. A growth control (inoculum alone) for each strain was also included in the test.

The microplates covered by their lids were packed in plastic bags abd incubated (5% $CO_2$, 37°C) for 24-48h. Then, 25 µL of Triphenyltetrazolium Chloride (TTC) or MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) solution at 2 mg/mL was added to each well, and the plate was incubated at 37°C. The indicator solution changes from clear to pink (TTC) indicating bacterial activity or from yellow to purple or dark blue (MTT) in the presence of fungal activity, while it remains clear when microbial growth is inhibited.

The MIC was defined as the lowest extract concentration inhibiting the test strain visible growth. The minimal bactericidal concentration (MBC) was determined by spreading 0.1 mL of clear tubes, which did not show any visible growth on appropriate agar plates and incubated according to the required conditions.

The MBC was defined as the lowest extract concentration concentration that killed 99.99% of the inoculum test bacteria. This corresponds to less than 100 CFU per 10 µL of well contents at concentrations below the MIC.

Tests were performed in triplicate on the same microplate for each sample, and the experiment was repeated three times.

**Table 1.** Incubation conditions of the used microbial strains.

| Microbial group | Strains | Incubation in oven |
|---|---|---|
| **Gram negative bacilli** | *Escherichia coli* ATCC 25922<br>*Pseudomonas aeruginosa* ATCC 27653 | Incubation of inoculated agar plates for 24 hours at 37°C |
| **Gram positive cocci** | *Staphylococcus aureus* ATCC 25923 | |

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| | | |
|---|---|---|
| | *Streptococcus pyogenes* ATCC 19615<br>*Streptococcus agalactiae* ATCC 13813 | The inoculated agar plates were initially placed in moist, $CO_2$-rich jars and incubated for 24-48 hours at 37°C |
| **Fungies** | *Candida albicans* ATCC 90028<br>*Candida tropicalis* ATCC 750 | Incubation for 48 hours at 25°C |

- *Interpreting antimicrobial test results*

The sensitivity of microbial strains to the fractions was interpreted based on the criteria established by Ponce et al. (2003) and their efficacy appreciated based on the scale proposed by Kuete (2010).

The BMC/MIC ratio was used to distinguish [26]:

- bactericidal extracts: BMC/MIC≤ 2
- bacteriostatic extracts : BMC/MIC > 2
- extracts with an inhibition diameter ≥10 mm ( selected for more investigation)

When the BMC/MIC ratio is ≥ 32, the strain is considered tolerant toward the extract.

2.7.2. Antioxidant Activity Assays

- *ABTS Radical Cation Scavenging Activity*

The antioxidant activity using ABTS radical cation (2,20-azino-bis(3-ethylbenzothiazoline)-6-Opisulphonic acid)) was assessed according to the trolox equivalent antioxidant capacity (TEAC) assay as previously described [27].

In brief, the ABTS radical cation ($ABTS^{\bullet+}$) solution was prepared by mixing of 19.2 mg of ABTS and 3.12 mg of potassium persulphate in 5 mL. The mixture was incubated at room-temperature in the dark for 16 h. The $ABTS^{\bullet+}$ solution was then diluted with 80% ethanol (4.5 mL $ABTS^{\bullet+}$ in 220mL ethanol). 20 µL aliquots of samples in varying amounts are added to 200 µL of $ABTS^{\bullet+}$ solution and mixed thoroughly. The reaction mixture was allowed to stand at room-temperature for 30 min and the absorbance at 734 nm was immediately recorded using a spectrophotometer (SHIMADZU). A standard curve was obtained by using Trolox standard solution at various concentrations in 80% ethanol. The calculation for radical scavenging activity was as follows:

$$I\ \% = [(\text{Abs } A_0 - \text{Abs } A_{1)}) / \text{Abs} A_0] \times 100$$

The $IC_{50}$ (concentration causing 50% inhibition) was determined graphically using a linear calibration curve by plotting the extract concentrations versus the associated scavenging action

The absorbance of the reaction samples was compared to that of the Trolox standard and the results were expressed as trolox equivalents (TE) per gram of dry lyophilized extract (mg TE/g dw extract).

- *DPPH Radical Scavenging Activity*

The antioxidant activity of *Opilia amentacea* extracts was evaluated using the 2,2-diphenyl-1-picrylhydrazyl radical scavenging method [28]. The process is based on the capacity of plant extracts to scavenge compared to the DPPH radical ($DPPH^{\bullet}$) to the Trolox in a dose-response curve. DPPH radical absorbs visible light at a maximum wavelength of 517 nm and disappears with reduction by an antioxidant compound [28].

In brief, 20 µL aliquots of samples in varying amounts are added to 200 µL of a DPPH-methanol solution. The 0.10 mM $DPPH^{\bullet}$ solution was created by dissolving 4 milligrams of $DPPH^{\bullet}$ in 100 milliliters of methanol. For the blank sample, 20 µL of methanol was added to 200 µL of $DPPH^{\bullet}$. After 30 minutes of incubation at room temperature in the dark, the spectrophotometric absorbance at 517 nm was measured (SHIMADZU). The calculation for radical scavenging activity was as follows:

$$I\ \% = [(\text{Abs } A_0 - \text{Abs } A_{1)}) / \text{Abs} A_0] \times 100$$

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The $IC_{50}$ (concentration causing 50% inhibition) was determined graphically using a linear calibration curve by plotting the extract concentrations versus the associated scavenging action.

- *FRAP Assay*

Ferric reducing antioxidant power (FRAP) assay was performed according to Paré *et al*., [29] with slight modifications. In brief, 0.5 mL of various concentrations of sample extracts were mixed with 1.25 mL of 200 mmol/L sodium phosphate buffer (pH 6.6) and 1.25 mL of 1% potassium ferricyanide. The mixture was shaken vigorously and incubated at 50°C for 30 min. After incubation, 1.25 mL of 10% trichloroacetic acid (w/ v) was added and then the mixture was centrifuged at 2000 rpm in a refrigerated centrifuge for 10 min. The upper layer (0.625 mL) was mixed with 0.625 mL of deionized water and 0.625 mL of 1% ferric chloride. The absorbance was measured using spectrophotometer at 700 nm. Ascorbic acid (200-0.4 µg/mL; $R^2 = 0.99996$) was used as standard curve. Reducing compounds (antioxidants) concentration of extract was expressed as moles equivalent of ascorbic acid (EAA) /g of dry extract according to the following formula:

$$\mathbf{C} = \frac{\mathbf{c} \times \mathbf{D}}{\mathbf{M} \times \mathbf{Ci}}$$

**C=** reducing compound concentration (mol EAA/g of dry extracts); **c=** sample concentration; **D=** dilution factor of the stock extract solution; **M=** molar mass of ascorbic acid (176 g/mol); **Ci=** concentration of the stock extract solution.

*2.8. Statistical Analysis*

Data were expressed as mean ± standard deviation (SD) of three experiments (n=3). The results were analyzed by GraphPad Prism 5. The one-way ANOVA was applied for comparison of *in vitro* antioxidant activities of the extracts. Level of significance was observed at $p \leq 0.05$ for correlation studies and $p \leq 0.01$ for *in vitro* studies.

## 3. Results and Discussion

*3.1. Phytochemical screening*

- ***Qualitative phytochemical screening***

The results of the qualitative phytochemical screening of the crude ethanol extracts of Opilia amentacea (OCFe, OCET, OCER) are summarized in Table 2 and Figure 1. Secondary metabolites such as steroidal and triterpenes glycosides (saponosides), tannins, reducing compounds, anthraquinones, flavonoids, coumarins and its derivatives were detected in the extracts (Table 2). Leaves and stem barks of Opilia amentacea were found more richer in secondary metabolites than the roots. Phytochemicals like anthocyanins, alkaloids and cardenolides were not detected.

**Table 2.** Phytochemical groups identified in *Opilia amentacea* extracts.

| Sample<br>Phytochemical groups | OCFe | OCET | OCER |
|---|---|---|---|
| **Steroidal and triterpenic glycosides (saponosides)** | (+) | (+) | (+) |
| **Anthraquinones** | (+) | (-) | (+) |
| **Anthocyans** | (-) | (-) | (-) |
| **Alkaloids** | (-) | (-) | (-) |
| **Flavonoids** | (+) | + | (+) |
| **Coumarins and derivatives** | (+) | (-) | (+) |
| **Tannins** | (+) | (+) | (+) |
| **Reducing compounds** | (+) | (+) | (+) |

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| Sample<br>Phytochemical groups | OCFe | OCET | OCER |
|---|---|---|---|
| **Cardenolids** | (-) | (-) | (-) |

OCFe: Hydroethanol extracts of leaves; OCET: Hydroethanol extracts of stem bark; OCER: Hydroethanol extracts of root bark;

The TLC analysis of the crude ethanol extract confirmed the presence of sterols, triterpenes, flavonoids and tannins.

Secondary metabolites are bioactive compounds involved in the biological properties of plants [30] and their presence in Opilia amentacea crude ethanol extract could explain their medicinal properties. The chemical composition (flavonoids, tannins, phenols, sterols and terpenes) of the plant despite the extraction process and solvent used is closed to that reported in literature [22,31]. Alkaloid salts, regularly reported from other studies [20,32] were not identified in the extracts. Biotic (the nature of the soil) and abiotic factors (the climate) might explain this difference [33].

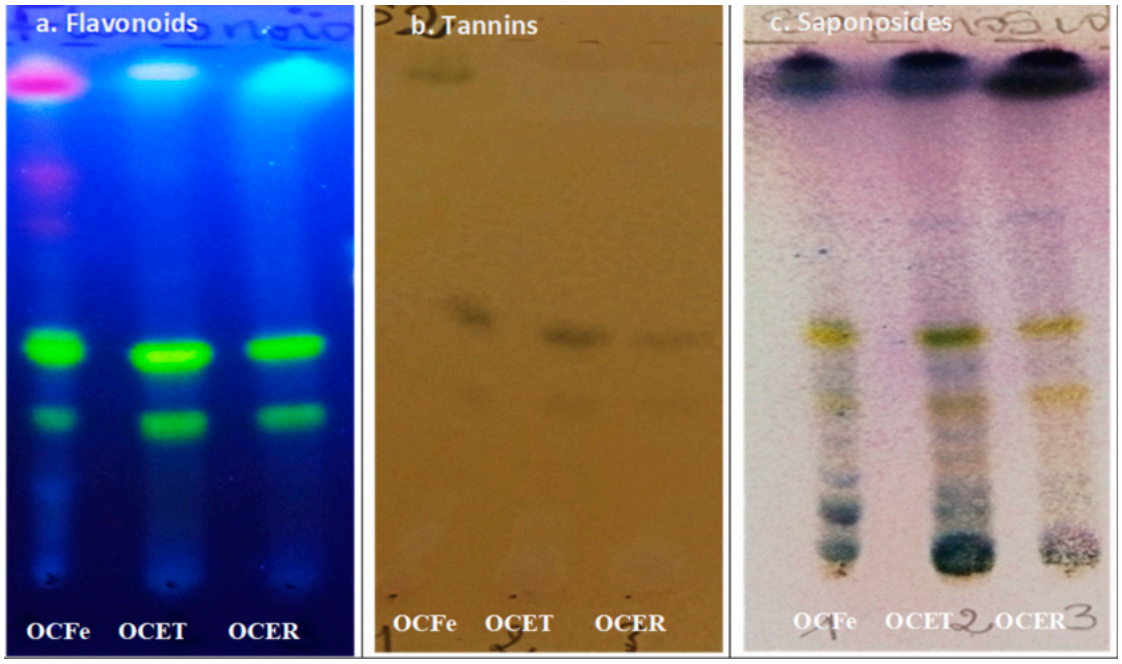


**Figure 1.** Chromatogram of extracts from Opilia amentacea. OCFe: Hydroethanol extracts of leaves; OCET: Hydroethanol extracts of stem bark; OCER: Hydroethanol extracts of root bark;.

- **Quantitative phytochemical assessment**

Quantitative assessment of phytochemical constituents is summarized in Figure 2.

Total phenolic content (TPC) of the ethanol extract of *Opilia amentacea* using the Folin–Ciocalteu's reagent ranged from 65.92 ± 0.93 for leaves to 65.71 ± 0.57 for stem bark and 55.08 ± 0. 45 mg Gallic Acid Equivalents/g dry weight. Leaves extract showed the highest total content in phenolic compounds followed by the stem bark and the root bark. It was previously recognized that the phenol content of any plant depends on intrinsic (genetic, extraction condition) and extrinsic (environment, handling and development stage) factors [34,35]. Plant polyphenols represent a large class of biologically active secondary metabolites [36]. Unfortunately, Folin-Ciocalteu method does not give different classes as well as the quantity of phenolic compounds.

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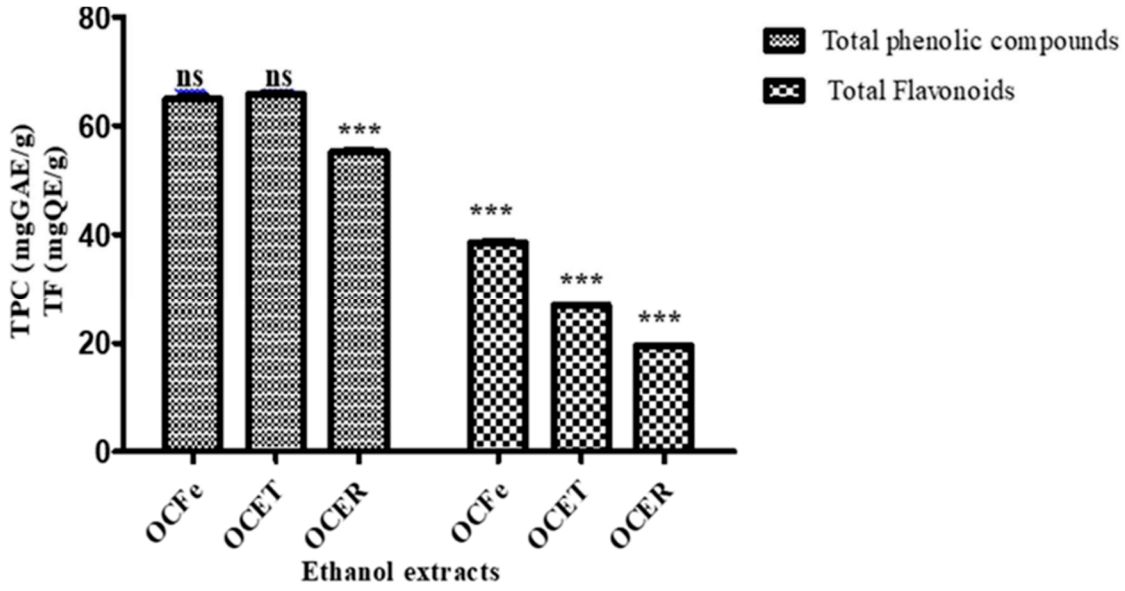


**Figure 2.** content of total phenolic compounds and total flavonoids. ns: no significant ($p>0.05$); ***: $p<0.0001$.

A previous study shown that leaves of the plant had the highest amount of polyphenol content (62.01 ±0.62 milligrams of gallic acid equivalents per gram (mg GAE/g) compared to leafy stems (53.23±0.18 mg GAE/g) and roots (39.45±0.01 mg GAE/g). In the same way, the total flavonoid content of the ethyl acetate fraction of leaves, leafy stems and roots were 28.32±0.01, 23.62±0.68 and 5.31±0.54 mg-rutin equivalent per gram (mg RE/g) respectively [37]. That confirms the richness of the plant in total polyphenols and flavonoids.

The total flavonoid contents (TFC) showed a similar trend ranged from 38.8 ± 1.05 for leaves to 26.81 ± 0.19 for stem bark and 19.54 ± 0.9 mg Quercetin Equivalents/g dry weight for root bark. As the total phenolic content, the total flavonoid contents ranked in the following order: Root bark < Stem bark < Leaves. Flavonoids are one of the largest classes of small molecular secondary metabolites produced in different parts of the plant [14]. They display a wide range of pharmacological effects beneficial for human health, which include, among others, antimicrobial activity, antioxidant activity, anti-inflammatory and anticancer activities [13,14].

It has been reported that light intensity, temperature and altitude could influence the biosynthesis of flavonoids [38].

### *3.2. Antimicrobial activity*

Twenty fractions of Opilia amentacea were screened for antimicrobial activity against a panel of Gram-positive cocci, Gram-negative bacilli and fungi and the results are presented in Tables 4 and 5. The hexane and dichloromethane fractions showed the best inhibitory activities. The most susceptible bacteria in this study were Gram-positive cocci. The most apolar fractions were the most active (4/5). By the disc diffusion method, the highest inhibitory effect was obtained with dichloromethane fractions (FDFe, FDET, and FDER) against Gram-positive cocci (S. aureus; S. pyogenes S. agalactiae) with inhibition diameters ranging from 12 to 15 mm on a standardized inoculum ($10^8$ CFU/mL). Their antimicrobial efficacy with MICs ranging from 0.96 to 4.64 mg/mL was very weak [25].

No effect of the extracts was found on Gram-negative bacilli (E. coli and P. aeruginosa).

Furthermore, a moderate fungicidal activity was found against Candida tropicalis with both hexane leaves (FHFe) and hexane root bark (FHER) fractions; with the absence of growth at 0.23 mg/mL and 0.43 mg/mL, respectively. However, no effect was found on Candida albicans

**Table 3.** Antimicrobial activity of *Opilia amentacea'* fractions (n=3).

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| *Strains Extracts* | *Test* | *Pa* | *Ec* | *Sa* | *Sp* | *Sag* | *Ca* | *Ct* |
|---|---|---|---|---|---|---|---|---|
| NC | **D** | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| PC | **D** | **27.33 ± 0.57** | **35.67 ± 0.57** | **31.33 ± 1.15** | **35.67 ± 0.57** | **37.67 ± 0.57** | **20.67 ± 0.57** | **21 ± 1** |
| *FHFe* | **D** | 0 | 0 | 0 | 0 | 0 | 9 ±0.3 | 13 ± 0.67 |
| *FHET* | **D** | 0 | 0 | 0 | 0 | 8 ± 0 | 8 ± 0 | 11.5 ± 0 |
| *FHER* | **D** | 0 | 0 | 0 | 0 | 9 ±0 | 8 ± 0 | 10 ± 0 |
| FDFe | **D** | 0 | 0 | 12 ± 0 | 15.2 ± 0.33 | 15.33 ± 1 | 0 | 0 |
| FDET | **D** | 0 | 0 | 0 | 14 ± 1.12 | 15 ± 1.67 | 0 | 0 |
| FDER | **D** | 0 | 0 | 0 | 11 ± 0 | 12 ± 0.33 | 0 | 0 |
| FaqER | **D** | 0 | 0 | 12.66 ± 0.28 | 14.5 ±0.5 | 14.5 ±0.5 | 0 | 0 |

**NC**: negative control (DMSO 1/5); **PC**: positive control (Erythromycin 15 µg for gram-positive cocci; Ciprofloxacin 5µg for gram-negative bacilli and Nystatin 100UI for fungi); **D**: inhibition diameter. **FHFe** *:* Hexane fraction of leaves*;* **FHET** *:* Hexane fraction of stem bark*;* **FHER** *:* Hexane fraction of root bark*;* **FDFe** *:* Dichloromethane fraction of leaves*;* **FDET** : Dichloromethane fraction of stem bark; **FDER** : Dichloromethane fraction of root bark**;** **FaqER:** Aqueous fraction of root bark**;** **Pa :** *Pseudomonas aeruginosa ;* **Ec** *: Escherichia coli ;* **Sa** *: Staphylococcus aureus ;* **Sp** *: Streptococcus pyogenes ;* **Sag** *: Streptococcus agalactiae ;* **Ca** *: Candida albicans ;* **Ct** *: Candida tropicalis.*

**Table 4.** Antimicrobial parameters (MIC, MBC) of active extracts (n=3).

| Extracts | MIC (mg/mL) | MBC (mg/mL) | MBC/MIC | Interpretation |
|---|---|---|---|---|
| | | ***S. aureus*** | | |
| **FDFe** | 4.64 ± 0 | 9.28 ± 0 | 2 | Bactericidal |
| **FaqER** | 12.51 ± 2.02 | 25.03 ± 3.1 | 2 | Bactericidal |
| | | ***S. agalactiae*** | | |
| **FDFe** | 2.03 ± 0 | 2.03 ± 0 | 1 | Bactericidal |
| **FDET** | 0.96 ± 0 | 0.96 ± 0 | 1 | Bactericidal |
| **FaqER** | 5.04 ± 0.9 | 10.08 ± 0.9 | 2 | Bactericidal |
| | | ***S. pyogenes*** | | |
| **FDFe** | 2.03 ± 0 | 2.03 ± 0 | 1 | Bactericidal |
| **FDET** | 0.96 ± 0 | 0.96 ± 0 | 1 | Bactericidal |
| **FaqER** | 5.04 ± 0.9 | 10.08 ± 0.9 | 2 | Bactericidal |
| | | ***C. tropicalis*** | | |
| **FHFe** | 0.23 ± 0 | 0.23 ± 0 | 1 | Bactericidal |
| **FHER** | 0.43 ± 0 | 0.43 ± 0 | 1 | Bactericidal |

**FHFe***:* Hexane fraction of leaves*;* **FHER***:* Hexane fraction of root bark*;* **FDFe:** Dichloromethane fraction of leaves*;* **FDET**: Dichloromethane fraction of stem bark; FaqER: Aqueous fraction of root bark.

Overall, the hexane fractions of leaves (FHFe) and roots (FHER) showed good antimicrobial effect. Hexane fractions of leaves and roots barks showed a moderate fungicidal activity on *Candida tropicalis* with MICs of 0.23 and 0.43 mg/mL, respectively.

Few investigation on the antimicrobial activity of *Opilia amentacea* have been reported. A study conducted by Owolabi *et al.*[20] Showed the susceptibility of *Escherichia coli* to the crude chloroform extracts of the plant with a MIC of 0.078 mg/mL. A moderate antibacterial activity was observed against *both Bacillus cereus and Pseudomonas aeruginosa* with MIC = 0.156 mg/m while a weak antibacterial activity was observed against *Staphylococcus aureus (*MIC = 625 µg/mL). A potent antifungal activity of the chloroform extracts of *O. amentacea* 'leaves against *Botrytis cinerea* and *Aspergillus niger* with respective MIC values of 0.156 µg/mL and 1.250 mg/mL [20]. However, the authors have reported any effect of the extracts against *Staphylococcus epidermidis* and *Serratia marcescens* using the chloroform extracts of the leaves [20].

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Nevertheless, the current results corroborate with the preliminary findings that showed significant inhibitory activity of ethanolic extracts of the plant on *Streptococcus pyogenes* and *Streptococcus agalactiae* strains (MIC = 30 µg/mL and 240 µg/mL)[39]. Moreover, the decocted leaves and stem bark extracts showed almost similar inhibitory activity on *C. albicans* (21.67 and 24.33 mm).to nystatin (100 UI)

Among the secondary metabolites identified in the plant extracts and fractions some are known to possess antimicrobial, antioxydant and anti-inflamatory activities. Endeed, the phytochemical analysis has detected the presence of sterols, triterpenes, flavonoids and tannins in the plant extracts and fractions.

It was already demonstrated mechanisms of action of phenolic compounds on bacteria and fungi among which the interaction with bacterial proteins and cell wall structures, damage to cytoplasmic membranes, decrease in membrane fluidity, inhibition of nucleic acid synthesis, cell wall synthesis or inhibition of energetic metabolism [36,40–42]. Among the many potential targets, the bacterial cell wall seems to be the primary molecular target for the antimicrobial action of most polyphenols. Gram-positive bacteria seem to be more susceptible to the phenolic compounds unlike Gram-negative bacteria which seem to be protected by their outer membrane acting as a barrier reducing the uptake of phenolic compounds [43,44].

Flavonoids are other groups of secondary metabolites known for their antimicrobial activity [55,56] although some of them have also shown less effect on Gram-negative bacteria and this because of the negatively charged LPS present in the outer bacterial membrane [14]; thus confirming our results which in general showed a high sensitivity of Gram-positive bacteria compared to Gram-negative bacteria. The antifungal activity of hexane fractions from leaves (MIC = 0.23 mg/mL) and hexane fractions from root bark (MIC = 0.43 mg/mL) against *Candida tropicalis* could be attributed to terpenoids, non-polar, biologically active compounds.

### *3.3. Antioxydant activity*

Results of the free-radical-scavenging effect of Opilia amentacea crude ethanol extracts and fractions by DPPH and $ABTS^{\bullet+}$ assay are summarized in Table 6 while the ferric reducing power (FRAP) results are presented in Figure 3. The lower the IC50 by DPPH and $ABTS^{\bullet+}$, the higher the antioxidant activity and the higher the FRAP value, and the better the ferric reducing power [59].

**Table 5.** Free radical scavening activity of ABTS and DPPH.

| Extrait | ABTS | DPPH |
|---|---|---|
| | $IC_{50}$ (µg/mL) | $IC_{50}$ (µg/mL) |
| **OCFe** | $23.87^{a} \pm 0.21$ | $715.88^{c} \pm 0.6$ |
| **OCET** | $69.81^{d} \pm 0.31$ | $990.33^{d} \pm 0,57$ |
| **OCER** | $76.14^{e} \pm 0.79$ | $>1000^{d}$ |
| **FHFe** | $>1000^{h}$ | $>1000^{d}$ |
| **FHET** | $>853.69^{g} \pm 0.01$ | $>1000^{d}$ |
| **FHER** | $>1000^{h}$ | $>1000^{d}$ |
| **FDFe** | $88.40^{f} \pm 0.30$ | $463.33^{b} \pm 0.32$ |
| **FDET** | $36.85^{b} \pm 0,52$ | $383.33^{a} \pm 0,4$ |
| **FDER** | $41.54^{c} \pm 0,18$ | $>1000^{d}$ |
| **Trolox** | $3.78^{***} \pm 0,0027$ | $6.34^{***} \pm 0.004$ |

Values are presented as mean ± SD (n = 3). Means with different superscript (a-e) letters in the columns are significantly ($P < 0.01$) different from one another; **OCFe:** hydroethanol extracts of leaves; **OCET:** hydroethanol extracts of stem barks; **OCER**: hydroethanol extracts of root barks; **FHFe**: Hexane fraction of leaves; **FHER**: Hexane fraction of root bark; **FDFe:** Dichloromethane fraction of leaves; **FDET**: Dichloromethane fraction of stem bark; FaqER: Aqueous fraction of root bark.

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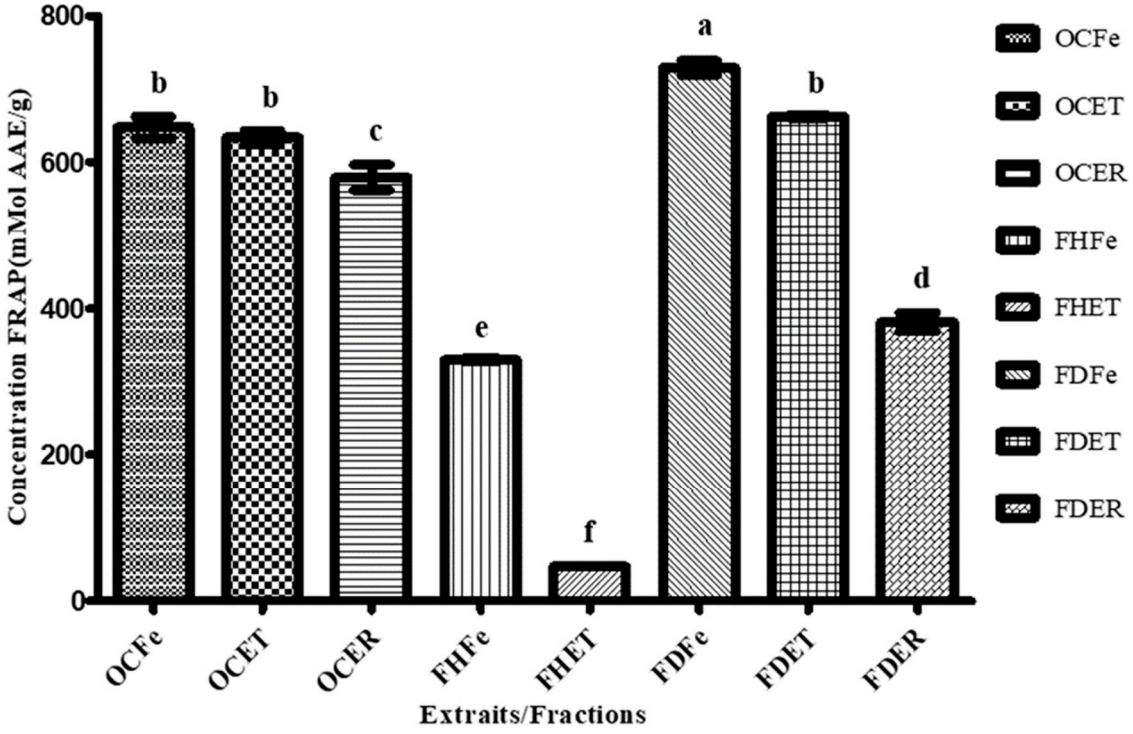


**Figure 3.** Ferric reducing antioxidant power (FRAP) of Opilia amentacea extracts/fractions. Bars with different superscript (a-e) letters are significantly ($P < 0.0001$) different from one another; Bars with same superscript (b) letter are no significantly ($P > 0.05$) different.

$IC_{50}$ obtained during the ABTS experiment ranged from 23.87 to 88,40 µg/mL for all extracts and fractions indicating low antioxidant activity compared to Trolox. Hexane fractions were devoid of ABTS free radical scavenging activity (IC50 between 853.68 and 1000 µg/mL). Only the dichloromethane fractions from leaves and stem barks (IC50 = 463.33 and 383.33 µg/mL) showed the lowest free radical scavenging values of the DPPH assay. The other extracts and fractions showed little to no DPPH activity ($IC_{50}$ = 715.88-1000 µg/mL). Our results are in agreement with those of Magid et al. [45], which reported a weak to moderate free radical scavenging power of Opilia amentacea. However, a strong antioxidant potential revealed by the hydrophilic and lipophilic assay has been reported for the flavonoid-rich fractions of the leaves of O. amentacea ($IC_{50}$= 10 µg/mL) [46].

A negative correlation meaning no relationship between total phenolic content of extracts/fractions and DPPH ($R^2$ = -0.458) and ABTS ($R^2$ = -0.534) scavenging activity was observed (Table 5); although, extracts were found relatively rich in phenolic compounds. These results do not support the statement that the antioxidant activity of plant extracts is correlated with the total phenolic content [47,48].

Dichloromethane fractions of leaves and stem bark of Opilia amentacea showed the highest ferric reducing antioxidant power (FRAP) (741.15 and 662.84 µMol, respectively), followed by crude ethanolic extracts.

Iron reducing power translate electron donating property of extracts, which have the ability to break the free radical chain by donating electrons and therefore decrease the oxidative damage by reduction of transition metals [16]. The efficiency of iron reduction implies the presence in the extracts of reducing mediators such as flavonoids which are the main electron donors [49,50]. During our experiment, high correlation ($R^2$ = 0.932) (Table 5) was found between total flavonoids content of extracts/fractions and ferric reducing antioxidant power. A number of concurring studies have shown a reciprocal correlation between phenolic and flavonoid contents and antioxidant activity by DPPH, ABTS and FRAP assays. In our study, we noted non-significant correlations between total flavonoids and the ABTS (p=-0.959) and DPPH (p= -0.931) tests, and between FRAP and ABTS (p=-

0.798) and FRAP and DPPH (p=-630) tests. This observation could suggest that the reducing power exhibited by the different fractions is not only a function of total phenolic compounds, but of the presence of other constituents with antioxidant potential [51].

According to Meng et al.[52], radical scavenging activity depends on the conjugation between the B- and C-rings which is affected by the structure, the number, and positions of hydroxyl groups, and the structural groups.

Dichloromethane fractions of leaves and stem bark of the plant wich combined the highest FRAP values (741.15 and 662.84 mMol AAE/g) and lowest $IC_{50}$ for both DPPH (463.33 and 383.33 µg/mL respectively) and ABTS (88.40 and 36.85 µg/mL) assays represent promising antioxidant candidates. These fractions were also found the best antibacterial fractions. The hexan fractions, although lacking antioxidant activity were the best antifungal fractions. A combination of these fractions could provide an effective broad-spectrum prototype against these susceptible microbial species.

**Table 6.** Correlation between antioxidant activity and the contents in total phenols and total flavonoids.

| P | CPT | CFT | $IC_{50}$ABTS | $IC_{50}$DPPH | $IC_{50}$FRAP |
|---|---|---|---|---|---|
| **CPT** | 1 | 0.749* | -0.534 | -0.458 | 0.919** |
| **CFT** | | 1 | -0.959** | -0.931** | 0.932** |
| **$IC_{50}$ABTS** | | | 1 | 0.503** | -0.798** |
| **$IC_{50}$DPPH** | | | | 1 | -0.630** |
| **$IC_{50}$FRAP** | | | | | 1 |

* indicate significance at P <0.05; **indicate significance at P < 0.01.

## 4. Conclusions

*Opilia amentacea* Roxb. Extracts/fractions were evaluated for phytochemical screening and their potential healing properties of dermatoses. This study revealed phenolic compounds such as flavonoids and tannins and non-polar compounds like sterols and triterpenes. *Opilia amentacea* was found rich in polyphenols and flavonoids. The dichloromethane and n-hexane fractions of the plant exhibited significant biological properties. Dichloromethane fractions demonstrated the best antioxidant and antibacterial activities while the hexan fractions showed the best antifungal activity. The antimicrobial properties of plant extracts are nowadays well documented. These results indicate that the plant may be used for the treatment of infectious dermatoses caused by *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus agalactiae* and *Candida tropicalis* species but also for non-infectious dermatoses regarding the antioxidant activity of the extracts and the richness of the plant in flavonoids.

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