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Evaluation of the Antimicrobial Activity of Cassia *Occidentalis Lin on Several Multi-Resistant Microbial Strains

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Abstract: The aim of this work is to assess the antimicrobial potential of cassia occidentalis flax (Caesalpiniaceae) leaf extracts on drug-resistant microbial strains, in order to bring phytotherapeutic prescriptions into line with the potential biological activities of the chemical constituents present. Aqueous and hydro-ethanolic extracts were prepared according to the methods of Zirihi and Kra (2003). The antimicrobial activities were determined by measuring the rate of inhibition of microbial growth, diffusion on a solid medium and dilution in a liquid medium. In addition, the mode of action of the active extracts was determined by the ratio of the minimum fungicidal concentration to the minimum inhibitory concentration (MFC/MIC). Aqueous and hydroalcoholic extracts from the leaves of C. occidentalis have been shown to have antimicrobial activity. These extracts inhibited the proliferation of C. albicans strains resistant to nystatin. The activity of the aqueous extracts was similar to that obtained with the hydroalcoholic extracts. However, both types of extract showed no significant efficacy against S. aureus, E. coli and K. pneumoniae strains at the doses used (200 mg/ml; 100 mg/ml and 50 mg/ml) in all the pharmacological tests repeated during this study.

Keywords: Pharmaco-Multi-Resistance, Antimicrobial Activity, Fungicide, *Cassia Occidentatlis*

1. Introduction

Antimicrobials remain the most preferred means of fighting infections. Among these antimicrobials, beta-lactams (antibiotics) are nowadays the most used throughout the world and particularly in developing countries like the Republic of Congo, due to the extent of their spectrum of action, safety, effectiveness and above all their less expensive costs [5].

On the other hand, the excessive self-medication of antimicrobials has nowadays caused the emergence of multi-resistant strains. Numerous cases of multidrug resistance have been reported for the Congo and other African countries [10].

Faced with therapeutic failures by reference antimicrobials, the antimicrobial molecules of tomorrow should target new targets of action in microorganisms. There are many avenues for research, but the exploration of the secondary metabolites of aromatic and medicinal plants appears to be the most promising, because these constitute, due to their biological diversity, the largest reserve of bioactive substances. According to the World Health Organization (WHO), almost 80% of populations depend on traditional medicine for primary health care WHO (2002) [8]. Significant economic benefits in the development of traditional medicine and in the use of medicinal plants for the

treatment of several diseases have been noted [7]. However, it was not until the beginning of the 20th century that scientists began to take an interest in it [12].

In this context, the objective of this study is to evaluate, in vitro, the antimicrobial activity of total aqueous and hydroalcoholic extracts of *Cassia occidentalis leaves* against a few pharmaco-multiresistant bacterial and fungal strains.

2. Material and Methods

2.1. Material

2.1.1. Plant Material

The plant material used consisted of the leaves of *Cassia occidentalis Flax* harvested in the Central Basin department, in the north of Congo-Brazzaville, from December 2016 to March 2018, the chemical study of which was carried out at the Institute Research in Health Sciences (IRSSA).

2.1.2. Microbial Strains

This study focused on four microbial species, three (3) bacterial and one (1) fungal. Each species consisted of two different strains. Among these strains, two were ordered from the Pasteur Institute (CIP strain) and six (6) strains provided free of charge by the National Public Health Laboratory (LNSP) in the Republic of Congo, as presented below:

The table below provides information on the origin and profile of each microbial strain used in this study.

Table 1. *Profile of the microorganisms tested.*

Strains	Band	Strain profile	Origins
Escherichia coli LNSP	Gram -	ESBL	Urine
Escherichia coli ESBL/ AmpC		ESBL	Urine
Klebsiella pneumoniae LNSP		Sensitive to C3G	Urine
Klebsiella pneumoniae C3GR		C3G resistant	Urine
Staphylococcus aureus CIP103429	Gram +	Sensitive to methicillin	Reference
Staphylococcus aureus MRSA/LNSP		Methicillin resistant	Pus
Candida albicans LNSP	Yeasts	Fungizone resistant	Vaginal sample
Candida albicans CIP2503		Sensitive to Fungizone	Reference

2.2. Methods

2.2.1. Preparation of Plant Extracts

Leaves of *Cassia occidentalis Flax* were cut into small pieces and dried at room temperature for two weeks, then made into a fine powder using an IKA Labortechnik MFC type grinder. This powder was used for the preparation of aqueous and hydro-ethanolic extracts according to the methods of Zirihi and Kra (2003).

The powder made from the leaves underwent extraction according to the method of Zirihi and Kra (2003), as described: 100 g of powder were macerated in one liter of distilled water by grinding in a blender (Blender). The homogenate obtained was first squeezed out of a square of cloth, then successively filtered twice through hydrophilic cotton and once through Whatman 3mm paper. The volume of the filtrate obtained was reduced using a Büchi type rotary evaporator at a temperature of 60°C. The dough was collected and freeze-dried. The extract thus obtained is the total aqueous extract noted Ex.aq. The ethanolic extract was produced by fractionation of the aqueous extract [14]: 10 g of Ex.aq were dissolved in 200 ml of a hydroalcoholic solution (V/V 30/70). This mixture was separated into two phases using a separating funnel for five hours. The upper alcoholic phase obtained was collected and dried in an oven at 50°C; the product thus obtained is the ethanolic extract (Ex.OH). This cycle of aqueous and ethanolic extraction was repeated three times. The extracts were placed in previously sterilized containers. Hermetically sealed, they were stored in the refrigerator at 4°C.

2.2.2. Study of the Antimicrobial Activity of Plant Extracts

(i). Measurement of the Rate of Inhibition of Microbial Growth

MH medium was used for culturing bacteria. The media were sterilized by autoclave at 121°C for 20 minutes. The incorporation of the different plant extracts into the culture medium was carried out according to the double dilution method of geometric bond of ratio ½ [1, 14]. Seven (7) concentrations were retained for Ex.aq (100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, 3.12 mg/ml and 1.56 mg/ml), compared to six for Ex.ETOH, including all those of Ex.aq, except the strongest (100 mg/ml). The witness did not receive any additional extracts. The different culture media were poured at 40°C into 90 mm diameter Petri dishes. Three Petri dishes were used for each concentration and the test was repeated three times under the same experimental conditions. The Petri dishes were sealed with adhesive film and incubated in an oven for 24 hours at 37 ± 2°C for the bacterial strains and 28 ± 2°C for the yeast strains. Microbial radial growth inhibition rates were measured daily for 5 days compared to the control. This inhibition rate was calculated according to the formula of Leroux and Credet (1978) [6]:

$$T\ (\%) = (D - d) / D \times 100$$

T: inhibition rate; D: bacterial/fungal growth in control Petri dishes,

d: bacterial/fungal growth in test boxes.

Determining the rate of inhibition of microbial growth of each strain made it possible to define, for each extract, the minimum inhibitory concentration (MIC) and the minimum

fungicidal concentration (MFC).

(ii). Sensitivity Test

The sensitivity of the strains to plant extracts was carried out by the diffusion technique in agar medium. Mueller Hinton media was inoculated by flooding for bacterial strains and Sabouraud chloramphenicol medium for yeast strains. Using a sterile Pasteur pipette, wells of approximately 5 mm in diameter were made in the nutrient agar. Each well received 80 µl of the extract to be tested at concentrations 100, 50 and 25 mg/ml. After 40 minutes of diffusion at room temperature, the Petri dishes were incubated at 37°C (bacteria) and 28°C (yeast) for 48 h. The presence or absence of a zone of inhibition was observed. The interpretation was made according to the method [3, 9] by measuring the diameters of the radial inhibition halos compared with the result obtained with the reference molecules.

(iii). Preparation of Inocula

The bacterial inoculum was prepared from colonies less than 24 hours old in Mueller Hinton Broth (BMH) while that of the yeasts was prepared in Sabouraud liquid medium. A colony isolated from each microbial culture was taken using a platinum loop and homogenized in 10 ml of culture broth, then incubated for 4 or 5 h at 37°C or 28°C to have a pre- culture. A volume of 1 ml was taken and added to 9 ml of each culture broth sterile. This microbial suspension produced is estimated at approximately 10^{6} cells/ml and constitutes the $10^{0\ \text{dilution}}$ or the pure inoculum.

(iv). Inoculum Count

The enumeration of the inoculum was carried out by a 10th dilution $^{\text{from}}$ the pure inoculum. 4 dilutions were obtained at 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}. These different dilutions as well as the pure inoculum were inoculated using a calibrated 2 µl automatic micropipette onto nutrient agar plates, then incubated at 37°C or 28°C for 24 h. This preparation constitutes box B for bacteria and F for yeast.

(v). Preparation of the Concentration Range of Plant Extracts

The concentration range of the hydro-ethanolic plant extracts was carried out in seven test tubes numbered from 1 to 7 by the double dilution method according to a geometric progression of ratio ½.

(vi). Inoculation

In a series of eight hemolysis tubes numbered from T1 to T8, 1 ml of the pure inoculum was introduced. Then, 1 ml of plant extract was added to the tubes according to the concentration range prepared. This distribution of extract was done so that 1 ml of plant extract of 200 mg/ml was transferred into tube T1, tube T2 received 1 ml of 100 mg/ml and so on until tube T7 which received 1 ml of the 3.1 mg/ml solution. The T8 tube received, instead of the plant extract, 1 ml of sterile culture broth which served as a growth control. Due to the volume/volume dilution thus achieved, the concentration in the tubes was reduced by half. All these tubes were incubated at 37°C or 28°C for 24 h.

(vii). Determination of the Minimum Inhibitory Concentration (MIC)

The MIC is the lowest concentration of the test substance for which there is no growth visible to the naked eye after an incubation time of 24 hours. Its determination was made by observation of the disorder induced by the growth of the microorganisms studied in each tube. The MIC was the lowest concentration for which no disturbance was observed.

(viii). Determination of the Minimum Bactericidal or Fungicidal Concentration (CMB/F)

The minimum bactericidal or fungicidal concentration (MBC/F) is the lowest concentration of substance that leaves at most 0.01% of microorganisms surviving. Using an automatic pipette calibrated 2 µl with sterile tips, the contents of the tubes in which no disorder was observed were taken and inoculated on agar plates starting with the CMI tube. Seeding was done in parallel streaks 5 cm long on the surface of the agar (Box B_1 or F_1). After 24 hours of incubation at 37°C or 28°C, the number of colonies on the streaks was compared to that in the inoculum counting box (Box B_2 or F_2). Thus, the first experimental tube whose number of microorganisms present on its streak is less than or equal to that of the $10^{4\ \text{dilution}}$ will correspond to the CMB/F.

3. Results

3.1. Evaluation of the Sensitivity of Strains on Solid Media

Table 2. *Aqueous extract of Cassia occidentalis leaves.*

Microbial strains	Origin	200 mg/ml	100 mg/ml	50 mg/ml
Candida albicans LNSP	PV	S (16mm)	S (13mm)	S (09mm)
Candida albicans CIP2503	CIP	S (19mm)	S (18mm)	S (12mm)
Escherichia coli LNSP	Urine	R	R	R
Escherichia coli ESBL/ AmpC	Urine	R	R	R
Klebsiella pneumoniae LNSP	Urine	R	R	R
Klebsiella pneumoniae C3GR	Urine	R	R	R
Staphylococcus aureus CIP103429	CIP	R	R	R
Staphylococcus aureus MRSA/LNSP	Pus	R	R	R

S: sensitive, R: resistant.

Table 3. *Hydroalcoholic extract of Cassia occidentalis leaves.*

Microbial strains	Origin	200 mg/ml	100 mg/ml	50 mg/ml
Candida albicans LNSP	PV	S (19mm)	S (15mm)	S (12mm)
Candida albicans CIP2503	CIP	S (18mm)	S (18mm)	S (13mm)
Escherichia coli LNSP	Urine	R	R	R
Escherichia coli ESBL/ AmpC	Urine	R	R	R
Klebsiella pneumoniae LNSP	Urine	R	R	R
Klebsiella pneumoniae C3GR	Urine	R	R	R
Staphylococcus aureus CIP103429	CIP	R	R	R
Staphylococcus aureus MRSA/LNSP	Pus	R	R	R

The above results showed that both the hydroalcoholic and total extracts used present good fungicidal activity on the two strains of *Candida albicans* studied, with inhibition halos varying between 12 and 19 mm. While they show almost no activity on all the bacterial strains studied, at concentrations 200 mg/ml, 100 mg/ml and 50 mg/ml.

3.2. Evaluation of the Sensitivity of Strains on Liquid Medium

For the study of sensitivity in liquid medium, only strains of *Candida albicans* were studied, with concentrations of 250 mg/ml, 200 mg/ml and 150 mg/ml.

Table 4. *Optical density with C. albicans strain CIP2503 NYSTA-S.*

	H_0	H6	H12	H18	H24	H30	H36	H42	H48
Witnesses	0.201	0.262	0.662	1,430	1,721	2,016	2,091	2,222	2,017
E1 (150 mg/ml)	0.210	0.270	0.665	0.698	0.612	0.554	0.454	0.339	0.301
E2 (200 mg/ml)	0.209	0.269	0.686	0.624	0.472	0.366	0.321	0.2	0.189
E3 (250 mg/ml)	0.207	0.257	0.661	0.502	0.311	0.287	0.201	0.178	0.161
NY (30µ)	0.22	0.255	0.656	0.321	0.187	0.095	0.075	0.042	0.021

The bacterial strains not being sensitive to the extracts were removed from this part of the study.

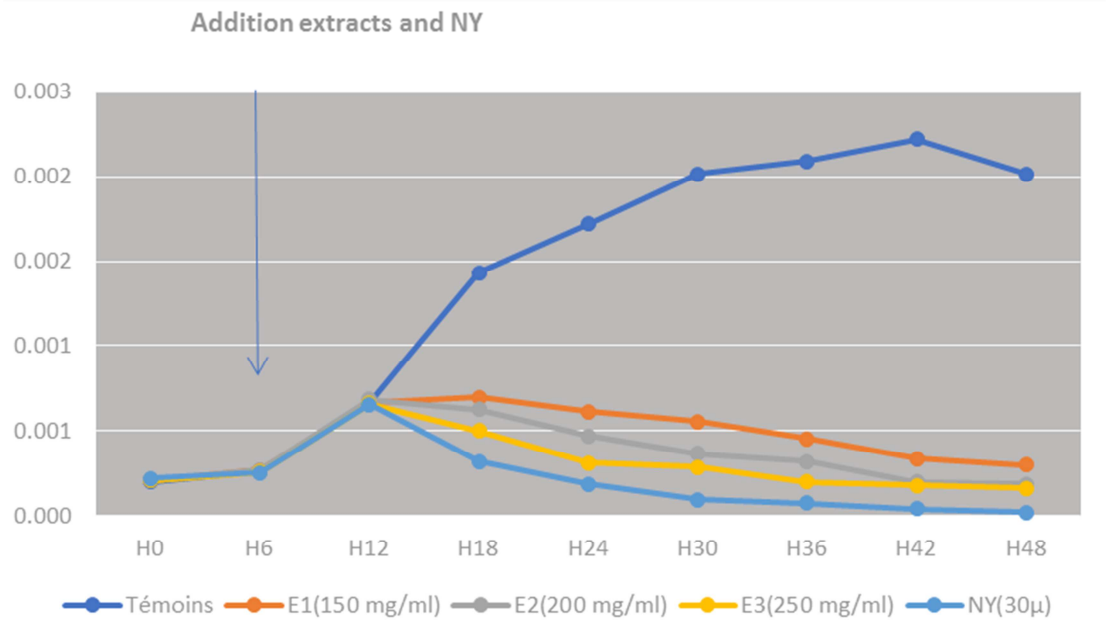

Figure 1. *Correlation of fungicidal activity-concentration of the hydroalcoholic extract on C. albicans CIP2503 NYSTA-S.*

The shape of the curves shows that the extracts used at the indicated concentrations have an antifungal activity on *Candida albicans* close to that of nystatin at 30µ. Furthermore, this effect is concentration-dependent.

Table 5. *Optical density with C. albicans strain LNSP NYSTA-R.*

	H_0	H6	H12	H18	H24	H30	H36	H42	H48
Witnesses	0.324	0.358	0.661	0.897	2,235	2,358	2,400	2,343	2,122
E1 (150mg/ml)	0.300	0.364	0.632	0.571	0.558	0.552	0.433	0.382	0.3
E2 (200mg/ml)	0.325	0.351	0.618	0.601	0.408	0.388	0.342	0.311	0.236
E3 (250mg/ml)	0.319	0.360	0.641	0.524	0.399	0.214	0.198	0.165	0.098
NY (30µ)	0.312	0.323	0.66	0.801	0.988	0.978	1,024	0.989	0.688

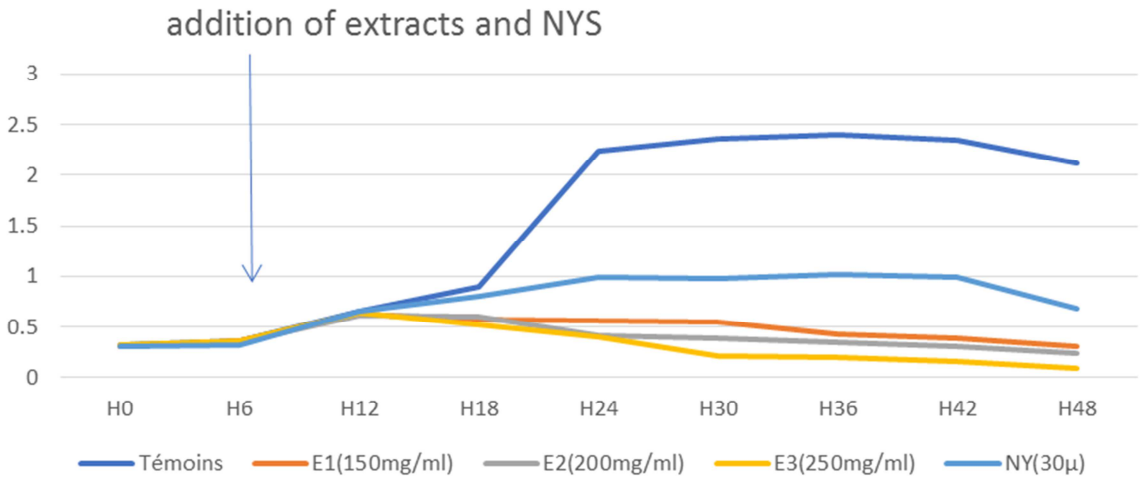

Figure 2. *Correlation of fungicidal activity-concentration of the hydroalcoholic extract on C. albicans LNSP NYSTA-R.*

The nystatin-resistant strain of *Candida albicans is sensitive to extracts used at concentrations ranging from 150 to 250 mg/ml, with a dose-dependent effect.*

3.3. Mode of Action of C. Occidentalis Extracts

Table 6. *Determination of antifungal parameters (MIC and CMF).*

Strains	Origin	MIC (mg/ml)	CMF (mg/ml)	CMF/CMI	Interpretation
Candida albicans LNSP	PV	12.5	25	2	Fungicide
Candida albicans CIP2503	CIP	6.2	6.2	1	Fungicide

After 48 hours of incubation, compared to the control tube, a progressive decrease in the number of *C. albicans* colonies is observed as the concentrations of the 70% FE extract increase in the experimental tubes.

4. Discussion

The inhibitory concentration values obtained attest that the extracts have more or less accentuated antifungal activities.

The curves of the three extracts show a decreasing pattern with steep slopes (Figure 1 and 2). The slope of FE70% is greater than that obtained with nystatin (NY). The decreases obtained with the different concentrations, respectively at 150, 200 and 250 mg/ml for FE 70%, clearly illustrate the dose-dependent sensitivity of the *C. albicans strains. to* the tested extract.

The tests with the aqueous fraction were not conclusive as to the desired activity compared with the ethanol/water fraction. This could be explained by the fact that the ethanol/water fraction made it possible to separate the active ingredients and concentrate them. This separation allows the active ingredients contained in the F E70% extract to better express their antifungal potential. The partition therefore makes it possible to improve the antifungal activity of the leaves *of C. occidentalis.*

The work of [13] on the antifungal activity of *B. abyssinca leaves* on *C. albicans* showed that the methanolic extract was the most active with a MIC of 780 µg/ml. Comparing this value to our results, the F E 70% extract (MIC = 620 µg / ml), show better antifungal activity compared to the methanolic extract of the predecessors.

The richness of *Cassia occidentalis leaves* in tannins could justify its antifungal activity, due to their affinity with fungal cell membrane ergosterol. Indeed, tannic acid resulting from the metabolism of tannins acts on membrane ergosterol by disrupting its permeability, resulting in the loss of vital intracellular elements [11].

The extract of *Cassia occidentalis leaves* would behave as an antagonist compared to nystatin on strains of *Candida albicans* resistant to nystatin, which is how this extract effectively inhibits the growth of the *C. albicans strain* NYSTA-R. Furthermore, previous studies have revealed that a handful of recently harvested leaves and flowers are beneficial for certain fungal skin conditions [4]. This is preferably done on non-open and non-oozing dermatological conditions.

Furthermore, the ineffectiveness of *Cassia occidentalis extract* against bacterial strains would confirm its aforementioned mechanism of action on *Candida albicans*. A successive dosage of ergosterol on a culture of *Candida albicans* in the presence of this extract could shed more light on its mechanism of action.

The same extract would inhibit the synthesis of glucans in the fungal wall like echinocandins.

The great variability of the phytochemical profile of *Cassia occidentalis* therefore explains the interest of African traditions on the usefulness of the organs of this plant. This richness has been confirmed by previous work [2].

5. Conclusion

The present work demonstrated the fungicidal activity of

aqueous and hydroalcoholic extracts of *Cassia occidentalis flax* on multiresistant strains of *Candida albicans*.

On the other hand, these extracts did not reveal any activity against Mult resistant strains of *Eshérichia coli, Klebsiella pneumoniae and Staphylococcus aureus*. This fungicidal activity would depend on the presence and richness of polyphenolic compounds, notably tannins and flavonoids.

This work also established that the antifungal activity observed is concentration-dependent, and that this activity hardly varies according to the extraction method.

Conflicts of Interest

The authors declare no conflicts of interest.

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