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In vitro evaluation of the antimicrobial properties of *Mallotus oppositifolium* decoction leaf extracts and fractions

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ABSTRACT

In order to verify the *in vitro* antimicrobial properties of *Mallotus oppositifolium* (Euphorbiaceae), the qualitative phytochemical screening and the antimicrobial activities on *Shigella dysenteriae* A2, *Salmonella typhi*, *Escherichia coli*, *Enterococcus faecalis*, *Staphyllococcus aureus* and *Candida albicans* strains of the aqueous decoction (DEMO) hexane (HEMO) and methanol (MEMO) fractions of leaves were assessed. The screening was performed using colorimetric methods. The antimicrobial activity was carried out using disc diffusion assays. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were determined by the dilution methods. The screening revealed the presence of metabolites such as alkaloids, flavonoids, phenolic compounds, tannins, saponins, lipids, carbohydrates, mucilages, proteins, triterpens and steroids. DEMO, HEMO, MEMO showed a concentration-dependent activity against *Shigella dysenteriae* A2 and *Salmonella typhi*, with inhibition zone ranging from 9.44±0.44 to 19.00±0.24 mm, 8.94±0.05 to 20.03±0.17 mm and 8.13±0.17 to 16.76±0.11 mm respectively. The MIC showed ranges from 0.25 to 1.00 $mg.mL^{-1}$, 0.20 to 0.50 $mg.mL^{-1}$ and 1.00 to 3.00 $mg.mL^{-1}$, while the MBC ranged from 1.00 to 5.00 $mg.mL^{-1}$, 0.50 to 1.00 and 3.00 to 10.00 $mg.mL^{-1}$ respectively. The leaves decoction and fractions activity on the two strains showed promising activities to justify the use of the plant against diarrhoea in folk medicine.

Keywords: Folk medicine, phytochemical analysis, *Mallotus oppositifolium* diarrhea, antimicrobial.

INTRODUCTION

In the southern countries biodiversity, plants are sources of traditional remedies that 80% of the population relies on, at least, for the treatment of the poverty related diseases (Tripathi and Tripathi, 2003) or in primary health care (WHO, 2006; Sule et al., 2010). The discovery of antibiotics provides efficient therapeutic agents against infectious diseases that so far are the leading causes of death (Chanda and Rakholiya, 2011). However, the clinical efficacy of many antibiotics is threatened by the emergence of multi-drug resistant pathogens (Barbour et al., 2004). This problem is further compounded in sub-Saharan Africa by the absence of systematic antibiotic susceptibility testing and thus the lack of appropriate guidelines for empiric treatment (Eyoh et al., 2014). In order to face the challenges of bacterial infections and the emerging drug resistance issues, the WHO has recommended evaluations of the

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quality, safety and efficacy of the medicinal plants through high throughput modern technics, and looking for new leads to develop better new chemical entities and drugs against microbial infections. It is in this line of alternative therapeutic approach that we have designed this study on *Mallotus oppositifolium* (Euphorbiaceae) that has been locally used to treat many diseases in Cameroon and the other equatorial and tropical countries of Africa. In other earlier studies, different parts of the plant (*cormus*) have been used in the treatment of diseases such as helminthiasis and gastro-intestinal disorder like dysentery and acute diarrhoea (Zintchem et al., 2009). The anti-inflammatory (Kamgang et al., 2004; Nwaehujor et al., 2012), antioxidant effects (Adedara et al., 2010) and other biological properties (Zintchem et al., 2013) of the plant have been carried out. Infectious diseases can be associated with the oxidative stress (Lykkesfeldt and Svenden, 2007; Jortzik and Becker, 2013). The present work aims at identifying active phytochemical compounds and to study the antimicrobial activity of the aqueous decoction leaves extract and fractions of *Mallotus oppositifolium*.

MATERIALS AND METHODS

Plant material and preparation of the extract and fractions

Mallotus oppositifolium (Mo) leaves were collected from the Gouife-Bep village (Bafia-Cameroon) on October 2012. The species was identified and authenticated in the National Herbarium of Cameroon by comparison with a voucher specimen deposited under the number HNC 16619. The leaves were thoroughly rinsed twice in running tap water and then in sterile water before being air-dried for 2 weeks at room temperature (22-26 °C). The dried leaves were ground into powder producing a fine texture using an electric blender, and kept in polyethylene bags ready for extraction. 1500 g of the sample powder in 5 L of distilled water was heated on water bath at 70 °C for 15 min. The decoction was filtered through cotton to remove debris. The resulting solution was filtered with Whatman N° 1 filter paper and concentrated *in vacuo* using rotatory evaporator at 45 ºC. 4/5 of the semi-solid decoction aqueous extract were re-dissolved in methanol and partitioned with n-hexane (3 × 200 mL) to obtain the hexane and methanol fractions that were concentrated in a rotary evaporator at 40-45 ºC. The 3 semi-solid parts of *Mallotus oppositifolium* leaves: the aqueous decoction extract (MEMO), the hexane (HEMO) and methanol (MEMO) fractions were stored at 4 ºC refrigerator for further use.

Chemicals reagent, solvents, growth indicators and positive control

The following chemicals and solvents (purchased from the Fortress Diagnostics Ltd, Antrim, United Kingdom) were used for the various experiments: picric acid, sodium hydroxide, copper sulfate, sulfuric acid, Fehling liquor, alcoholic potassium hydroxide, phenolphthalein, ferric chloride, chloroform, acetic anhydride, alcohol.

Four culture media were used for the microbial growth: Mueller Hinton agar (from Oxoid, Basingstoke, England), Sabouraud-Chloramphenicol agar (from Fortress Diagnostics Ltd, Antrim, United Kingdom), Salmonella-Shigella agar and MacConkey agar, (purchased from Liofilchem srl, Italy).

Antimicrobial susceptibility testing was performed by the disc diffusion method with commercially available disks of Ciprofloxacin (30 µg) and Nystatin (100 UI, Novadina Pharmaceutical Ltd, London, United Kingdom) used as reference antibiotics.

Microorganism stains

Microbial strains were obtained from the bacteriology unit of "Centre Pasteur", Cameroon. Five bacteria strains: *Escherichia coli* ATCC 35218, *Entroccocus feacalis*, *Salmonella typhi*, *Shigella dysenteriae* A2, *Staphylococcus aureus* and a fungus isolate: *Candida albicans*. The pure cultures of bacteria and fungus were maintained respectively in Muller-Hinton and Sabouraud-Chloramphenicol dextrose agar and stored at 4 °C under anaerobic conditions.

Phytochemical screening

The plant extract and fractions were subjected to qualitative chemical screening to

identify different phyto-constituents (Trease and Evans, 2009; Anyasor et al., 2010).

Antimicrobial susceptibility test

The susceptibility screenings of the test extract and fractions were done according to the methods described by Sheela and Ramani (2011). The sterilized (autoclaved at 121 °C for 15 min) medium (40 °C) was inoculated (1 mL.100 mL^{-1} of medium) with the suspension (10^5 CFU. mL^{-1}) of the microorganisms (matched to McFarland barium sulphate standard) and poured into a Petri dish to give a depth of 3-4 mm. Every disc (6 mm in diameter) was impregnated with 10 µL of each dilution (0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100 and 200 mg. mL^{-1}) of aqueous decoction extract, hexane and methanol fractions of *Mallotus oppositifolium* and placed on the surface of the inoculated Muller-Hinton agar. The inoculated plates were pre-incubated for 1 h at room temperature and later incubated at 37 °C for 18-24 h for bacteria and at 25 °C for 48 h for the fungal isolate. Ciprofloxacin (30 µg/disc) and Nystatin (100 µg/disc) were used as standards for antibacterial and antifungal activities respectively. Antimicrobial activity was evaluated by measuring the inhibition zone of the tested microorganisms. All inhibition assays were made in triplicate.

Determination of Minimal Inhibitory Concentration (MIC)

The Determination of minimal inhibitory concentration (MIC) of the aqueous decoction extract, hexane and methanol fractions of *Mallotus oppositifolium* leaves was carried out using the method of Akinyele et al. (2011). Two-fold serial dilutions of each extract and fractions were prepared and 2 mL aliquot of different concentrations of the solution were added to 18 mL of pre-sterilized molten Muller-Hinton agar at 40 °C to give final concentration regimes of 50 to 0.05 mg/mL. The medium was then poured into sterile Petri dishes and allowed to solidify. The surfaces of the media were streaked with 18 h old microorganisms cultures. The plates were later incubated at 37 °C for 18-24 h for bacteria and at 25 °C for 48 h for the fungal isolate. The inhibitory concentration was measured after 24 h of incubation. The MICs were taken as the lowest concentration of extracts that inhibited the visible growth of the tested microorganisms.

Determination of Minimum Bactericidal Concentration (MBC) and Minimum fungicidal Concentration (MFC)

The Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) of aqueous decoction extract, hexane and methanol fractions of *Mallotus oppositifolium* against the pathogens were determined using the method of Spencer and Spencer (2004). The plates of the MIC that showed no growth of the microbes were sub-cultured by streaking using wire loop on sterile Muller Hinton agar plates. The plates were incubated at 37 °C for 18-24 h and at 25 °C for 48 h respectively for bacteria and fungi. The Bactericidal and Fungicidal Concentration were measured after 24 h of incubation. The MBC and MFC were taken as the lowest concentration of the extract or fraction that showed no microbial growth on the agar plates.

All the antimicrobial assays were performed under strict aseptic conditions using axenic culture media, to ensure consistency of all findings.

Statistical analysis

Results of the three replicates were pooled and expressed as mean ± SEM. ANOVA was used to analyse the data, followed by adequate *post hoc*. A $p \leq 0.05$ was considered statistically significant.

RESULTS

Phytochemical screening

The phytochemical screening of *Mallotus oppositifolium* leaves using different standard tests as shown in Table 1 revealed that the aqueous decoction showed the presence of alkaloids, proteins, flavonoids, saponins, phenolic compounds, anthraquinones, tannins, triterpens, glucosides, and others as indicated in Table 1. Volatile and fixed oils were absent in the methanol fraction while gums and

mucilages were all absent in the hexane fraction.

Antimicrobial susceptibility test

The antimicrobial activity of the aqueous decoction extract, hexane and methanol fractions of *Mallotus oppositifolium* leaves are shown in Table 2. The plant extract and fractions significantly ($p \leq 0.05$) inhibited the growth of *Shigella dysenteriae* A_2, *Salmonella typhi* and did not show any significant antimicrobial activity against *Escherichia coli*, *Entroccocus feacalis*, *Staphylococcus aureus* and *Candida albicans* at concentrations ranging from 0.39 to 200 $mg.mL^{-1}$, using the agar disk diffusion method. Unlike the standard drugs Ciprofloxacin and Nystatin that showed significant inhibition ($p \leq 0.05$) the bacteria and yeasts. In that ranges, the diameter inhibitions zone (DIZ) was increased gradually with the increase of extract concentration. At 200 $mg.mL^{-1}$, maximum DIZs of DEMO, HEMO and MEMO were respectively 19.00±0.24, 20.03±0.24 and 16.76±0.11 mm ($p \leq 0.05$) against *Shigella dysenteriae* A_2, 18.50±0.25, 18.89±0.11, and 14.84±0.12 mm ($p \leq 0.05$) against *Salmonella typhi* (Table 2). At ranges 0.39 $mg.mL^{-1}$ to 0.78 $mg.mL^{-1}$, there were no inhibitions of DEMO, MEMO and HEMO on *Salmonella typhi.*

Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) presented in Table 3 indicated that the aqueous decoction extract as well as hexane and methanol fractions inhibited the growth of *Shigella dysenteriae* A_2 (1.00, 3.00, 0.50 $mg.mL^{-1}$ respectively) and *Salmonella typhi* (0.25, 1.00, 0.20 $mg.mL^{-1}$ respectively). The MIC/MBC ratio results of the three extract and fractions with *Shigella dysenteriae* A_2 were 5.00, 2.00 and 3.33 respectively whereas those with *Salmonella typhi* were 4.00, 2.50 and 3.00 respectively.

Table 1: Phytochemicals constituents of Mo leave aqueous decoction leave extract, methanol and hexane aqueous decoction leave fractions using different standard tests.

Name of the phytochemicals	Aqueous decoction	Methanol fraction	Hexane fraction
Alkaloids	+	+	+
Proteins	+	+	+
Flanonoids	+	+	+
Saponins	+	+	+
Phenolic compounds	+	+	+
Catecho-tannins	+	+	+
Galli-tannins	-	-	-
Anthraquinones	+	+	+
Steroidal compounds	+	±	+
Phlobatannins	+	+	+
Glucosides	+	+	+
Reducing substances (sugar)	+	±	+
Volatile oils	+	-	+
Fixed oils	+	-	+
Gums and Mucilages	+	+	-
Triterpens	±	±	±
Coumarins	+	+	+
Anthocyanones	+	-	-
Anthracenic glycosides	-	-	-
Poly-oses	-	-	-

+: present; -: absent;

Table 2: Antimicrobial activity of *Mallotus oppositifolium* aqueous decoction and fractions in comparison with standard drugs.

A		**Extract and fractions**									
		Concentrations of the extract and fractions (mg. m L^{-1})									
Fractions	**Microorganisms**	**200**	**100**	**50**	**25**	**12.50**	**6.25**	**3.12**	**1.56**	**0.78**	**0.39**
DEMO	*Shigella dysenteriae* A_2	19.00±0.24	18.39±0.08	16.96±0.38	15.41±0.32	13.97±0.14	12.65±0.18	11.42±0.32	10.84±0.08	9.37±0.45	9.42±0.33
	Salmonella typhi	18.50±0.25	17.09±0.39	15.03±0.33	13.53±0.35	12.02±0.01	11.38±0.35	10.83±0.44	9.44±0.44	-	-
	Escherichia coli	-	-	-	-	-	-	-	-	-	-
	Pseudomonas aeruginosa	-	-	-	-	-	-	-	-	-	-
	Staphylococcus aureus	-	-	-	-	-	-	-	-	-	-
	Candida albicans	-	-	-	-	-	-	-	-	-	-
HEMO	*Shigella dysenteriae* A_2	20.03±0.17	17.90±0.12	16.71±0.13	15.04±0.18	14.02±0.15	12.98±0.03	11.93±0.27	10.84±0.12	9.37±0.12	8.97±0.05
	Salmonella typhi	18.89±0.11	16.72±0.24	15.76±0.22	14.89±0.10	13.82±0.18	12.83±0.11	11.19±0.12	9.95±0.05	8.94±0.05	-
	Escherichia coli	-	-	-	-	-	-	-	-	-	-
	Pseudomonas aeruginosa	-	-	-	-	-	-	-	-	-	-
	Staphylococcus aureus	-	-	-	-	-	-	-	-	-	-
	Candida albicans	-	-	-	-	-	-	-	-	-	-
MEMO	*Shigella dysenteriae* A_2	16.76±0.11	14.88±0.12	13.55±0.08	12.72±0.16	11.90±0.08	10.60±0.34	10.36±0.12	9.78±0.11	8.93±0.17	-
	Salmonella typhi	14.84±0.12	13.53±0.30	12.51±0.25	11.30±0.05	10.92±0.04	10.21±0.07	9.42±0.13	8.13±0.09	-	-
	Escherichia coli	-	-	-	-	-	-	-	-	-	-
	Pseudomonas aeruginosa	-	-	-	-	-	-	-	-	-	-
	Staphylococcus aureus	-	-	-	-	-	-	-	-	-	-
	Candida albicans	-	-	-	-	-	-	-	-	-	-

B	Standard drugs	
Ciprofloxacin 30 µg	*Shigella dysenteriae* A_2	27.80±0.17
	Salmonella typhi	29.80±0.20
	Escherichia coli	26.35±0.24
	Pseudomonas aeruginosa	34.40±0.20
	Staphylococcus aureus	30.70±0.30
Nystatin 100 µg	*Candida albicans*	19.15±0.15

DEMO: decoction extract of *Mallotus oppositifolium*; HEMO: Hexane fraction of *Mallotus oppositifolium*; MEMO: Methanol fraction of *Mallotus oppositifolium*. Values are expressed as mean ± standard error of triplicates means within a column followed by same letters (s) do not differ significantly ($p< 0.05$) according to DMRT.

Table 3: Minimum inhibitory concentration (MIC), Minimum bactericidal concentration (MBC) in mg. m L^{-1} and MBC/MIC ratio.

	DEMO			MEMO			HEMO		
Microbial strains	**MIC**	**MBC**	**MBC/MC**	**MIC**	**MBC**	**MBC/MIC**	**MIC**	**MBC**	**MBC/MIC**
Salmonella typhi	1.00	5.00	5.00	3.00	10.00	3.33	0.50	1.00	2.00
Escherichia coli	-	-	-	-	-	-	-	-	-
Shigella dysenteriae A_2	0.25	1.00	4.00	1.00	3.00	3.00	0.20	0.50	2.50
Staphylococcus aureus	-	-	-	-	-	-	-	-	-
Pseudomonas aeruginosa	?								
Candida albicans	-	-	-	-	-	-	-	-	-

DISCUSSION

The objective of the present study was to identify some active phytochemical constituents and to study the antimicrobial activities of the decoction leaf extracts and fractions of *Mallotus oppositifolium* (Mo).

The results obtained showed that the leaves inhibited the growth of microbial causative agents of diarrhoea such as *Shigella dysenteriae* A_2 and *Salmonella typhi*. Extract of different parts of the plant have been shown to have anti-inflammatory, antioxidant, antidiarrheal, antibacterial, antifungal and antitrypanosomal properties (Kabran et al., 2012). In Cameroon for instance, the Mo leaves are used as decoction or heated in the traditional treatment of infectious diseases such as dysentery and acute diarrhoea (Zintchem, 2009). In this study, it was important to assess the use of water, methanol and hexane solvents to determine the therapeutic potential of the disease as the case of earlier studies conducted by Aiyelaagbe et al. (2007), to determine the higher degree of extracting capacity of the plant extract.

The phytochemical analysis of the plant decoction and fractions globally indicates the presence of secondary metabolites known as alkaloids, flavonoids, phenols, tannins, anthraquinones, saponins, sterols and glycosides. The lack of volatile and fixed oils in the methanol fraction and of gums and mucilages in the hexane fraction may have been due to the polarity and the extracting capacity of the solvents used in this study. The therapeutic effects of plants mostly ranged between those metabolites (Okigbo et al., 2009), acting individually or synergistically to exert the associated activities. Phenolic compounds, anthraquinones and tannins have been reported to possess antioxidant activities (Okwu and Elenike, 2006; Ozgen et al., 2010) and properties (Zintchem et al., 2013).

Antimicrobial properties of plants extract may be due to the presence of alkaloids, steroids, tannins, flavonoids, saponins and/or terpenoids (Oben et al., 2006; Teke et al., 2011). The antimicrobial effect of alkaloids may be through the lysis and the morphological changes of cells (Sawer et al., 2005) or through DNA intercalating or inhibiting synthesis (Guttat et al., 2003). Antimicrobial effects of flavonoids may be due to their capacity to increase the fluidity of the lipids membrane or to complex the extracellular proteins (Prasad et al., 2004). That of tannins can be attributed to the complex of transport proteins (Souza et al., 2008) or the coagulation of fungal protoplasm (Adekunle and Ikumapayi, 2006). Saponins are glycosides of steroids characterized by their foaming and lytic effect on cells (Okwu, 2005; Okigbo, 2009). They can serve as defence mechanism against microorganism's predation (Rai and Kon, 2013).

In Cameroon and several Central and West African's countries, *Mallotus oppositifolium* decoction leaves are used against dysentery and acute diarrhoea (Kamgang et al., 2001). In this investigation, *in vitro* antimicrobial effect of the plant against diarrhoea and related diseases were quantitatively assessed on the basis of the diameter of inhibitory zone (DIZ), the minimum inhibitory concentration (MIC) and the polarity of the extract solvents. The ratio between minimum bactericidal concentration (MBC) and MIC determined the bacteriostatic or bactericidal properties of the extract or fractions.

The DEMO has an antimicrobial effect against *Shigella dysenteriae* A_2 and *Salmonella typhi.* In traditional medicine where fresh water is the plant major extracting agent, decoctions may be more efficient in infectious diseases. That can justify the traditional use of Mo decoction leaves to treat infectious diarrhoea. As the bacteriostatic activity has been defined as a ratio of MBC/MIC of > 4 (Pankey and Sabath, 2004), That of DEMO in *Shigella* and *Salmonella*, 5 and 4 respectively can suggest that the extract was bacteriostatic.

The plants extract and fractions showed some level of concentration-dependent effect against standard strain of *Shigella dysenteriae* A_2 and *Salmonella typhi* by inhibiting their growth. Without any specific cut-off values as reference for categorizing antimicrobial effect of the plant, a lower MIC values indicated the effectiveness of the extract or fraction as antimicrobial agent.

The MEMO has less antimicrobial activity against *Shigella dysenteriae* A_2 and

Salmonella typhi than DEMO and HEMO. This may be due to the global number of antimicrobial heterogeneous phytochemicals in the decoction or fractions, functioning synergistically to combat the pathogens (Nanthakumar et al., 2014). The increase of heterogenicity of phytochemicals or secondary metabolites generally enhances their antimicrobial synergistic effect (Alam, 2009; Zakaria et al., 2010).

Conclusion

This current study gives an insight to some scientific justification for the utilization of extracts from *Mallotus oppositifolium* by the local indigenes from the equatorial and the humid tropical forest regions for the treatment of diarrhoea. The antimicrobial activity of the aqueous decoction extract and fractions (hexane, methanol) of the leaves may be due to the identified phytochemicals compounds in this study. However, it is important to note that despite the promising anti diarrhoea properties recorded by the fractions in this study there is a need for further study to purify the fractions, *in vitro*-antimicrobial activity guided fractionation to isolate and identify the compounds responsible for biological activity on pathogen models. Furthermore, those active secondary metabolites need to be tested on *in vivo*-induced diarrhoea on animal models. All research efforts to solve bacterial resistance are geared towards the promotion of plants of pharmaceutical importance such as *Mallotus oppositifolium.*

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