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Inhibitory Effect of Methanolic and Methanolic-Aqueous Mixture Extract of Leaves of *Plectranthus neochilus Schltr* (Lamiaceae) *and Bauhinia rufescens Lam* (Fabaceae) on Two Strains of Enterobacteria Producing Beta-lactamases

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Authors' contributions

This work was carried out in collaboration among all authors. Author MH designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors BD, BMP and SM managed the analyses of the study. Author DDR managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The objective of the study was to evaluate *in vitro* inhibitory effect of methanolic and methanolic-aqueous mixture extracts of *Plectranthus neochilus* Schltr (*P. neochilus*) and *Bauhinia rufescens* Lam (*B. rufescens*) on the growth of *Escherichia coli* 25922 and *Proteus mirabilis.* A phytochemical screening was carried out to highlight compounds (phenolic compounds, flavonoids, alkaloids) with antibacterial activity. Then, an antibiogram was Carried out to investigate the enzymes rendering the resistance. Finally, the E-test was used to evaluate the antibacterial activity of the extract mixture. The Screening results showed that both plants contain total phenolics, flavonoids and

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alkaloids compounds. The antibiogram has made it possible to establish the sensitivity profile of the strains tested with regard to certain antibiotics. The extract mixture showed antibacterial activity on both strains tested. In the present work, the different mixtures of extracts showed an inhibitory effect on *Escherichia coli* 25922 [a strain sensitive to almost all the antibiotics tested, in particular the three classes: beta-lactams (Ceftazidine, Ceftriaxone, Meropenem), quinolones (Levofloxacin, Ciprofloxacin) and aminoglycosides (Gentamicin, Amikacin)] and on *Proteus mirabilis* (a multiresistant strain with almost all the antibiotics tested).

Keywords: Bauhinia rufescens Lam.; Plectranthus neochilus Schltr.; antibacterial activity; Enterobacteriaceae; β-lactamase.

1. INTRODUCTION

Enterobacteria are gram-negative bacilli belonging to the family Enterobacteriaceae. These bacteria are generally mobile and facultative anaerobic [1,2] and are responsible for nosocomial infections, food poisoning, urinary tract infections, gastroenteritis, pneumonia, typhoid [3].

Significant resistance was found for several bacteria that have spread to hospitals and communities [4]. These resistances are also noticeable in enterobacteria [5,6,7]. In enterobacteria, these resistances are due to the acquisition and dissemination of extended-spectrum beta-lactamases (ESBL) [8]. Some Enterobacteriaceae are resistant to almost all antibiotics, including fourth-generation of cephalosporins [9]. These multidrug resistances are related not only to genetic and environmental factors of the microorganism but also to the improper and inappropriate use of antibiotics [10,11].

In Cameroon, at the regional hospital of Maroua, multi-resistant bacteria are increasingly isolated. The spread of these bacteria causes public health problems because it is difficult to treat the associated infections. The consequences of these resistances are associated with increased mortality, increased health care costs and the need to use expensive drugs [12,13].

However, traditional medicine around the world is either the primary mode of health care delivery, or a complement to it [14,15]. The use of this medicine is widespread and of great health and economic interest [16]. In most of the developing countries, medicinal plants are the most widely used means, especially in the rural areas, for health problems [17]. According to the World Health Organization, more than 80% of the African population uses plants for their health care. These medicinal plants are also important for pharmacological research and drug development. They are not only used directly as therapeutic agents, but also as raw materials for drug synthesis. They represent a significant source of new drugs; especially since they have lower side effects [18].

For this purpose, *Plectranthus neochilus* Schltr. and *Bauhinia rufescens* Lam. are herbs used in traditional medicine to treat several cases of illness (liver failure, dyspepsia, respiratory infections, malaria, gout, diarrheal diseases, dysentery). They belong respectively to the family of Lamiaceae and Fabaceae [19,20,21]. Both of these plants are known for their broad-spectrum antimicrobial activity and ubiquitous nature [22]. These are easily accessible sources with high antimicrobial activity. In view of the high prevalence of enterobacterial infections, of their increasing antimicrobial resistance, of the expensive cost of the last generation of antibiotics; the exploration of medicinal plants is an appropriate alternative. The aim of this work was to evaluate the antibacterial activity of the mixture of extracts from *Plectranthus neochilus* Schltr. and *Bauhinia rufescens* Lam. on two strains of enterobacteria.

2. MATERIALS AND METHODS

2.1 Plant Material

The plant material consisted of *Bauhinia rufescens* Lam's leaves. (Fig. 1A) of *Plectranthus neochilus* Schltr's leaves (Fig. 1B). These plants have been authenticated by the Scientific Committee of the Faculty of Sciences of the University of Maroua. The harvest of the plant was made in KAKATARÉ district located in the district of Maroua 2 division. The various parts that were used to produce the extracts were harvested in June 2018 between 5.30am and

6.30am. The choice of this period was based on the principle that the morning harvest corresponds to the most favorable moment when the active principles of the plant are generally preserved and concentrated [23,24].

2.2 Biological Material

The microorganisms used were, *Escherichia coli* 25922 and *Proteus mirabilis* provided by the National Insurance of Social Fund hospital of Maroua.

2.3 Preparation of Extracts

2.3.1 Maceration with methanol from the leaf powder of *Plectranthus neochilus* Schltr. and *Bauhinia rufescens* Lam

The leaves were dried in the oven at 55°C for 24 hours until a constant mass was obtained. These leaves were then crushed to obtain a fine powder. Extraction by maceration from the powder was carried out according to the principle described by Matias [25]. Thus, 10 g of leaves powder were introduced into 200 ml of methanol and stirred for 2 h using a magnetic stirrer. The homogenate was filtered using Whatman n°1 paper. Finally, the methanol was evaporated using the oven at 77°C. A fine powder was recovered and stored in a refrigerator at 4°C until the further use.

2.3.2 Maceration in methanol-aqueous mixture of fresh leaves

Extraction by maceration in methanol-aqueous mixture of fresh leaves was performed according to the protocol described by Roumeissa and Maya [24]. 10 g of fresh leaves for each of the two plants. Each of this plant material was individually ground in the mortar. The paste obtained for each was introduced into 200 mL of aqueous methanol (70% v / v) preheated in a 500 mL beaker until boiling. The mixture was stirred until cooling and then allowed to stand for 24 hours. After 24 hours, the mixture was filtered with Whatman filter paper (No. 1) and the filtrate recovered in a 1000 mL of Erlenmeyer flask. The procedure was repeated 3 times. After maceration, the solvent was evaporated using the water bath at 77°C until the volume of 25 mL was obtained. The concentrate was stored at 4°C in a refrigerator until use.

2.4 Phytochemical Screening

The bioactive compounds of the methanolic and aqueous-methanolic mixture extracts were sought by using standard procedures [26,27]. The extracts were qualitatively tested for the presence of chemical constituents such as total phenolics compounds, flavonoids and alkaloids.

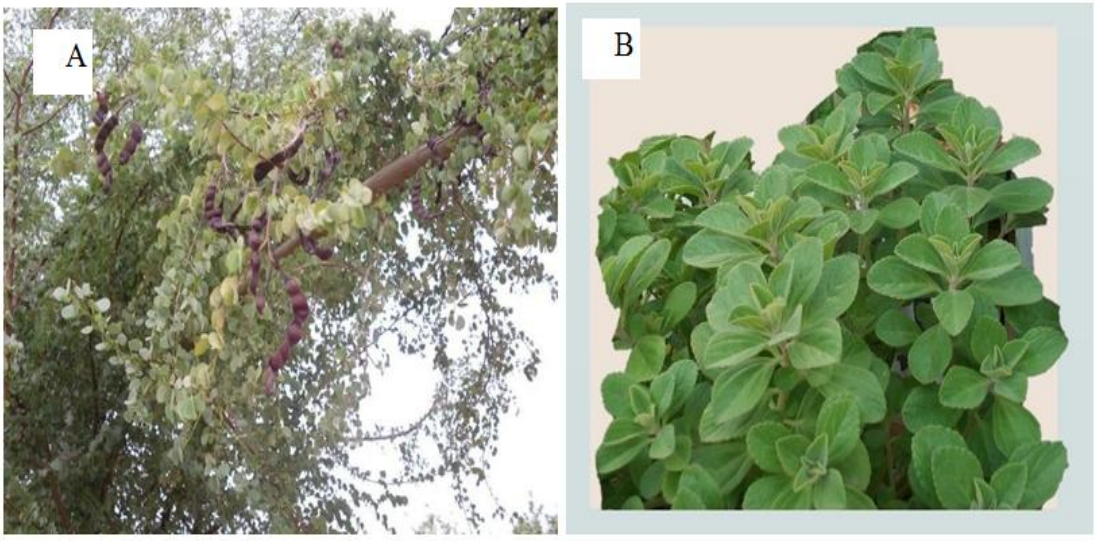

Fig. 1. *Bauhinia rufescens* Lam (A) and *Plectranthus neochilus* Schltr. (B) (Personal snapshot, Mamoudou Hamadou, 2018)

2.4.1 Phenolic compounds

A qualitative test was carried out according to the protocol described by Békro et al. [27]. 2 mL of each solution was added to a test tube and 5 drops of 10% $FeCl_3$ were added. The appearance of the dark green or blackish green color has been interpreted as the abundance of phenolic compounds [14,24]. The decrease in intensity has been interpreted as the average presence of these compounds. The lack of coloring has been translated as the absence of the compound.

2.4.2 Flavonoids

The method described by Quettier-Deleu et al. [26] was used to detect flavonoids. This method uses aluminum trichloride as a reagent. It is based on the oxidation of flavonoids by this reagent, resulting in the formation of a yellow complex by the introduction of 1 mL of a solution of $AlCl_3$ in 1 mL of each extract (prepared in 80% methanol). The intensity of the coloration was interpreted as a quantitative marker of the presence of flavonoids.

2.4.3 Alkaloids

The Wagner's Test described by Shah and Seth, [14] was used to identify the alkaloids. 5 drops of de Wagner's reagent (diluted iodine solution) are added to a test tube containing one milliliter (1 mL) of extract. The presence of the alkaloids is marked by the formation of a reddish-brown precipitate. The quantitative interpretation was based on the color and density of the precipitate. An intense precipitate refers to the abundance of alkaloids and vice versa.

2.5 Antibiogram

The antibiogram was performed according to the recommendations of the CA-SFM [28]. The antibiotic discs used in this work are: Ceftazidine 30 µg (CAZ), Ceftriaxone 10 µg (CTR), Levofloxacin 5 µg (LE), Gentamicin 10 µg (GEN), Amikacin 30 µg (AK), Ciprofloxacin 5 µg (CIP) and Meropenem 10 µg (MRP).

2.6 Antibacterial Activity

The antibacterial activity of the extract mixtures was evaluated by the diffusion method on strips: the E-test was used. It allowed to determine the Minimum Inhibitory Concentration (MIC) of the different extracts [29]. Different concentrations were prepared for methanolic-aqueous extract mixture from the leaf powder of both plants: 0.78125; 1.5625; 3.125; 6.25; 12.5; 25; 50; 100; 200 and 400 mg mL^{-1}. For the methanol extract from the leaves powder of both plants, the concentrations were: 1.71; 3.42; 6.84; 13.67; 27.34; 54.68; 109,375; 218.75; 437.5; 875 mg mL^{-1}. Finally 10 µL of each solution was dropped on the strips.

3. RESULTS AND DISCUSSION

3.1 Phytochemical Screening

Table 1 shows the observations relating to the detection of total phenolic compounds, flavonoids and alkaloids in the various extracts *of Plectranthus neochilus Schltr. and Bauhinia rufescens Lam.*

From this table, various distributions of phenolics, flavonoids and alkaloids were observed in *Plectranthus neochilus* Schltr and *Bauhinia rufescens* Lam. In *Plectranthus neochilus* Schltr, the methanol and methanol-aqueous mixture extracts of the leaves averagely contain phenolics, flavonoids and alkaloids compounds. In *Bauhinia rufescens* Lam., phenolic compounds were abundant in the aqueous and methanol extracts of the leaves. These compounds were moderately present in the methanol extract. As for the flavonoids, they were very abundant in the methanolic and methanolic-aqueous extracts of the leaves. Alkaloids, were moderately present in the leaves regardless of the extraction method used.

Table 1. Qualitative phytochemical analysis of *the extracts*

Plants	Compounds	Relative quantity
Plectranthus neochilus	Total phenolic compounds	Intermediate
	Flavonoids	Low
	Alkaloids	Intermediate
Bauhinia rufescens	Total phenolic compounds	High
	Flavonoids	High
	Alkaloids	Intermediate

The low presence of flavonoids in *Plectranthus neochilus* Schltr observed during phytochemical screening can be explained by the fact that species of the *Plectranthus* genus are characterized by a complete absence of flavonoids as highlighted by the works of Matias [25]. The presence of phenolic compounds in abundance in *P. neochilus* observed in Table 1, was in agreement with the results of Matias [25] which shown that the genus of *Plectranthus* is characterized by the presence of 17 flavonoids and an abundance of phenolic and alkaloid compounds.

In *Bauhinia rufescens* Lam. these three compounds are in abundance. This strong presence of these compounds (phenolic compounds, alkaloids and flavonoids) is in agreement with the results obtained by Usman et al. [30] and Works have actually highlighted the presence in large quantities of phenolic compounds, flavonoids and alkaloids in this plant.

This result is consistent with the work of [31,32,33]. Works have shown that methanol-aqueous and methanol are the solvents of choice for the extraction of compounds with antibacterial activity.

3.2 Antibiogram

The CA-SFM manual [28] was used for interpretative reading of inhibition diameters. The different antibiotics in Table 2 are grouped into three classes: beta-lactams (Ceftazidime, Ceftriaxone, Meropenem), quinolones (Levofloxacin, Ciprofloxacin) and aminoglycosides (Gentamicin, Amikacin).

Results obtained from Table 2 show that *Escherichia coli* is sensitive to all antibiotics tested except ceftazidime, a cephalosporin. While *Proteus mirabilis* is resistant to almost all antibiotics tested except Amikacin, an aminoglycoside.

These observations show that *E. coli* is sensitive to almost all antibiotics tested except Ceftazidime with which an Intermediate resistance was observed. Ceftazidime is a third-generation cephalosporin [34]. The resistance of this bacteria could be explained by its membership in the class 1 enterobacterium. Class which is characterized by a natural resistance of bacteria to cephalosporins by production of a low-level Cephalosporinase [35]. This enzyme hydrolyzes penicillins, the first generation and the specific cases the third generation of cephalosporins.

Proteus mirabilis has different sensitivity profile even though it belongs to the enterobacterium class 1. This strain developed resistance to the beta-lactams tested (Ceftazidine, Ceftriaxone, Meropenem) as well as quinolones (Levofloxacin, Ciprofloxacin) and an intermediate sensitivity to Gentamicin and total amikacin. *Proteus mirabilis* resistance can be justified by mutations due to the abusive and inappropriate use of antibiotics [36]. In addition, the ß -lactam resistance profile proves the activation of enzymes of resistance in *Proteus mirabilis* [37,38,39]. expanded Spectrum ß-lactamase (ESBL) that hydrolyzes penicillins, with the exception of the clavulanic acid combination - amoxicillin; cephalosporins with the exception for carbapenems; high-level cephalosporinases that act by hydrolyzing all penicillins including clavulanic acid - amoxicillin; all cephalosporins but no detectable activity on carbapenems; the carbapenemases of which there are several, hydrolyze all the penicillins, all the cephalosporins, and according to their chromosomal or plasmidic origin presents a variant profile of substrate in the carbapenems.

The presence of one of these enzymes in an enterobacteria is often accompanied by resistance to other classes of antibiotics including quinolones and aminoglycosides. This helps to understand the resistance of *Proteus mirabilis* to quinolones and aminoglycosides [40].

As for the resistance profile of *Proteus mirabilis* vis-à-vis of quinolones (Levofloxacin, Ciprofloxacin) generally involves a series of genetic mutations that cumulative effects lead to the expression of resistance [40].

The resistance of *Proteus mirabilis* to aminoglycosides (Gentamicin) involves the intervention of acetylases, nucleotidases and phosphorylases. Enzymes which, by transferring a radical to the hydroxyl groups at different positions at the aminoglycoside levels, can cause inability to bind to the 30S subunit. Therefore Aminoglycosides will become unable to inhibit protein biosynthesis in bacteria [41].

Table 2. Results of the antibiogram

Bacterial strains	Antibiotics	D (mm)	Sensitivity		
			S≥*	R<*	Observations
Escherichia coli	Ceftazidim	19	22	19	I
	Ceftriaxon	30	25	22	S
	Gentamicin	21	17	14	S
	Amikacine	21	16	13	S
	Lévofloxacin	30	23	19	S
	Ciprofloxacin	28	26	24	S
	Méropénèm	30	22	16	S
Proteus mirabilis	Ceftazidim	10	22	19	R
	Ceftriaxon	24	25	22	I
	Gentamicin	16	17	14	I
	Amikacin	21	16	13	S
	Lévofloxacin	15	23	19	R
	Ciprofloxacin	23	26	24	R
	Méropénème	16	22	16	I

** Reference values provided by the CA-SFM manual. S: Sensitive; R: Resistant; I: Intermediate ≥: higher; <: lower*

Table 3. MIC of the mixture of methanol-aqueous extracts of leaves of *Plectranthus neochilus* Schltr. *and Bauhinia rufescens Lam*

Concentration of the methanol-aqueous mixture (mg mL^{-1})	Strains tested	
	E. coli	***P. mirabilis***
0,78	-	-
1,56	-	-
3,13	-	-
6,25	-	-
12,50	-	-
25,00	+	-
50,00	+	+
100,00	+	+
200,00	+	+
400,00	+	+

-: No inhibition; +: inhibition of bacteria

This resistance profile observed in *Proteus mirabilis* is increasingly present in hospitals and often leads to therapeutic dead ends and high mortality. This phenomenon is all the more worrying because these enzymes diffuse very rapidly throughout the world via the movements of living beings, and plants used as food but also through the inter- and intraspecific transmission of genes via a plasmid [42].

3.3 Antibacterial Activity

The antimicrobial activity of the plant extracts and the antibiotic were evaluated on Gram-negative bacterial strains using the Mueller Hinton E-test® banding method. This method allowed us to determine the MICs presented in Tables 3 and 4.

Aqueous and methanol extracts of leaves and stems of *Plectranthus neochilus* Schltr. and leaves of *Bauhinia rufescens* Lam. were individually tested on strains of *Escherichia coli, Proteus mirabilis* and *Shigella flexneri.* The results obtained show no antibacterial activity on the bacterial strains tested. However, the extract mixtures showed antibacterial activity (Tables 3 and 4).

The results presented in Tables 3 and 4 showed that the extract mixtures have antimicrobial activities on *Escherichia coli* and *Proteus mirabilis*. The MICs of the different mixtures were read from the concentration where the microbial growth is not observed. The MICs of the methanol-aqueous leaf extracts are 25 and 50 mg mL^{-1}, respectively, for *E. coli*, *Proteus*

mirabilis. Those of the methanol mixture of the leaves were respectively 27.34 and 54.68 mg mL^{-1} for *E. coli* and *P. mirabilis*.

These values are higher than the MICs obtained by Okou et al. [42] although the work of the researchers was done on the extracts taken individually.

This great difference can be justified by the fact that the concentration of the active ingredient is low that it required a large amount of extracts to observe an effect. In addition, this high minimum inhibitory concentration is probably due to the fact that the active ingredient (bioactive molecule) is complexed by other compounds of the mixture. These can thus limit or alter the effectiveness of the active ingredient. This explanation may be justified by the work of Roumeissa and Maya [24], in which the aqueous phase of the extracts had no effect on the *Escherichia coli* strain but the isolated flavonoids showed strong inhibitory activity.

Table 4. MIC reading of the mixture of methanol extracts of leaves of *Plectranthus neochilus Schltr.* and *Bauhinia rufescens Lam*

Concentration of the mixture with pure methanol (mg mL^{-1})	Strains tested	
	E. coli	***P. mirabilis***
1,71	-	-
3,42	-	-
6,84	-	-
13,67	-	-
27,34	+	-
54,68	+	+
109,40	+	+
218,75	+	+
437,50	+	+
875,00	+	+

-: No inhibition; +: inhibition of bacteria

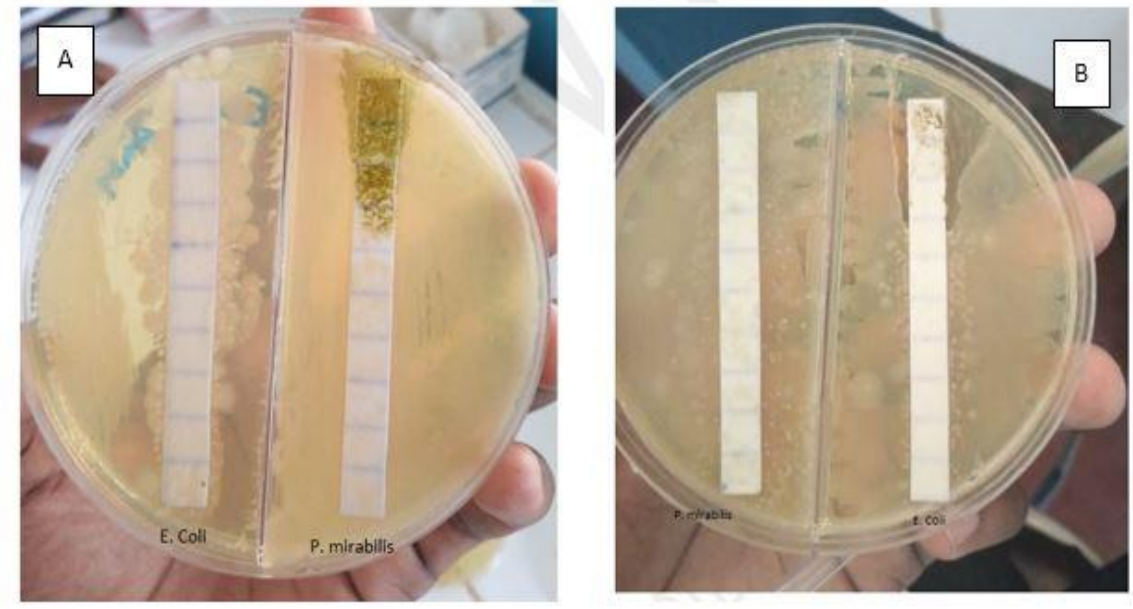

Fig. 2. Results of mixture of methanol extracts of leaves of *Plectranthus neochilus* Schltr. *and Bauhinia rufescens Lam.* of E-test (A) and the results of mixture of methanol-aqueous extracts of leaves of *Plectranthus neochilus* Schltr. *and Bauhinia rufescens Lam.* of E-test (B)

4. CONCLUSION

Finally, *Plectranthus neochilus Schltr* and *Bauhinia* rufescens Lam contain compounds with antibacterial properties. The strains tested, *E. coli* and *P. mirabilis* are the strains that have developed multidrug resistance. However, the mixtures of extracts showed an inhibitory effect vis-à-vis of these strains. It is noted that methanolic-aqueous extract (70% v / v) is the best solvent for extracting bioactive compounds. However, analysis must be carried out to explain the synergy of the extracts and to purify the active principle of the mixtures.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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