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Der Pharmacia Lettre, 2015, 7 (11):62-67
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Phytochemical screening and in vitro evaluation of the antibacterial properties of *Terminalia macroptera* stem bark extracts against selected pathogenic bacteria

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ABSTRACT

Terminalia macroptera alstone belonging to the Combretaceae family have been investigated for evaluation of their phytochemical composition and antibacterial properties. Aqueous and ethanolic_{70%} extract of plant were tested against three clinical strains of *Escherichia coli* (ESBL) and three clinical strains of *Klebsiella pneumonia* (ESBL), by well plate and dilution methods. Selected antibacterial agents (ceftazidim and ceftriaxon) were used as positive reference standards in the tests. The screening revealed the presence of metabolites such as flavonoids, phenolic compounds, tanins, saponins, sterols, terpens and cardiac glycosids. The aqueous extract has presented a bacteriostic power against almost tested strains through a ratio MBC/MIC=4. However, ethanolic_{70%} extract showed strong antibacterial activity against *E. coli* with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) ranged from 6.25 to 25 mg/ml and 12.50 to 50 mg/ml, respectively. It also showed on *K. pneumonia* strains MIC varying between 6.25 and 50 mg/ml and MBC 12.50 to 50 mg/ml. So ethanolic_{70%} extract was found to be potential bactericidal against all strains tested.

Keywords: *Terminalia macroptera*, aqueous and ethanolic extracts, phytochemical analysis, antibacterial properties

INTRODUCTION

In recent decades, there is a progressive increase in antibiotic resistant strains of clinically important pathogens [1, 2]. Despite the advancement in science and technology on the discovery of many natural and synthetic drugs, infectious diseases are still the leading cause of morbidity and death, especially in developing countries [3, 4]. The outlook for the use of antimicrobial drugs in the future is still uncertain. Alternative actions must be taken as to reduce the incidence of conventional therapeutic failure to treatments. Among the potential sources of new agents, plants have long been investigated. They are known to produce a variety of compounds to protect themselves against a variety of pathogens [5].

Thus, in order to face the challenges of bacterial infections and emerging drug resistance issues, many scientific researchers try to evaluate the quality, safety and efficacy of the medicinal plants through high throughput modern techniques and looking for new leads to develop better new chemical entities and drugs against microbial infection. It is in this line of alternative therapeutic approach that we have designed this study on *Terminalia macroptera* (Combretaceae) that has been locally used to treat many diseases in Ivory Coast and tropical countries of Africa. In

other earlier studies, different parts of plant have been used in the treatment of diseases such as gastro- intestinal, disorder like dysentery and acute diarrhea [6], the eczema [7]; stomach aches [8] and other biological properties of the plant have been carried out.

The present work aims at identifying active phytochemical compounds and to study the antimicrobial properties of the aqueous and ethanolic_{70%} stem bark extracts of *Terminalia macroptera*

MATERIALS AND METHODS

Plant Material

It consists of bark *Terminalia macroptera* Guill. et Perr. (*combretaceae*). These barks were collected in April 2012 in Niakara (north of Ivory Cost). Their authentication was performed by professor Ake-Assi of National Center Floristic (NCF), University Felix Houphouet Boigny of Cocody-Abidjan where a sample is retained.

Bacterial strains

The bacteria used for the biological tests are *Escherichia coli* (EC) and *Klebsiella pneumoniae* (KP) ESBL. All the bacteria strains were provided by the department of bacteriology and virology, Institute Pasteur of Ivory Cost. (I.P.IC). These strains come from to organic product are : urine (EC 326, EC 529, KP 421), pus (EC 792, KP 322) and sputum (KP 314).

Preparation of plants extracts

The stem barks of *T. macroptera* collected were washed cut and has been dried shelter powder by a type IKAMAG-RCT grinder. According to the methods described by [9,10], 100 g of plant powder have been macerated in 1 L of distilled water then homogenized under magnetic agitation for 24 hours at 25° C with a IKAMAG-RCT type agitator. The homogenate obtained, has been filtered successively two times through hydrophilic cotton (cotton wool) then once through whattman paper n°2. The volume of filtrate obtained is first reduced with a rot vapor Büchi at 60° C. Then, the rest of the filtrate is evaporated with a med center vent cell drying oven at 50° C to give a brown powder which is the aqueous extract. The same process was carried out by using ethanol 70% instead of distilled water to obtain ethanolic extract 70% [11]. The end, all the plant extracts obtained are kept in refrigerator until used for testing antibacterial.

Study of the antibacterial activity of different extracts

For each bacterial strain, inoculums was prepared by homogenizing 0.3 ml of a suspension opalescent 3 hours in to 10 ml of Muller-Hinton broth concentrate twice in order to obtain a bacterial load estimated at 5.10^6 CFU/ml and constitute the dilution 10^0 .

Determining zones of inhibition of growth

The susceptibility test have been carried out on Muller-Hinton agar (Bio-Rad, France) by using wells [12] so , like in the case of classic anti-biogram realization, each well or hole of 6 mm in diameter has been filled with 80 µl of extract concentration 200 mg/ml. Taking care to separate two holes at least 20 mm. A control well was carried out for each bacterial strain with 80 µl of the solution mixture of DMSO/Sterile distilled water V/V [13, 14]. After a pre-release of 45 minutes at room temperature under the hood, the whole was incubated in an even at 37° C for 18 to 24 hours. Meanwhile, the Ceftazidim (CAZ) and Ceftraxon (CRO) used as positive controls. After incubation; the action of extracts is determined by measuring an area of growth inhibition (lack of colonies) around the well.

Determination of Minimum inhibitory concentration (MIC) and Minimum Bactericidal concentration (MBC)

Dilution method in liquid medium was used to determine these antimicrobials parameters [15]. This, in a series of 8 hemolytic tubes numbered C₁ to C₉ was introduced 1 ml of the bacterial inoculums esteemed to 5.10^6 UFC. Then 1 ml of a plant extract with a known concentration according to the range of prepared concentration has added in the same tubes. This distribution of plant extract is made so that 1 ml of plant extract 200 mg/ml is transferred to the tube C₁, that of 100 mg/ml, to the tube C₂ and so on until to C₇ tube which receive 1 ml of plant extract of 3.125 mg/ml. The C₈ receive, instead of plant extract, 1 ml of DMSO/distilled water (1/9, V/V) was used as control. This distribution of plant extract concentration is well known to each tube containing 1 ml of inoculums already reduced the concentration of the plant extract in the middle half. So the tube C₁ concentration increased from 200 mg/ml to 100 mg/ml, 100 mg/ml to 50 mg/ml for C₂ so on until a concentration of 3.125 mg/ml for T₇. This experiment was performed identically for each extract tested. The eight (8) first tubes (C₁ to C₈) are collected "experimental tubes" and the last tube C₈ is rated "growth control tube or T_C". These loaded tubes are incubated at 37°C for 24 hours. The experience has been reported three times.

The MIC is the concentration of the first tube where it finds no trouble visible to the naked eye. From the MIC lowest concentration that allows at most 0.01% survival of bacteria in the first suspension to survive within 24 hours corresponds to MBC. Therefore, the calculation of the ratio MBC/MIC of the extracts has permitted to determine their antibacterial **power**.

Phytochemical analysis

The phytochemical analysis of the different extract of *Terminalia macroptera* have based on the coloration and precipitation test [16, 17].

Test for alkaloids

0.5 g of extract was diluted into 10 ml with acid alcohol, boiled and filtered. To 5 ml of the filtrate was added 2 ml of dilute ammonia. 5 ml of chloroform was added and shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 10 ml of acetic acid. This was divided into two portions. Mayer's reagent was added to one portion and Draggen dorff's reagent to the other. The formation of a cream (with Mayer's reagent) or reddish brown precipitate (with Draggen dorff's reagent) was regarded as positive for the presence of alkaloids.

Test for polyphenols and tannins

About 0.5 g of the extract was boiled into 10 ml of water in a test tube and then filtered. A few drops of 0.1% of ferric chloride was added and observed for brownish green or a blue-black coloration.

Test for terpenoids (Salkowski test)

To 0.5 g each of the extract was added 2 ml of chloroform. Concentrated H_2SO_4 (3 ml) was carefully added to form a layer. A reddish brown coloration of the interface indicates the presence of terpenoids.

Test for glycosides

Extracts was treated with 2 ml of glacial acetic acid, add 1 drop of FeCl_3 and 1 ml of concentrated H_2SO_4 appearance of brown coloration indicates the glycosides.

Test for flavonoids

Three methods were used to test for flavonoids. First, dilute ammonia (5ml) was added to a portion of an aqueous filtrate of the extract. Concentrated sulphuric acid (1ml) was then added. A yellow coloration that disappears on standing indicates the presence of flavonoids. Secondly, a few drops of 1% aluminium solution were added to a portion of the filtrate. A yellow coloration indicates the presence of flavonoids. Next, a portion of the extract was heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. A yellow coloration indicates the presence of flavonoids.

Test for saponins

To 0.5 g of extract was added 5 ml of distilled water in test tube. The solution was shaken and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken after which it was observed for the formation of an emulsion.

Fehling's test:

Filtrates were hydrolysed with dil. HCL neutralized with alkali and heated with fehling's A and B solution. Formation of red precipitate indicates the presence of reducing sugars.

Test for steroids and terpenoids:

9 ml of ethanol was added to 1 g each of the extracts and refluxed for a few minute and filtered. Each of the filtrates was concentrated to 2.5 ml in a boiling water bath. Distilled water, 5ml was added to each of the concentrated solution, each of the mixtures was allowed to stand for 1 h and the waxy matter was filtered off. Each of the filtrates was extracted with 2.5 ml of chloroform using a separating funnel. To each 0.5 ml of the chloroform extracts in a test tube was carefully added 1 ml of concentrated sulphuric acid to form a lower layer. A reddish-brown interface showed the presence of steroids. To another 0.5 ml each of the chloroform extract was evaporated to dryness on a water bath and heated with 3 ml of concentrated sulphuric acid for 10 min on a water bath. A grey color indicates the presence of terpenoids.

Statistical analysis

The data are presented as mean \pm S.E.M. All the data were analyzed by one-way ANOVA and differences between the means were assessed with Newman-Keuls Multiple comparison test. Differences were considered significant at $p < 0.05$. All analyses were carried out using Graph pad software, version 5.01 (USA).

RESULTS

The antimicrobial activity of the aqueous and ethanolic_{70%} extracts of *T. macroptera* against three strains of *E. coli* (ESBL) and *K. pneumoniae* (ESBL) are shown in table 1. Comparatively to standard antibacterial agents, the plant extracts have shown significantly ($p < 0.05$) maximum zone of inhibition against all tested bacteria varying 12.33 to 18.33 mm for aqueous extract and 15.67 to 22.33 mm for ethanolic extract. If the plant extracts have proved the best activities against all the tested microorganisms, it's not the case of the standard antibiotics: ceftazidim (CAZ) and ceftriaxon (CRO). These antibiotics have given the lowest activities. The diameters of zone inhibition of these antibiotics have varied from 6.67 to 16.00 mm. On the basis of these inhibition diameters, ethanolic extract has been proved as the most active as aqueous extract with maximum diameter 22.33 mm against 18.33 mm for aqueous extract.

Table 1: Sensitivity of aqueous and ethanolic_{70%} extracts of stem bark on bacterial strains: zone of inhibition diameters (mm)

Bacterial strains	Aqueous	Ethanolic _{70%}	Ceftazidim	Ceftriaxon
<i>E. coli</i> 792	16.00±1.00 ^a	20.67±0.66 ^a	8.00±0.66 ^b	10.33±0.88 ^b
<i>E. coli</i> 326	18.33±0.88 ^a	22.33±0.33 ^a	16.00±0.33 ^a	14.00±0.88 ^a
<i>E. coli</i> 529	16.33±0.88 ^a	20.33±0.33 ^a	12.33±0.88 ^a	12.67±0.88 ^a
<i>K. pneumoniae</i> 421	12.33±0.33 ^a	16.33±0.33 ^a	16.00±0.57 ^a	9.33±0.57 ^b
<i>K. pneumoniae</i> 322	14.00±0.57 ^a	19.00±0.58 ^a	14.00±0.66 ^a	9.33±0.66 ^b
<i>K. pneumoniae</i> 314	13.33±0.66 ^a	15.67±0.66 ^a	10.00±0.58 ^b	6.67±0.66 ^b

Note: values are the mean of three tests ± S.E.M; Mean values with the same superscript within a row do not differ significantly ($p < 0.05$), in each Column for the same strain..

Phytochemical screening

The phytochemical screening of *T. macroptera* stem bark extract, using different standard tests shown in table 2, revealed that the aqueous extract showed the presence of polyphenolic compounds, flavonoids, saponins, galic tannins, cardiotonic glucosides. but alkaloids, sterols, terpens, coumarins and cathechic tannins were absents. Also table 2, showed that ethanolic extract contains all the compounds of aqueous extract with the presence of sterol and terpens.

Table 2: Phytochemical constituents of aqueous and ethanolic_{70%} extracts of stem bark *T. macroptera*

Chemical class	Alc	Polyph	Flav	ST	TGal	TCat	Coum	Sap	GlC
Aqueous extract	-	+	+	-	+	-	-	+	+
Ethanolic _{70%} extract	-	+	+	+	+	-	-	+	+

Key: Alc: Alcaloides, Polyph: Polyphenols, Flav: Flavonoides, ST: Sterols & Terpens, TGal: Tanin Galique, TCat: Tanin Catechique, Coum: Coumarine, Sap : Saponosides, GlC : Glucosides cardiotoniques

Antibacterial activity

The antimicrobial parameters of the aqueous extract on the tested germs are presented in table 3. Minimum bactericidal concentration (MBC) has advanced 25 to 100 mg/ml, for all strains. But the strains of *Klebsiella pneumonia* recorded a MBC equal to 100 mg/ml. The Minimum inhibitory concentration (MIC) recorded equal to 25 mg/ml and the ratio MBC/MIC= 4, indicated that aqueous extract is bacteriostatic against *K. pneumonia*. Moreover in the same table 3, MIC recorded on the strains of *Escherichia coli* equal to 12.50 mg/ml and MBC varied 25 to 50 mg/ml. The ratio MBC/MIC= 2 for two strains of *E. coli* indicated a bactericidal power. MBC of ethanolic extract were ranged enter 12.50 and 50 mg/ml (table 4). Table 4 showed the ratio MBC/MIC≤2 for almost strains studied, indicated the bactericidal power of ethanolic extract.

Table 3: Antibacterial activity of aqueous extract of *T. macroptera*

Antimicrobials parameters (mg/ml)				
Bacterials strains	MIC	MBC	MBC/MIC	Interpretation
<i>Escherichia coli</i> 792	12.50	50	4	Bacteriostatic
<i>Escherichia coli</i> 326	12.50	25	2	Bactericidal
<i>Escherichia coli</i> 529.	12.50	25	2	Bactericidal
<i>Klebsiella pneumonia</i> 421	25	100	4	Bacteriostatic
<i>Klebsiella pneumoniae</i> 322	25	100	4	Bacteriostatic
<i>Klebsiella pneumonia</i> 314	25	100	4	Bacteriostatic

Key: MIC (Minimum Inhibitory Concentration); MBC(Minimum Bactericidal Concentration).

Table 4: Antibacterial activity of ethanolic_{70%} extract of *T. macroptera*

Bacterials strains	Antimicrobials parameters (mg/ml)			Interpretation
	MIC	MBC	MBC/MIC	
<i>Escherichia coli</i> 792	25	50	2	Bactericidal
<i>Escherichia coli</i> 326	12.50	25	2	Bactericidal
<i>Escherichia coli</i> 529.	6.25	12.50	2	Bactericidal
<i>Klebsiella pneumonia</i> 421	25	50	2	Bactericidal
<i>Klebsiella pneumoniae</i> 322	50	50	1	Bactericidal
<i>Klebsiella pneumonia</i> 314	6.25	12.50	2	Bactericidal

Key: MIC (Minimum Inhibitory Concentration); MBC(Minimum Bactericidal Concentration).

DISCUSSION

Aqueous and ethanolic_{70%} extracts tested in the present study displayed antibacterial activity on the bacteria strains tested. This suggests that these extracts possess broad spectrum activities. Moreover, differences were observed between their antibacterial activities. The antibacterial activity of aqueous extract was found to be moderate in almost the tested bacteria. These differences could be due to the difference in the chemical composition of these extracts as revealed by phytochemical analysis. Also in over studies, it has proved that the compounds like tannins, flavonoids, sterols and terpenoids posed some antibacterial properties [18, 19, 20, 21]. In our study, we have found all these compounds in the extracts studied. Thus we can confirm that the antibacterial activity of two extracts studied. This result confirms again *T. macroptera* importance in traditional medicine. However in the recent studies [22], showed antistaphylococcal activity of *T. macroptera*. The ethanolic extract improved the antibacterial activity where ceftazidim (CAZ) and ceftriaxon (CRO) well known broad spectrum antibacterial agent fail to be active. This result shows that ethanolic extract concentrated the active principle and it could be the best candidate for the treatment of diseases associated with these microorganisms than the aqueous extracts. Similar results were reported by [23], when evaluating the activity of *P. laxiflora* against *E. coli* ESBL and *K. pneumonia* ESBL. From this result, we can deduce that unlike water, ethanolic is solvent that allow a better extraction of antimicrobial compounds virtues as those identified in the corresponding extracts. These results confirm those of [24], who showed that the antimicrobial activities are related to the origin of the sample and the test strain as well as the nature of the solvent. Besides that, it has been reported that *E. coli* is responsible for 40-70% of urinary tract infection in hospitals [25, 26, 27]. Hepatobiliary infections and neuro-meningeal postsurgical are caused by *K. pneumonia* [28, 29]. The sensitivity of these strains to *T. macroptera* extract studied is of great importance because these strains are highly resistant to antibiotics used in clinical practice. Also, any antibacterial agent to which they are sensitive it deserves special attention.

CONCLUSION

This work has allowed us to highlight the antibacterial properties of *Terminalia macroptera* on *E. coli* and *K. pneumoniae*, two bacteria that produce beta-lactamase and extended-spectrum involved in a large number of bacterial infections. The ethanolic_{70%} extract showed the best bactericidal powers on *E. coli* (ESBL) and *K. pneumonia* (ESBL).

In view of the results obtained in the present work, *T. macroptera* could be used in phytomedicine to fight the germs tested involved in pathologies related. To this end, it would be interesting to undertake studies of toxicity of the extract which are found to be active and to consider the development of improved traditional medicine (ITM) after purification.

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