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Antimicrobial activity of compounds from *Acanthospermum hispidum* DC and Caesalpinia bonduc (L.) ROXB: Beninese plants used by healers against HIV-associated microbial infections

Alban G. Houngbèmè[1], Habib M.Y.Ganfon[1], Sédami Medegan [1,2], Boniface Yèhouénou[3], **Bouraïma Bambola[1],** Clément Gandonou[1], Fernand A. Gbaguidi[1, 2, *]

[1]Laboratoire de Pharmacognosie/Institut de Recherche et d'Expérimentation en Médecine et Pharmacopée Traditionnelles (IREMPT) / Centre Béninois de la Recherche Scientifique et Technique (CBRST)/ UAC, 01 BP 06 Oganla Porto-Novo. [2] Laboratoire de Chimie Pharmaceutique Organique, Ecole de Pharmacie, Faculté des Sciences de la Santé, Université d'Abomey-Calavi, Campus du Champ de Foire, 01 BP 188, Cotonou, Bénin. [3]Laboratoire d'Etude et de Recherche en Chimie Appliquée (LERCA)/Ecole Polytechnique d'Abomey-Calavi/Université d'Abomey-Calavi, 01 BP 2009 Cotonou.

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ABSTRACT

In this study, we have tested alcoholic extracts (60%) from four Beninese plants: *Ocimum gratissimum* L., *Acanthospermum hispidum* DC, *Caesalpinia bonduc* (L) Roxb and *Calotropis procera* W. T. Aiton. They are used by the healers to prevent opportunistic diseases associated to HIV-AIDS; on six strains such as: *Escherichia coli* O 157H7, *Staphylococcus aureus* ATCC 25923 which resist to methicillin (MRSA), *Salmonella typhi*, *Klebsiella pneumonia*, *Candida albicans* ATCC 10231, and *Mycobacterium bovis* BCG 040812 which cause microbial infections associated with HIV-AIDS. The results show that all the extracts are bacteriostatic and fungistatic but only the hydro-ethanolic extracts of *Acanthospermum hispidum* (HE_2) and of *Caesalpinia bonduc* (HE_3) presented antibiotic power (respectively ap = 2 and ap = 4) on *Candida albicans* ATCC 10231. The *Mycobacterium bovis* BCG shown resistance to tested extracts (CMI > 250 µg/mL). The two fungicidal extracts HE_2 and HE_3 did not show harmful effects on the cells WI–38 with an IC_{50} > 100 µg/mL for HE_2 and IC_{50} = 50 µg/mL for HE_3. The successive bio-guided purifications of extracts HE_2 and HE_3 permitted isolation of three antibacterial compounds: Flavanone (M_1); stigmasterol (M_2); and quercetin (M_3). The three isolated compounds possess antibiotic power (ap 3±1) on tested strains and are not toxic on shrimp larvae (LC_{50}: 0.30 ± 0.17 mg/mL).

INTRODUCTION

Bacterial infections are a most common cause for morbidity among the patients infected by HIV with a major impact in the population (Bonnet *et al.*, 2007). Respiratory infections are tracts and lungs diseases that cause cough and/or a labored breathing. According to the World Health Organization (WHO), all infections with brutal beginning, affecting ears, nose, throat, larynx, trachea, windpipe, bronchus, the bronchioles or lungs are respiratory infections (Sanogo, 2010).

* *Corresponding Author*
Fernand Ahokanou GBAGUIDI, Laboratoire de Pharmacognosie/Institut de Recherche et d'Expérimentation en Médecine et Pharmacopée Traditionnelles (IREMPT) / Centre Béninois de la Recherche Scientifique et Technique (CBRST)/ UAC, 01 BP 06 Oganla Porto-Novo.Email: ahokannou@yahoo.fr

Skin problems are among the first symptoms indicating the presence of HIV in the organism, and about 90% of the seropositive people present symptoms of cutaneous eruption (generalized dermatitis, bacterial, fungal, viral and parasitic infections, skin tumors) during their illness (Verville, 2013). Moreover, tuberculosis causes a number of deaths in the world and represents a major public health issue (Pavan *et al.*, 2009). It is an infectious, contagious and endemic disease, with higher respiratory tropism caused by the *Mycobacterium tuberculosis* (or Kock bacillus), which is responsible of the death of two millions people per year (OMS, 2006).

The Human Immunodeficiency Virus (HIV) causing the Acquired Immunodeficiency Syndrome (AIDS) and tuberculosis are related (OMS, 2004). In Africa, tuberculosis is often the first manifestation of infection by the HIV, and appears to be the main cause of death (Grant *et al.*, 1997; Rana *et al.*, 2000).

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The treatments for all these infections (urinary, respiratory, pulmonary, cutaneous and tuberculosis), which are related to HIV, are based on a regular taking of antibiotics coming up from western medicine (Fournet *et al.*, 2008). The lower efficiency and the high costs of these medicines associated to chemo-resistance problems often limit their under-spread application. This situation highlights the urgent need to develop from medicinal herbs new efficient and inexpensive a toxic drugs to treat microbial infections associated with HIV-AIDS. An ethnopharmacology study was recently performed by the Health Minister of Benin and shows that 13 Beninese plants were mostly used by endogenous medicine to fight against sexually transmitted infections associated to HIV–AIDS (Houngnihin *et al.*, 2010). Our study is focused on antimicrobial activity of these plants extracts on various strains associated to HIV-AIDS. The previous study realized by our team (Houngbeme *et al.*, 2014) has shown that only 4 of the hydro-ethanolic extracts of *Ocimum gratissimum* L; *Acanthospermum hispidum* DC, *Caesalpina bonduc* (L) Roxb and *Calotropis procera* W.T.Aiton), presented activity against bacteria (*Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922).

The present work fits into the scope of our long-term continuing investigations and has for main purpose to evaluate antimicrobial activity of these hydro–ethanolic extracts on germs implied in microbial infections associated to HIV-AIDS in an effort to isolate the bioactive molecules and evaluate their toxicity.

MATERIAL AND METHODS

Material

The fresh samples of the plants used in this study (Table 1) were dried for about fourteen days in a room at constant room temperature (22°C). They were then carefully powdered using an electric grinder (of Flour mills Nigeria, EL MOTOR N°1827).

The microbial support used is made up of: *Escherichia coli* ATCC O: 157H7, *Salmonella typhi* (isolated), *Klebsiella pneumoniae* (isolated) *Staphylococcus aureus* ATCC 25923 resisting to methicillin (MRSA), *Candida albicans* ATCC 10231 and *Mycobacterium bovis* BCG 040812. These strains were provided by the National Laboratory of Public Health of the Health Ministry of Benin.

The brine shrimp larvae (*Artemia salina*, LEACH) are commercialized by the German company JBL GmbH & Co.KG and the cells WI–38 are used for assessing the toxicity tests.

Methods

Preparation of crude extracts **(**Houngbeme *et al.*, 2014)**:**

Fifty grams (50 g) of each powdered plant were mixed with 500 mL of water–ethanol 95° (4:6; v/v). The mixture is stirred for (72h) and the steeping obtained is successively filtered three times on hydrophilic cotton. The filtrate was then evaporated to dryness at 40°C using a rotary evaporator (Heidolph Laborota 4000 efficient) coupled to a water chiller (Jubalo FL 300) to give the crude extracts and the yield was determined.

Test on bacteria and yeasts

The micro-dilution method in 96 wells microplates was used throughout (Houngbeme *et al.*, 2014)**.** Two blank samples were performed: a negative one with extract and a positive one with bacterial suspension in order to control the growth of germs. The negative control (on the line A of the plate) is composed by various successive dilutions (two fold) from 100 µL of the main extract solution up to 20 mg/mL of DMSO (1%) and 100 µL of Mueller Hinton Broth (MHB) containing 0,02g/L of red phenol. The positive control (on line B) is performed in the same way as the negative control but the extract solution is replaced by 100 µL of bacterial suspension at 10^6 colony forming units per milliliter (CFU / mL) (Density equal to Scale 2 of MC Farland). In the wells of lines C and D, the strains were incorporated into the extracts by adding 100 µL of microbial suspension to each well containing extract dilutions.

Cotrimoxazol (*Bio-Rad*) which have action on various bacteria was used as reference's antibiotic at 0.5 µg/mL for the stock solution. The microplate was recovered and connected to the sterilizer at 37°C for 24 h duration. We determined the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). The knowledge of MIC and MBC were used to calculate the antibiotic power (ap) of the tested extract: ap = MBC/MIC. When ap is lower or equal to 4, this means that the tested extract shows some antibiotic power (Yèhouénou, 2012).

In vitro* test on *Mycobacterium bovis (M. bovis)

The method developed with success by Yemoa *et al.* (2011) to test the *Mycobacterium ulcerans* (pathogenic agent of the ulcer of Burili) sensitivity was adapted herein on hydro-ethanolic extract of plants. It is a method of liquid spot microdilution in microplates of 96 wells, using the technical << Resazurin Microtiter Assay: REMA >> (Palomino *et al.*, 2002). Moreover, we also performed successive dilutions starting from 100 µL of the extract solution at 500 µg / mL and 100 µL of the spot 7H9 in the wells of columns 2 to 10 (line B to G). We also performed negative controls (200 µL of the middle 7H9 in the wells B_6, C_6, D_6) and positive controls (100 µL of bacterial suspension at 10^8 CFU/ mL and 100 µL of the middle 7H9 in the wells E_6, F_6, G_6). 200 µL of sterilized distilled water were introduced into the external wells (columns 1, 7 to 12; lines A and H). Over the dilutions, the concentration of DMSO in the middle of the culture is lower than the threshold of 0.625 % (Cos *et al.*, 2006).

After dilution, 100µL of bacterial suspension of *M. bovis* at 1mg / mL containing approximately 10^8 CFU / mL (Portaels *et al.*, 1998) were inoculated in the wells containing the extracts. The plates are then incubated at 31 ± 1 °C for 15 days and were added in the first well (positive control), 30 µL of aqueous solution of resazurin (Acros organic N.V) at 0,02% w/v (initially blue) prepared and stored at 4°C for two weeks before use. The plates are afterwards put in a sterilizer at 31 ± 1 °C. The development of

a color after 24 h evidences the growth of *M. bovis*. The whole plate is then revealed by adding 30 µL of resazurin in each well and assessed 24 h later. The results can be interpreted and are conclusive if and only if the negative controls remain blue and the positive controls remain pink. Based on the previous works, an extract is considered being active when CMI ≤ 128 µg / mL (Tosun *et al.*, 2004; Gu *et al.*, 2004) and not active, if the CMI > 250 µg / mL.

Larval toxicity assay **(**Houngbeme *et al.*, 2014)

The test is performed against *Artemia salina* Leach by Fatondji *et al.* (2010); Sakirigui *et al.* (2012a, 2012b) and proposed in the literature as a simple bioassay method for assessment of preliminary toxicity of natural active products (Pelka *et al.*, 2000). The eggs of *Artenia salina* were incubated in sea water until hatching of young larvae (48 h). We prepared under various concentrations a series of solutions of each tested crude extracts. A defined number of larvae (16) were introduced in each solution. All solutions and control solutions without active component were put under stirring for 24 h. A counting of the dead larvae number (under a microscope) in each sample was performed to evaluate the toxicity of the solution. In case of death (during the control), the data were corrected by Abbott's formula:
% death = [(test - control) / control)] x 100 (Carballo *et al.*, 2002)**.** Data (dose-response) were subsequently transformed by logarithm and the LC_{50} were determined by linear regression (Mousseux, 1995). Tests were carried out in triplicate.

Cytotoxicity assay **(**Block *et al.*, 2004; Ganfon *et al.*, 2012)

The cytotoxicity of the extract on J774 and WI-38 cells was evaluated as described by Stevigny and *al.* (2002), using the tetrazolium MTT salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma)) colorimetric method based on the cleavage of the reagent by dehydrogenases in viable cells (Mosmann, 1983).

Camptothecin (Sigma) was used as positive cytotoxic reference's compound at 0.25 µg/mL. Stock solution of compounds and active fractions were prepared in DMSO at 20 mg/mL. The solutions were diluted in order to provide 200, 100, 50, 25, 12.5 and 6.25 µg / mL concentrated solutions. The highest concentration of solvent which was shown to be non-toxic at which the cells were exposed was ≈1%. Each extract was tested in a series of six dilutions in 96-wells microtiter plate. Each experiment was duplicated to confirm the results.

Chromatographic methods

Thin layer chromatography (TLC)

The pure material was obtained by standard preparative TLC method using stationary phase: plate 10 x10 cm, TLC Silicagel F254S, Merk ®; mobile phase: toluene - ethyl acetate - formic acid (36-12-5, v/v/v). The bioautographic method (derived from analytic TLC) was used. in order to detect the presence or not of clear zones of inhibition for 24 h (Haouat *et al.*, 2013).

Fractionation on column at atmospheric pressure

This technique was used to achieve coarse fractions and to mark which fraction recovers the most active compounds after the biological tests. The operating conditions are:

- stationary phase: Silica frost, 0,062 mm-0,2 mm and the frost of polyamid SC6 MN.
- elution: 300 mL of eluant in the following order: n-hexan , dichloromethane, ethyl acetate, n–butanol and isopropanol

The MPLC (Medium pressure Liquid Chromatography)

The active samples obtained by fractionation on open column are put on the MPLC column in order to fractionate them again until the separation and the isolation of the most compounds using a glass column: omnifit (OM 6427 15 x750 mm); stationary phase: Lichroprep Si RP-18.

Separation on dextran frost: sephadex® LH 20.

With this frost, the compounds are separated according to their molecular weight using stationary phase: sephadex LH20 and eluent: ethanol 95°.

RESULTS AND DISCUSSION

Antimicrobial activity of extracts

The Table 2 summarizes the values of different biological parameters measured for hydro-ethanolic extracts of plants. The biological screening took into account sixbacterial strains implied in microbial infections associated with HIV-AIDS. The fourth tested extracts showed bacteriostatic and fungistatic activities on our studied strains; this result comes to reinforce our preliminary works for which these same extracts were revealed bacteriostatic and bactericidal on *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 (Houngbeme *et al.*, 2014).

In this study, the *Mycobacterium bovis* (*M. bovis*) BCG 040812 remained non-sensitive to the extracts. *M. bovis* resistance's could be explained by the mycobacteria complex chemical composition. Indeed, they are rich in lipids and often permeable to less polar compounds (Connell and Nikaido, 1994). On the over hand, the well known property of *Mycobacterium* is the capacity of its cellular inner side to resist to bleaching by diluted acid and alcohol characteristic related to the high composition in lipid of the cellular inner side (Boukary *et al.*, 2011).

Two extracts are indeed fungicidal, precisely the hydro-ethanolic extracts of *Acanthospermum hispidum* (HE_2) and of *Caesalpinia bonduc* (HE_3) have some antibiotic power (respectively ap = 2 and ap = 4) on *Candida albicans* ATCC 10231. Comparing to the Cotrimoxazol, it can be assumed that there is no significant difference between, Cotrimoxazol (ap = 1) and HE_2 activities (ap = 2). Nevertheless, HE_3 activity (ap = 2) appears to be lower than Cotrimoxazol's. The activity demonstrated by the extract of *Caesalpinia bonduc* corroborates the previous works which emphasizes the antibacterial and

fungicidal activities of the plant (Singh and Raghav, 2012). From this result, it can be deduced that the hydro-ethanolic extracts of *Acanthospermum hispidum* (HE_2) and *Caesalpinia bonduc* (HE_3) can contribute to the slowing of candidoses which are highly associated to HIV-AIDS. The calculated selectivity index showed that the extracts are more toxics on the shrimp larvae than active because the SI is lower to 1 (Tiuman *et al.*, 2005). Only the HE_3 extract possesses a weak selectivity (SI=1.12) on *E. coli* O 157H7.

Cytotoxicity of HE_2 and HE_3 extracts

For this test, the results are expressed by the inhibitory concentration 50 % (IC_{50}) when superior to 100 µg / mL for the hydro-ethanolic extract of *Acanthospermum hispidum (A. hispidum)* (HE_2) against 0,4 (± 0,2) µg / mL for the witness (the comptothecin).

The IC_{50} is equal to 50 µg / mL for the hydro-ethanolic extract of *Caesalpinia bonduc* (HE_3). The limit of toxicity being fixed at 20 µg / mL (Ganfon *et al.*, 2012), it can be concluded that the HE_2 and HE_3 extracts do not present any harmfulness on the cells WI-38. They can be considered therefore not toxic. This result reinforces the previous result on larval toxicity which allowed to highlight the non-toxic character of these extracts on the shrimp larvae (Houngbeme *et al.*, 2014), and therefore, on Human cells of carcinoma and colon in accordance to the works of Pelka *et al.* (2000) and Carballo *et al.* (2002).

These results are similar to those of Ganfon *et al.*, when they evaluated in 2012, the *in vitro* cytotoxicity of acid aqueous extracts of *A. hispidum* on the same cell line WI-38. The two fungicidal and bacteriostatic extracts are not toxic, which motivated our submitting to successive fractionation in an effort to isolate some molecules.

Bio-guided purification of fungicidal extracts HE_2 and HE_3

Antimicrobial activities of obtained fractions

The two extracts are firstly submitted to a fractionation on column at atmospheric pressure and the different fractions obtained are tested on the previous strains. The results are summarized in the tables 3 and 4.

All fractions from the two extracts showed bacteriostatic and fungistatic actions on the different microbial strains. For the fractions from extract HE_2, the MIC vary from 0.3125 mg /mL to 10 mg / mL for *Escherichia coli* O 157H7, from 0.625 mg / mL to 5 mg / mL for both MRSA and *Salmonella typhi*, and to finish, from 1.25 mg / mL to 10 mg / mL for both *Klebsiella pneumoniae* and *Candida albicans*.

Concerning the values of MIC of the fractions from HE_3 extract, they vary from 0.3125 mg / mL to 10 mg/mL for both *E. coli* O 157H7 and *Salmonella typhi*, from 0.625 mg / mL to 10 mg / mL for MRSA; from 2.5 mg / mL to 10 mg / mL for *Klebsiella pneumoniae* and from 1.25 mg / mL to 5 mg / mL for *Candida albicans*. At the end of this biological screening, three fractions (FBut of HE_2, FBut and FHex of HE_3) appeared to be bactericid and fungicidal on three microbial strains: *E. coli* O 157H7 (gram-); *MRSA* (gram+) and *Candida albicans* (yeast). The difference between antibiotic power of fractions and positive reference wasn't significant for the hexanic fraction of HE_2 and the butanolic fraction of HE_3 respectively on MRSA and *C. albicans* (ap= 2). We became interested in these three fractions in order to isolate the molecules responsible of the antimicrobial activity.

Identification of active spots by bioautography

In this experiment, we have studied only three germs on which the fractions have presented activity. The results are presented at Table 5.

The diameter of inhibitory area varies from microbial strain to another one and according to the nature gram+ or gram- of the latters. Otherwise, there is no significant difference between the inhibition diameters of spots and the one of nystatin so those fractions could be good candidates for the elaboration of new fungicidal medicines.

Isolation of M_1, M_2 and M_3 compounds

-The n-butanol fraction (90 mg) of HE_2 extract of *A. hispidum* is put on the polyamid frost flown at atmospheric pressure glass in order to get rid of colours (chlorophyll). The obtained residue has undergone a first fractionation by MPLC in reverse phase using acetonitrile / ethyl acetate gradient (6-4 to 8-2, v/v) to give ten fractions. The sub-fraction $Fb_{2.3}$ is separated on the Sephadex® LH20 with ethanol 95° to obtain the M_1 compound (9 mg) which is a white amorphous powder which absorbs at the UV at 366 nm and becomes yellow with the NEU/ PEG reagent on a silica slab CCM similar to a flavonoid.

-100 mg of hexanic fraction $FHex_3$ are split up on MPLC with silica gel 60 F254 nm, 15-25µm, Merck and a mixture composed of toluene-ethyl acetate (8-2 ; v/v). Then, we performed a preparative CCM followed by a filtering on SPE cartridge. The filtered product obtained is concentrated and separated by chromatography on sephadex gel LH20. We obtained a white amorphous powder called M_2. The M_2 compound is neither visible on CCM slab with eyes or with UV at 254 nm or on 366 nm without revelation by sulfuric anisaldehyde, which colors the compound M_2 in blue then it can be predicted a terpenoid or a steroid.

-The $Fbut_3$ fraction (85 mg) is submitted at a fractionation by MPLC in reverse phase (Lichroprep RP18; 40-60 µm, Merck) with acetonitrile / ethyl acetate (2-8; v/v) to have eight fractions ($Fb_{3.1}$ à $Fb_{3.8}$). The sub-fraction $Fb_{3.6}$ (47.52 mg) is then split up on MPLC with the same stationary phase and a mixture of acetonitrile / methanol (8-1;v/v). Then, the under fraction $Fb_{3.6.3}$ (23.15 mg) is separated on Sephadex® gel LH20 with ethanol 95° to have 9.63 mg of a yellow amorphous powder named M_3 which absorbs in the visible and is coloured in yellow with NEU / PEG reagent; M_3 can be a flavonoïd.

Table 1: List of Studied Plants (Houngbeme *et al.*, 2014).

N°	Species	Local names	Familly	Part used	Locality of crop
1	*Ocimum gratissimum* L.	**Fon and Goun** :*tchayo ;* **Yoruba** : *simonua*	LAMIACEAE	leaves	Danto/ Ouémé
2	*Acanthospermum hispidum* DC.	**Fon :** *kpononmi ;***Goun**: *ahwanglon ;* **Yoruba** : *tchakatoun*	ASTERACEAE	A .P	Danto/ Ouémé
3	*Caesalpinia bonduc (L.)* Roxb.	**Fon**: *adjikwin ;***Yoruba** : *ewé ago, itchè*	CAESALPINIACEAE	leaves	Adjohoun/ Ouémé
4	*Calotropis procera* (Aiton)	**Fon and Goun** :amouman, kpènto ; **Yoruba** : boum boum, kpamon	ASCLEPIADACEAE	leaves	Abomey-calavi/ Atlantique

A .P: aerial parts

Table 2: Results of antimicrobial activity and toxicity of extracts.

Extracts	LC 50 (mg/mL)	Strains											
		E. coli O 157H7				*MRSA*				*Salmonella typhi*			
		MIC	SI	MBC	ap	MIC	SI	MBC	ap	MIC	SI	MBC	ap
HE1	0.05	2.5	0.02	-	-	2.5	0.02	-	-	0.625	0.08	-	-
HE2	0.39	0.625	0.624	-	-	0.625	0.624	-	-	1.25	0.312	-	-
HE3	0.35	0.3125	1.12	-	-	1.25	0.28	-	-	2.5	0.14	-	-
HE4	0.02	2.5	0.008	-	-	1.25	0.016	-	-	1.25	0.016	-	-
Cotrimoxazol	-	0.125	-	0.125	1	0.0312	-	0.0312	1	0.125	-	0.125	1

Extracts	LC 50 (mg/mL)	Strains											
		Klebsiella pneumoniae				*Candida albicans*				*M. bovis*			
		MIC	SI	MBC	ap	MIC	SI	MBC	ap	MIC	SI	MBC	ap
HE1	0.05	1.25	0.04	-	-	1.25	0.04	10	8	>250	-	nd	nd
HE2	0.39	1.25	0.312	-	-	1.25	0.312	2.5	2	>250	-	nd	nd
HE3	0.35	2.5	0.14	-	-	0.625	0.56	2.5	4	>250	-	nd	nd
HE4	0.02	1.25	0.016	-	-	0.625	0.032	10	16	>250	-	nd	nd
Cotrimoxazol	-	0.125	-	0.125	1	0.0625	-	0.0625	1	>250	-	-	-

MIC: Minimum Inhibitory Concentration (mg/mL but µg/mL for Cotrimoxazol and Mycobacterium bovis); MBC: minimum bactericidal concentration (mg/mL), LC_{50}: Lethal concentration50%; ap: antibiotic power,HE: hydro-ethanolic extract, SI = LC50 / MIC: Selectivity index; nd: no determining. The indices 1, 2, 3 and 4 indicate respectively O.gratissimum, A.hispidum, C. bonduc and C. procera.

Table 3: Results of antimicrobial activities and toxicity of he_2 fractions .

Fractions of HE_2	LC 50 (mg/mL)	Strains											
		E. coli O 157H7				*MRSA*				*Salmonella typhi*			
		MIC	SI	MBC	pa	CMI	SI	MBC	ap	MIC	SI	MBC	ap
F_{Hex}	0.12	0.3125	0.02	-	-	2.5	0.02	-	-	0.625	0.08	-	-
F_{DCM}	0.07	0.625	0.62	-	-	0.625	0.62	-	-	1.25	0.312	-	-
F_{ACE}	0.18	0.3125	1.12	-	-	1.25	0.28	-	-	2.5	0.14	-	-
F_{But}	0. 78	2.5	0.008	10	4	2.5	0.02	5	2	5	0.016	-	-
F_{Ipr}	0.21	10	0.021	-	-	5	0.04	-	-	5	0.042	-	-

Fractions of HE_2	LC 50 (mg/mL)	Strains							
		Klebsiella pneumoniae				*Candida albicans*			
		MIC	SI	MBC	ap	MIC	SI	MBC	ap
F_{Hex}	0.12	1.25	0.04	-	-	5	0.024	-	-
F_{DCM}	0.03	1.25	0.312	-	-	2.5	0.012	-	-
F_{ACE}	0.18	2.5	0.14	-	-	1.25	0.144	-	-
F_{But}	0. 78	2.5	0.016	-	-	2.5	0.312	5	2
F_{Ipr}	0.21	10	-	-	-	10	0.021	-	-

MIC: Minimum Inhibitory Concentration (mg/mL); MBC: minimum bactericidal concentration (mg/mL), ap: antibiotic power, HE: hydro-ethanolic extract, SI = LC50 / MIC: Selectivity index; LC_{50}: Lethal concentration 50%; F_{Hex} : n-hexane fraction; F_{DCM} : dichloromethane fraction; F_{ACE} : ethyl acetate fraction ; F_{But} : n-butanol fraction ; F_{Ipr} : isopropanol fraction

Table 4: Results of antimicrobial activities and toxicity of he_3 fractions

Fractions of HE3	LC 50 (mg/mL)	Souches microbiennes											
		E. coli O 157H7				MRSA				Salmonella typhi			
		MIC	SI	MBC	ap	MIC	SI	MBC	ap	MIC	SI	MBC	ap
F Hex	0.12	0.3125	0.02	1.25	4	2.5	0.02	5	2	0.312	0.08	-	-
F DCM	0.03	10	0.624	-	-	5	0.62	-	-	10	0.312	-	-
FACE	0.18	10	1.12	-	-	10	0.28	-	-	10	0.14	-	-
FBut	0. 78	2.5	0.008	10	4	0.625	0.02	2.5	4	2.5	0.016	-	-
FIpr	0.21	10	0.021	-	-	5	0.04	-	-	10	0.021	-	-

Fractions of HE3	LC 50 (mg/mL)	Souches microbiennes							
		Klebsiella pneumoniae				Candida albicans			
		CMI	SI	CMB	pa	CMI	SI	CMB	pa
F Hex	0.12	5	0.04	-	-	1.25	0.04	5	4
F DCM	0.03	10	0.312	-	-	5	0.312	-	-
FACE	0.18	10	0.14	-	-	5	0.56	-	-
FBut	0.78	2.5	0.016	-	-	2.5	0.032	5	2
FIpr	0.21	10	0.021	-	-	5	0.042	-	-

MIC: Minimum Inhibitory Concentration (mg/mL); MBC: minimum bactericidal concentration (mg/mL), ap: antibiotic power, HE: hydro-ethanolic extract, SI = LC50 / MIC: Selectivity index; LC50: Lethal concentration 50%; F Hex : n-hexane fraction; FDCM : dichloromethane fraction; FACE : ethyl acetate fraction ; FBut : n-butanol fraction ; FIpr : isopropanol fraction.

Table 5: Values of Inhibitory diameter of Spotlights.

Fractions spotlights / antibiotic	Diameter of inhibitory area (mm)		
	E.coli **O 157H7**	***MRSA***	***C. albicans***
M_1	5	7	8
M_2	5	7	7
M_3	4	6	8
Nystatin (T_1)	-	-	10
Bactrim (Trimétoprime-sulfamétroxazole, T_2)	11	-	-
Pénicillin G (T_3)	-	12	-

M_1, M_2 and M_3 indicate the compounds that showed a inhibitory area respectively in F_{But} of HE_2 , F_{Hex} and F_{But} of HE_3

Table 6: Result of biological activities of isolated compounds.

isolated molcules	LC_{50} (mg/mL)	Strains											
		E. coli O 157H7				*MRSA*				*Candida albicans*			
		MIC	SI	MBC	ap	MIC	SI	MBC	ap	MIC	SI	MBC	ap
M1	0.28	2.5	0.11	10	4	2.5	0.11	10	4	5	0.06	10	2
M2	0.13	0.625	0.21	2.5	4	2.5	0.05	5	2	0.625	0.21	2.5	4
M3	0.48	2.5	0.19	10	4	0.625	0.77	2.5	4	2.5	0.19	10	4

MIC: Minimum Inhibitory Concentration (mg/mL); MBC: minimum bactericidal concentration (mg/mL), ap: antibiotic power, HE: hydro-ethanolic extract, SI = LC50 / MIC: Selectivity index; LC_{50}: Lethal concentration 50%; M_1, M_2 et M_3 indicate the three isolated molecules.

Structure of isolated compounds

The treatment of the information's provided by spectral techniques helps to define the molecular structure of isolated compounds.

Spectroscopic data of M_1 Compound

MS (m/z): 225.09073 ($[M+H]^+$) ; 210 ($[M+H]^+$-CH_3) ; 207 ($[M+H]^+$-H_2O); 147 ($[M+H]^+$-C_6H_6); 121($[M+H]^+$- C_8H_8).

^{1}H NMR (DMSO-d_6, 400 MHz, δ en ppm): δ 2.82 (*dd*, H-3b); 3.25 (*dd,*H-3a); 5.5 (*dd*, H-2); 5.85 (d, H-5, H-8); 7.1 (dd, H-6, H-7); 7.50 (*dd,* H-3',5'); 7.6 (*d,* H-2',6').

^{13}C NMR (DMSO, 100 MHz, δ en ppm) : δ 191.55(C=O) ; 161.06 (C-9) ; 138.90 (C-1') ; 136.27 (C-7) ; 128.50 (C-5) ; 128.54 (C-3', C-5) ; 126.61 (C-4') ; 126.31 (C-2', C-6') ; 121.45 (C-10) ; 120.65 (C-6) ; 118.03 (C-8) ; 78.80 (C-2) ; 43.5 (C-3).

The mass spectrum shows that the formula of M_1 compound is $C_{15}H_{12}O_2$ whose molecular mass is 224.09073 g / mol (calculated mass is 224.08376 g/mol) and corresponds to non-substituted flavanone. The presence of peaks between 7 and 8 ppm on the ^{1}H-NMR spectrum shows that the flavonoïd possesses non substituted cycle (Mabry *et al.*, 1970). Two non equivalent protons at δ 3.10 ppm and 2.85 ppm correspond to H-3 protons of a flavanone (Mabry *et al.*, 1970). A peak at δ 5.43 ppm corresponds to H-2 (CH) proton of a flavanone (Mabry *et al.*, 1970). We find some aromatic protons at δ 7.6 ppm (*d*, *J* = 2.1 Hz), 7.50 ppm (*dd*, *J* = 8.3, *J* = 2.1 Hz) which are respectively attributed to H-2', 6'and H-3', 5'.On the other hand, we don't observe a peak between δ 12.5 et 13 ppm what shows the absence of phenolic proton in the compound (Harborne, 1993). The ^{13}C-NMR spectrum shows thirteen peaks which correspond to thirteen carbons. The absence of peaks at δ 161.7 ppm and 160.6 ppm shows that it's a non substituted cycle (Portet, 2007). Those pieces of information are in accordance with the flavanone non-substituted molecule (figure 1).

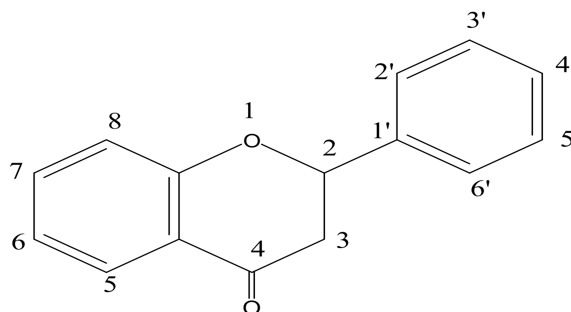

Fig. 1: Structure of Flavanone (M_1)

Spectroscopic data of M_2 compound

MS (m/z): 413, 3608 ($[M+H]^+$) ; 435,35924 ($[M+Na]^+$)

^{1}H NMR ($CDCl_3$, 400 MHz, δ en ppm): δ 7.30 (*s*, OH-3); 5.2 (*m,*H-6); 4.9 (*s*, H-22); 5.1 (s, H-23); 3.51 (tdd, H-3); 1.21 (*s,* H-19); 1.17 (*s,* H-28); 1.03 (*s,* H-27); 0.99 (*s,* H-26); 0.97 (*s,* H-24); 0.90 (*s,* H-29).

^{13}C NMR ($CDCl_3$, 100 MHz, δ en ppm) : δ 140.76 (C-22) ; 138.33 (C-5) ; 129.28 (C-23) ; 121.72 (C-6) ; 76.71 (C-3) ; 56.87 (C-4) ; 55.96 (C-5) ; 51.25 (C-24) ; 50.40 (C-17) ; 42.30 (C-9) ; 42.22 (C-13) ; 39.69 (C-10) ; 37.27 (C-10) ; 36.52(C-20) ; 31.9(C-25) ; 31.66(C-21) ; 28.94 (C-23) ; 25.43 (C-12) ; 24.36 (C-11) ; 21.23 (C-25) ; 21.11(C-26) ; 19.41(C-29)

The peak $[M+H]^+$ at m/z = 413.3608 and the peak $[M+Na]^+$ at m/z = 435.35924 correspond to brute formula $C_{29}H_{48}O$ (calculated mass is 412.37074 g/mol) which is the one of stigmasterol. The H-3 proton appears in form of tripled splitted at δ 3.51 ppm (J = 4.5 and 1.0 MHz) and H-6 olefinic proton shows a multiplied at δ 5.2 ppm. Moreover, two olefinic protons appear at δ 5.16 ppm (m) and δ 5.14 ppm (m) corresponding respectively to

H-20 and H-21 protons of stigmasterol (Habib *et al.*, 2007 ; Jain *et al.*, 2009). Six methyl protons also appear at δ 0.90-1.21 ppm. The chemical shift values of carbons correspond well to the ones of stigmasterol carbons.

These spectrometric data are in accordance with the reported values in literature for the stigmasterol (Jain and Bari, 2010; Hartati *et al.*, 2008; Rajput and Rajput, 2012). On the basis of this, we can attribute to M_2 the structure of stigmasterol (figure 2).

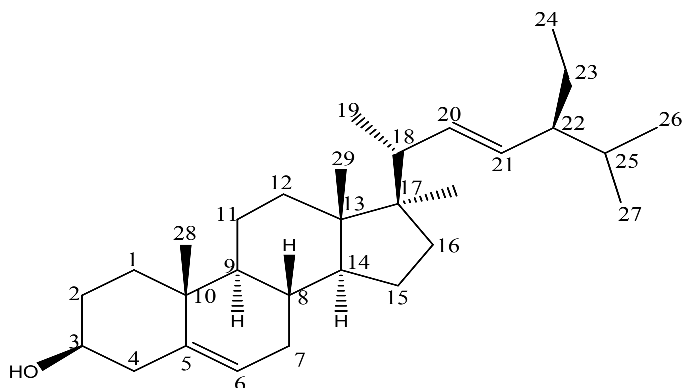

Fig. 2 : Structure Du Stigmasterol (M_2).

Spectroscopic data of M_3 compound

MS (m/z): 303.04967 ($[M+H]^+$) ; 325.03159 ($[M+Na]^+$); 285 ($(M+H]^+-H_2O$); 257 ($[M+H]^+$-HCOOH).

^{1}H NMR (DMSO, 400 MHz, *δ* en ppm): *δ* 7.9 (*d*, H-2', H-6'); 6.9 (*d,* H-5'); 6.5 (*d*, H-8); 6.2 (d, H-6); 3.5 (s, OH-3',4',5,7); 12.5 (*s,* OH-3).

^{13}C NMR (DMSO, 100 MHz, *δ* en ppm) : *δ* 175.8 (C-4) ; 163.84 (C-7) ; 160.68 (C-5) ; 156.09 (C-2) ; 147.66 (C-9) ; 146.75 (C-4') ; 145.01 (C-3') ; 135.70 (C-3) ; 121..92 (C-1') ; 119.94 (C-6') ; 115.57 (C-2') ; 115.02 (C-5') ; 102.97 (C-10) ; 98.14 (C-6) ; 93.31(C-8).

The molecular ion $[M+H]^+$ at m/z = 303.04967 responds to the brute formula $C_{15}H_{10}O_7$, the one of the quercetin. The calculated mass is 302.0426 g/ mol. On the ^{1}H-NMR spectrum of M_3 compound, we observe two aromatic protons forming two doubled at δ 6.20 and 6.50 ppm (*J* = 2.0 Hz), characteristic of cycle A of a flavonoid substituted in C-5 and C-7 and can be attributed respectively to H-6 and H-8 protons (Markham, 1982). The presence of three aromatic protons at δ 7.54 ppm (*d*, *J* = 2.1 Hz), 7.50 ppm (*dd*, *J* = 8.3, *J* = 2.1 Hz), 6.92 ppm (*d*, *J* = 8.3 Hz) corresponding respectively to H-2', H-6' and H-5'. Moreover we notice the presence of a peak at 12.5 ppm which is a phenolic proton (Harborne, 1993).

The ^{13}C-NMR spectrum of M_3 compound shows ten quaternaries carbons, one carbonyle group (C=O, δ 175.8 ppm) and C-3 carbon characteristic of a flavonol (δ 135.7 ppm), and five aromatic CH (Mabry *et al.*, 1970; Nancy *et al.*, 1999)**.** All these information help us to identify the M_3 compound as quercetin (figure 3).

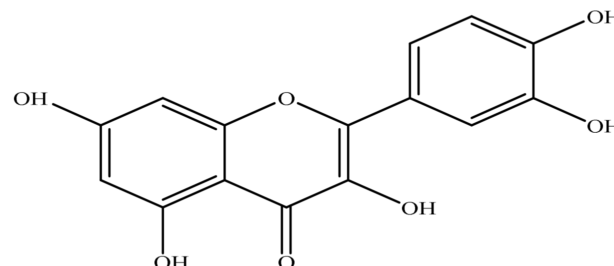

Fig. 3: Structure Of Quercetin (M_3)

Antimicrobial activities and toxicity of isolated compounds

We undertook to determine the MIC and the MBC of these molecules by microdilution. Their toxicity was also evaluated on the larvae of shrimps. The obtained results are summarized at Table 6. The three isolated compounds present antibiotic power on the studied strains. Their antibiotic power values whose vary between 2 and 4 were compared with the antibiotic power of Cotrimoxazol (ap = 1). There is no significant difference between the Cotrimoxazol activity's (ap = 1) and the Flavanone and the Stigmasterol activities whose present the same antibiotic power (ap = 2) on *C. albicans* and MRSA respectively. These results come to reinforce the ones shown by the inhibition areas. The antimicrobial activity of M_1 and M_2 compounds finds its justification in the fact that the flavonoids are used for a range of pharmacological activities as their antimicrobial actions (Sannomiya *et al.*, 2005; de Rijke *et al.*, 2006). The highest antibiotic power (ap = 2) is obtained on *Candida albcans* ATCC 10231, what suggests that the M_1 compound has affinity with chemical composition of the inner side of *C. albicans*. The compounds are not toxic on the larvae of shrimps (LC_{50} > 0.1 mg/mL).

CONCLUSION

The present study permitted to value the plants used by Beninese healers in the treatment of microbial infections associated with HIV-AIDS. The hydro-ethanolic extracts of *A. hispidum* and of *C. bonduc* are fungicidal on pathogenic yeast (*Candida albicans* ATCC 10231). Two flavonoids (flavanone and quercetin) and one steroid (stigmasterol) are identified from these extracts using chromatographic and spectroscopic methods. The three isolated molecules of these extracts possess antibiotic power on the tested strains (*Candida albicans* ATCC 10231, *E. coli* O 157H7, *MRSA*), what justify the derived fractions antimicrobial activity. These molecules are not toxic on some human cells like the carcinome and colon. Based on these results, we expect to be able to conceive formulae of new medicines and imprint new progresses to phytotherapy of microbial infections associated to HIV-AIDS. It is essential how to go further in this work by studying the mechanism of these molecules.

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