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Research Article
Total Phenolics, Flavonoids and *in vitro* Antioxidant Properties of *Lophira lanceolata* TIEGH

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Abstract

Background and Objective: Oxidative stress is linked to many illnesses like cancer, diabetes, inflammatory disorder and aging. Thus, the search for plant reaches in polyphenolic compounds is apt to ameliorate these diseases. The present study aims at screening various solvent fractions of the methanol extract of *Lophira lanceolata* to ascertain it's *in vitro* antioxidant potentials. The antioxidant metabolites like phenolics and flavonoids were also quantified in the various solvent fractions. **Materials and Methods:** The methanol extract of *Lophira lanceolata* was purified using column chromatographic technique to afford *n*-hexane (HF), ethyl acetate (EF) and methanol (MF) factions respectively. The *in vitro* antioxidant activity of the solvent fractions was also determined while the Total Phenolics Content (TPC) was quantified using the Folin-Ciocalteu method and the Total Flavonoids Content (TFC) quantified colorimetrically using the aluminum chloride method. The total yield of the fractions and preliminary phytochemical analysis of the solvent fractions were also determined. **Results:** The phytochemical screening of the fractions revealed the presence of antioxidant metabolites like phenolics and flavonoids. The Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) of the fractions showed that EF had the highest value while HF had the least value. The half-maximal effective concentration (EC_{50}) of the various fractions was generally lower showing that the fractions have potential antioxidant properties. **Conclusion:** The data obtained showed the solvent fractions of *Lophira lanceolata* act as an antioxidant due to its free radical scavenging potentials.

Key words: *Lophira lanceolata*, antioxidant, DPPH, total phenolics, total flavonoids, phytochemical analysis

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The continued search for natural antioxidants is currently of great interest[1]. These natural antioxidants help in reducing damages to cells which results in disease conditions like cancer, diabetes, arthritis and ageing[2]. Plant polyphenols like flavonoids are good examples of natural antioxidants and act as a natural anti-oxidant by hydrogen transfer and/or single electron transfer[3].

Lophira lanceolata is a small to medium-sized deciduous tree growing up to 16 meters tall. It is widely distributed in the sub savannah zone. An infusion of young twigs of *L. lanceolata* is used for the treatment of fever, respiratory troubles and to relieve the gripping of dysentery in Nigeria[4].

In traditional medicine, the edible oil called '*meni*' is used to treat dermatosis, toothache, inflammation and rheumatism[5] while concoction prepared from the roots is used to alleviate menstrual pain in women[6]. The anti-plasmodial and antioxidant activities of the methanol leaf extract of *L. lanceolata* have been reported[5].

Cameroon traditional healers use the plant for the treatment of human onchocerciasis caused by *Caenorhabditis elegans*[7]. Steroids and steroidal glycosides isolated from the methanol extract of the roots exhibited remarkable anti-mycobacterial activity against the growth of *Mycobacterium tuberculosis*[8].

Qualitative phytochemical analysis of the ethanol extract of the leave of *L. lanceolata* showed the presence of polyphenolic compounds like flavonoids and phenols[9].

The present study is aimed at determining the total phenolics and flavonoids and *in vitro* antioxidant assay of the solvent fractions of the methanol leaf extract of *L. lanceolata*.

MATERIALS AND METHODS

Study area: The study was carried out at the Department of Pharmacology and Toxicology, University of Nigeria, Nsukka from October, 2018-June, 2019.

Chemicals: Methanol, ethyl acetate, *n*-hexane were obtained from Sigma Aldrich (Germany). Folin-Ciocalteu phenol reagent was obtained from Loba Chemie (India). Ferric chloride ($FeCl_3.6H_2O$), potassium ferric cyanide ($K_3Fe(CN)_6$) and ascorbic acid were obtained from JHD (China), hydrogen peroxide was bought from BDH (England), Trichloroacetic acid was procured from Qualikems (India) and phosphate buffer (pH 6.6 and 7.4) from JHD (China). Sodium Hydroxide (NaOH), Quercetin, sulphuric acid (BDH, England), sodium nitrite ($NaNO_2$) and gallic acid (qualikems, India). Sodium carbonate (Na_2CO_3), aluminum chloride ($AlCl_3.6H_2O$), sodium phosphate (NaH_2PO_4) and ammonium molybdate were bought from JHD (China). Sodium nitroprusside and Griess reagent was procured from Sigma Aldrich, Germany. All chemicals used were of analytical grade.

Plant collection: The fresh leaves of *Lophira lanceolata* were collected from Kogi State, Nigeria in January, 2015. The plant was identified and authenticated by Mr. Ozioko, Alfred, a taxonomist and staff of the International Centre for Ethnomedicine and Drug Development (InterCEDD) Nsukka, Nigeria.

Extract preparation: The air-dried leaf (500 g) of *L. lanceolata* were macerated with 2.5 L methanol and extracted by cold maceration at room temperature for 48 hrs with agitation. The liquid extract was filtered and the filtrate concentrated under reduced temperature (40°C) using a rotary evaporator to yield the dry extract.

Column chromatographic fractionation of the extract: The methanol extract of *L. lanceolata* was purified by column chromatographic method[10]. Briefly, twenty grams (20 g) of the extract was purified on silica gel (60-200 mesh, 500 g) packed into a glass column (1.5×150 cm). The column was eluted with *n*-hexane (2.5 L), ethyl acetate (2.5 L) and methanol (2.5 L) to afford three solvent fractions viz: *n*-hexane (HF), ethyl acetate (EF) and methanol (MF) respectively. The solvent fractions were concentrated *in vacuo* at a reduced temperature (40°C) to yield the dried fractions.

Qualitative phytochemical analysis of the fractions: The qualitative phytochemical analyses of the fraction were done to determine the presence of flavonoids, tannins and phenolics according to standard methods[11].

Test for flavonoids: A total of 0.50 g of the fractions were boiled in 5 mL of distilled water and filtered. 5 mL of 10% (v/v) ammonia solution was added to the filtrate followed by three drops of concentrated H_2SO_4. The presence of flavonoids was confirmed by yellow coloration which disappears on the addition of H_2SO_4.

Test for tannins/phenolic: About 0.50 g of the fractions were boiled in 5 mL of distilled water and filtered. Two drops of 0.1% (v/v) $FeCl_3.6H_2O$ was added to the filtrate and observed for color change. A blue-black coloration was taken as evidence for the presence of tannins or phenolics.

Quantitative phytochemical analysis

Total Phenolics Content (TPC) determination: Folin-Ciocalteu method was used for the determination of the total phenolics content of the *L. lancelata* fractions using gallic acid as an internal standard[12]. Briefly, 1 mL (1 mg mL^{-1}) of the fractions was mixed with 9 mL of distilled water in a 25 mL volumetric flask. A 2.5 mL of a 10-fold diluted Folin-Ciocalteu phenol reagent was then added. After 5 mins, 10 mL of 7.5% (w/v) Na_2CO_3 solution was added to the mixture and made up to the mark with distilled water. The mixture was incubated in the dark for 90 mins at room temperature. A set of standard solutions of gallic acid (10-100 µg L^{-1}) were prepared in the same manner as described for the fractions. The absorbencies of the fractions and standard solutions were read against the reagent blank at 760 nm with a UV/visible spectrophotometer. The total phenolics content was determined from the calibration curve and expressed as milligram of Gallic Acid Equivalent (GAE) per gram of the fractions. The determination of the total phenolics in the fractions was carried out in triplicates.

Determination of the Total Flavonoids Content (TFC): Aluminum-chloride colorimetric assay was used to determine the total flavonoid content in the fractions as previously reported[13]. Briefly, 1 mL (1 mg mL^{-1}) of the fractions was mixed with 4 mL of distilled water in a 10 mL volumetric flask. A 0.3 mL of 5% (w/v) sodium nitrite was added to the flask. After 15 min 0.30 mL of 10% (w/v) $AlCl_3.6H_2O$ solution was added to the mixture, followed by the addition of 2 mL of 1.0 M NaOH after 5 min and diluted to the mark with distilled water. A set of standard solutions of quercetin (10-100 µg L^{-1}) were prepared in the same manner as described for the fractions. The absorbencies of the fractions and standard solutions were measured against the reagent blank at 510 nm with a UV/Visible spectrophotometer. The total flavonoids content was determined from the calibration curve and expressed as milligram of Quercetin Equivalent (QE) per gram of fractions[14]. The determinations of the total flavonoids in the fractions were carried out in triplicates.

In vitro antioxidant assays

DPPH radical scavenging activity: The ability of solvent fractions to scavenge 1, 1-diphenyl-2-picrylhydrazine (DPPH) free radicals was assessed using standard method[12]. Briefly, 3 mL of the DPPH solution (4.5 mg/100 mL of methanol) was added to 1 mL of different concentrations of the fractions (2-10 µg mL^{-1}) and the mixture was incubated at room temperature for 30 min in the dark. Control was prepared as described above but without the fractions and methanol used for the baseline correction. The absorbance of the mixture and the standard (ascorbic acid) were determined at 517 nm against a blank with a UV/Visible spectrophotometer (UV-1800, Shimadzu, Japan). The assay was carried out in triplicate. The percentage inhibition was determined using the formula[15]:

$$\text{Inhibition (\%)} = \frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} \times 100$$

The EC_{50} (the microgram of the fraction that can scavenge 50% of the radicals) values were estimated from the percentage inhibition versus concentration plot, using a non-linear regression algorithm. A lower IC_{50} value indicates the greater antioxidant activity of the fractions.

Nitric oxide scavenging activity: Nitric oxide scavenging activity was determined according to the method described by Senguttuvan *et al.*[16] with slight modification. Briefly, 2 mL of 10 mM sodium nitroprusside in 0.5 mL phosphate buffer saline (pH 7.4) was added to 0.5 mL of the various concentrations (2-10 µg L^{-1}) and the mixture was incubated at 25°C for 30 min. Thereafter, 1.5 mL of Griess reagent (1% sulphanilamide and 0.1% N-(1-napthylethyl) enediamine dihydrochloride in 3% (v/v) *ortho*-phosphoric acid) was added. The absorbance of the mixtures was measured immediately at 546 nm. A reaction blank was prepared for each measurement by replacing the Griess reagent with water. The percentage inhibition of nitric oxide of the fractions was calculated using the formula DPPH. The EC_{50} values were calculated from the percentage inhibition versus concentration plot using a non-linear regression algorithm.

Ferric Reducing Antioxidant Power (FRAP): The Ferric Reducing Antioxidant Power (FRAP) of the fractions was determined by the method as described by Sahreen *et al.*[17]. Briefly, 2 mL of the various concentrations (2-10 µg L^{-1}) of the fractions were mixed with 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 2 mL of freshly prepared potassium ferric cyanide (0.1% w/v). The mixture was incubated in a water bath at 50°C for 20 min. Then 2 mL of trichloroacetic acid (10% w/v) was added to the mixture and centrifuged at 3000 rpm for 10 min. The supernatant (2.0 mL) was mixed with 2 mL of distilled water and 0.5 mL of 0.1% w/v ferric chloride. The absorbance of the mixture was measured at 700 nm. Ascorbic acid was used as the control. All the tests were performed in triplicate. The percentage inhibition of ferric

reducing activity power was measured by comparing the absorbance values of control and test samples using the formula[15]:

$$\text{Inhibition (\%)} = 1 - \frac{\text{Absorbance}_{\text{control}}}{\text{Absorbance}_{\text{sample}}} \times 100$$

EC_{50} values were estimated from the % inhibition versus concentration plot using a non-linear regression algorithm.

Total Antioxidant Capacity (TAC) by phosphomolybdate assay: The total antioxidant capacity of the fractions was determined by the phosphomolybdate method as described by Jan *et al.*[18]. Briefly, 0.1 mL aliquot of various concentrations of the plant fractions (2-10 μg L^{-1}) was mixed with 1 mL of reagent solution (600 mM sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate 1:1:1). The sample tubes were covered with aluminum foil and incubated in a water bath at 95°C for 90 min. The mixtures were cooled to room temperature and the absorbencies of the mixture were determined at 765 nm against a blank containing 1 mL of the reagent solution. Ascorbic acid was used as a control. The assay was carried out in triplicate. The antioxidant capacity was estimated by using the formula DPPH. The EC_{50} values were then determined was determined from the regression curve.

Hydrogen peroxide scavenging assay: Scavenging activity of Hydrogen peroxide (H_2O_2) of *Lophira lanceolata* fractions was determined by the method described by Ruch *et al.*[19]. Briefly, 4 mL of the fractions prepared at various concentrations (2-10 μg L^{-1}) were mixed with 0.6 mL of 4 mM H_2O_2 solution prepared in phosphate buffer (0.1 M, pH 7.4) and incubated for 10 min. The absorbance of the solution was measured at 230 nm against a blank. Ascorbic acid was used as the control. The percentage of inhibition was calculated by comparing the absorbance value of the control and test samples using the formula as DPPH. All the tests were performed in triplicate. EC_{50} values were estimated from % inhibition versus concentration plot, using a non-linear regression algorithm.

Data analysis: All experiment was performed in triplicates and data reported as mean±standard deviation. Statistical analyses were performed using GraphPad Prism 5 software was performed followed by Dunnett's *post hoc* test. Differences between test and control treatments are considered significant at $p<0.05$.

RESULTS

Percentage yield: The percentage yield of the different solvent fractions is shown in Table 1. The percentage yield of the fractions varied from 2.08-2.71% with the methanol fraction having the highest yield.

Qualitative phytochemical analysis: The phytochemical screening of the solvent fractions showed the presence of antioxidant secondary metabolites like flavonoids and tannins/phenolics.

Total phenolic determination: The total phenolics content of the fractions varied widely between 124.90-347.50 mg GAE g^{-1} (Table 2). Ethyl acetate fraction had the highest total phenolics content (496.10±3.10 mg GAE g^{-1}) than the other solvent fractions.

Total flavonoids determination: The total flavonoid content was highest in the ethyl acetate fraction (315.0±1.23 mg GAE g^{-1}) followed by the methanol fraction (267.0 mg GAE g^{-1}) and *n*-hexane fractions (98.0±3.90 mg GAE g^{-1}) as shown in Table 2.

Radical scavenging activity: The radical scavenging ability of the fractions was in the following order EF>MF>HF. The EC_{50} values of the fractions of *L. lanceolata* were 2.05, 0.61 and 1.25 μg mL^{-1}, respectively (Table 3) for HF, EF and MF. The

Table 1: Percentage yield of different solvent fractions of *L. lanceolata*

Solvent fractions	Yield (%)
n-hexane	2.59
Ethyl acetate	2.08
Methanol	2.71

Percentage yield of the extracts is based on dried methanol extract

Table 2: Total phenolic and flavonoid contents of the solvent fractions of *L. lanceolata*

Solvent fractions	Total phenolics (mg GAE g^{-1})	Total flavonoids (mg QE g^{-1})	Flavonoids/phenolics (F/P ratio)
n-hexane	124.90±3.70	98.0±3.90	0.79
Ethyl acetate	496.10±3.10	315.0±1.23	0.64
Methanol	347.50±1.78	267.0±9.0	0.77

Values are expressed as mean±SD (n = 3). The absorbance against the reagent blank was determined at 710 and 510 nm with a UV/Visible spectrometer for phenolics and flavonoids, respectively. Total phenolics content was expressed as mg Gallic Acid Equivalents (GAE) and total flavonoid content expressed as mg Quercetin Equivalents (QE)

Table 3: Radical scavenging activities of *L. lanceolata* fractions at different concentrations

	EC$_{50}$ values (µg mL^{-1}) of radical scavenging				
Fractions	DPPH	Nitric oxide	FRAP	TAC	Hydrogen peroxide
n-hexane	2.05	0.05	1.10	3.34	6.76
Ethyl acetate	0.61	1.09	1.86	7.14	7.89
Methanol	1.25	2.36	1.10	2.51	4.63

DPPH: DPPH radical scavenging activity, FRAP: Ferric reducing antioxidant power, TAC: Total antioxidant capacity

nitric oxide scavenging ability of the fractions was in the following order HF>EF>MF. The EC$_{50}$ values of the fractions were 0.05, 1.09 and 2.36 µg mL^{-1} for HF, EF and MF respectively (Table 3).

DISCUSSION

Therefore, this study was carried out to evaluate the antioxidative effect of the solvent fractions of *L. lanceolata*. Various *in vitro* antioxidant models were used to assess the antioxidant activities of the solvent fractions. A previous study showed that the methanol leaf extract of *L. lanceolata* exhibited strong anti-plasmodial and antioxidant activity[5].

The lower EC$_{50}$ values of EF and MF could be attributed to the high level of flavonoids and phenolics in the fractions. The mechanism of antioxidants with DPPH radical scavenging activity results from the donation of hydrogen to free radicals *in vitro*, thus ameliorating its scavenging effect[20]. Polyphenolic compounds present in the solvent fractions could be exerting their antioxidative properties via this mechanism. The ability of the solvent fractions of *L. lanceolata* to scavenge oxygen, thus producing nitrite ions was determined using nitric oxide assay as shown in Table 3.

Nitric oxide plays a major role in the prolongation of inflammation and immunological responses which results from oxidative stress[21]. The synthesis and release of NO promote inflammation; an ailment caused by free radicals. It has been reported that plant extracts or fractions could act as scavengers of NO or inhibitors of its production[22], thus acting as a good anti-oxidant. The solvent fractions exhibited an ability to inhibit the production of nitrite radical with EC$_{50}$ values of 0.05, 1.09 and 2.36 µg mL^{-1} for HF, EF and MF respectively. Our findings agree with the previously reported study that catechin (a standard antioxidant) exhibited good nitrite radical scavenging ability (IC$_{50}$ = 1.66 µg mL^{-1}) which compares favorably with the nitrite scavenging ability of the solvent fractions[23]. We postulate that the solvent fractions of *L. lanceolata* inhibited the nitrite radical formation competing with oxygen in the reaction[24].

The ferric reducing power of the solvent fractions was determined by determining the conversion of ferric ion (Fe^{+3}) to ferrous ion (Fe^{+2}). The reducing ability of the solvent fractions increased with an increase in concentration. The *n*-hexane fraction showed the highest reducing ability followed by the methanol fraction. The finding is in agreement with previous research that reported that the ability of plant extracts to reduce Fe^{+3} to Fe^{+2} could be attributed to the presence of polyphenolic compounds[25]. The EC$_{50}$ values of the fractions were 1.104, 1.859 and 1.102 µg mL^{-1} for HF, EF and MF respectively. The antioxidant capacity of the various solvent fractions was found to increase in this order: EF>HF>MF (Table 3). The mechanism of ferric reducing antioxidant power is based on electron transfer reducing ferric ion to the ferrous ion[26]. The reducing ability of the solvent fractions increased with an increase in concentration. The *n*-hexane fraction showed the highest reducing ability followed by the methanol fraction.

The antioxidant capacity of the various solvent fractions was found to increase in this order: EF>HF>MF (Table 3). The solvent fractions showed dose-dependent antioxidant activity. The EC$_{50}$ value o f antioxidant capacity were 3.34, 7.14 and 2.1 µg mL^{-1} respectively for HF, EF and MF. The hydrogen peroxide scavenging ability of the various fractions was concentration-dependent as shown in Table 3. Methanol fraction showed high scavenging ability (EC$_{50}$ = 4.63 µg mL^{-1}) followed by the *n*-hexane fraction with IC$_{50}$ value of 6.67 µg mL^{-1}. Extracts and compounds are considered to have high antioxidant capacity if the IC$_{50}$ value is less than 10 or 1 µg mL^{-1} respectively for extracts or compounds[27]. The result showed that the solvents fractions exhibited high antioxidative capacity since their EC$_{50}$ values were less than 10 µg mL^{-1}.

The H_2O_2 scavenging ability of the various fractions were in the order: EF>MF>HF, indicating that the methanol fraction has the highest H_2O_2 scavenging ability. Hydrogen peroxide scavenging ability of the solvent fractions could be attributed to the electron donating ability of the polyphenolic present in the fractions, which converts the H_2O_2 to H_2O, thus reducing its toxic effects to the cells since it decomposes to hydroxyl radicals *in vivo* unlike water[28].

To evaluate the correlation between the polyphenolic compounds, present in the solvent fractions the flavonoids/phenolics was for the fractions. The result showed that the flavonoids/phenolics was highest in the *n*-hexane fraction (0.79), followed by the methanol fraction (0.77) with the ethyl acetate fraction having the least value (0.64) (Table 2). The present study has shown that solvent fractions of *L. lanceolata* could be a source of drugs for the management of oxidative stress ailments. Research is on-going aimed at isolating and characterization of the bio-active compounds responsible for the activity.

CONCLUSION

The presence of polyphenolic constituents in *L. lanceolata* has explained its use in the traditional medicine in the management of inflammation and rheumatism. Oxidative stress has been reported to cause inflammatory disorders. Our present study showed that the solvent fractions of *L. lanceolata* possess *in vitro* antioxidant properties and this is attributed to the presence of polyphenolic compounds in the fraction. Further research is recommended for the isolation and characterisation of the active principles responsible for this activity.

SIGNIFICANCE STATEMENT

This study discovers the antioxidant potentials of the solvent fractions of *Lophira lanceolata* that can be beneficial for the management of oxidative stress-related ailments prevalent in our society. This study will help the researcher to uncover the critical areas of an anti-oxidative assay that many researchers were not able to explore. Thus, a new theory on isolation and characterization of the bioactive principles responsible for this activity may be arrived at.

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