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Research Paper

Study on biological activities and chemical composition of extracts from *Desmodium adscendens* leaves

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ABSTRACT

Phenolic compounds in *Desmodium adscend*ens leaves extract were isolated, identified characterized by HPLC, NMR and MS. Five phenolic compounds were identified, caffeic acid, *p*-coumaric acid, epicatechin, rutin, quercetin. The essential oil was obtained by distilled method. The major compounds found in the essential oil were phytone (14,72 %), caryophyllene oxide (11,32%), esdesma (7,41%), geraniol (5,42 %), linalool (5,33%), palmitic acid (5,06 %), α-caryophyllene (4,76 %), scytalone (3,83%), ß-ionone (3,47%), 2,2-dimethyl-hexanale (3,37 %), pelargonaldehyde (3,26 %), hyperforine (3,27%), 2-pentyl furan (2,71), oleic acid (2,68 %), and 4- azidoheptane (2,02 %). The antimicrobial properties evaluate on Water extract (WE) and methanol water extract (MWE) showed that MWE has the better activities on *Escherichia coli*, *Aspergillus niger*, *Pseudomonas aeruginosa*; *Bacillus subtillis*, *Staphylococcus aureus* than WE.

Keywords: Polyphenolic compounds; Flavonoids; Anthocyanins; RP-HPLC.

INTRODUCTION

Desmodium adscendens (Family-*Fabaceae*) is a vine which grows wild in the Amazon rainforest of Peru, in South American contries and as well on the West Coast of Africa. Native people use this plant as juice or tea. Also, *D. adscendens* is a medecinal plant which is widely used in popular medicine in different parts of the world. In Brazilian traditional medicine, the leaves of *D. adscendens* are used in the treatment of several diseases: leucorrhoea, body aches, pains, ovarian inflammations, excessive urination, gonorrhea, and diarrhoeas (Muanda, et al., 2010). Its positive effect against hepatic infection was also verified in vivo (Wong, et al., 2006). In previously studied, we have identified, quantified and evaluated the antioxidant capacity of the leaves of *D. adscendens* (Muanda, et al., 2010).

The aims of this study are to determinate the antimicrobial properties and to isolate bioactive compounds from the leaves of *D. adscendens*.

MATERIALS AND METHODS

Chemicals: All the chemicals used were analytical grade, gallic acid, rutin, procatechiuc acid, orientin, catechin, p-coumaric acid, caffeic acid, isovitexin, vitexin, chlorogenic acid, homoorientin,quercetin, quercetin dehydrat, quercetin glucosyl, epicatechin were purchased from Across organics (Geel, Belguim), n-hexan, methanol and dichloromethane were obtained from Sigma and Roth (Strasbourg, France).

***Apparatus*:** *The RP-HPLC* analyses were performed with a Waters 600E pump coupled to a Waters 486 UV visible tunable detector and equipped with a 20µl injection loop and an Alltech Intertsil ODS column (RP C18 size 4.6 mm x 150 mm; particle size, 5µm).

The GC-MS*:* The essential oils were analysed and identified by GC/ MS. An HP model 6890 GC equipped with a 30m x 0.25 mm i.d. (d_f: 0.25µm) DB-5 boded-phase fused-silica capillary column (Agilent, Folsom, CA) and a flame ionization detector (FID) were used. Injector and temperatures were 200 and 300°C, respectively. The oven temperature was programmed from 35 to 250°C at 5°C/ min and held for 50 min. The linear velocity of the helium carrier gas was 30 cm/s. Injections were in the split-les mode. An HP model 6890 GC interfaced to an HP 5791A mass selective detector (GC/MS) was used for mass spectral identification of the GC components at a MS ionization voltage of 70 eV. A 30 mx0.25 mm i.d (df= 0.25µm) DB wax bonded-phase fused-silica capillary (Agilent, Folsom, CA) was used for GC. The linear velocity of the helium carried gas was 30 cm/s. The temperature of the injector and detector was 250°C. The oven temperature was programmed from 50°C to 250°C at 5°C / min and held for 50°C. The percentage of each compound in the oil is determined from peak areas without correction factors account assuming that all components have coefficients of neighboring rethinking. Identifications of constituents were performed by coupling an HP model 6890 GC gas to a mass spectrometer type an HP 5791A mass selective detector (GC-MS). The volume injected is 1µl of a pure oil solution diluted to 1% in dichloromethane. Quantitative analysis was based on the comparison of retention times and the computer mass spectra libraries using Wiley GC/MS Library and Nist, Tutore Libraries. The percentage composition was computed from the GC peak areas.

Plant Materials: *D. adscendens* were obtained from Nigeria, and the biological authentication was carried out by Professor Max Henry in the Botanic and Mycology Laboratory, of Nancy University (France). All plant materials were dried at room temperature and were ground and sifted in a sieve (0.750µm).

Antimicrobial properties*:* The following bacterial strains were employed in the screening: *Staphylococcus aureus* (ATCC29213), *Bacillus subtilis* (ATCC6059), *Escherichia coli* (ATCC25922), *Pseudomonas aeruginos* (ATCC6059) and fungi: *Aspergillus niger* (135550/99), *Candida albicans* (ATCC90028) given by IBISE Laboratory (UIT Thionville-Metz University).

The antimicrobial properties were examined by the disk-diffusion method (Bauer, et al., 1966). The bacterial cell suspension was prepared from 24h culture and adjusted to an inoculation of 1x 10^6 CFU/ml. Sterile nutrient agar (Immun präparate, Berlin, D, 26g agar/L distilled water) was inoculated with bacterial cells (100µl of bacterial cell suspension in 25 ml medium) and poured into dishes to give a solid plate. Yeasts and fungi (1x 10^7 CFU/ml) were inoculated into sterile Mueller-Hinton-agar (Becton

Dickinson, Heidelberg) according to DIN E 589440-3 for the agar disc-diffusion assay (Al-Fatimi, et al., 2007). 20µl of test material (250g/ 500ml) dissolved in the same solvent like for the extraction, were applied on sterile paper discs (6mm) diameter. Ampicillin, gentamicin and nystanin, were used as positive control, and the solvents (Water and Methanol-water 50/50 (v/v)) were used as negative control. The solvent were evaporated in a stream of air and the discs were deposited on the surface of inoculated agar plates. Plates were kept in a refrigerator for 1 h to permit good pre-diffusion of substances into the agar. The plates with bacteria were incubated for 24 h at 34°C, the plates with yeast for 36°C and the plates with fungi for 72h at 30°C. Inhibition zone diameters around discs (diameter of inhibition zone plus diameter of the disc) were measured. An average zone of inhibition was calculated for the three replicates. Minimal inhibitory concentration (MICs) was determined by the agar diffusion technique as described by Muanda et al., 2010b.

The highest concentration of the extract tested was 1.5mg/ml. The MIC corresponds to the lowest concentration of the tested extracts able to inhibit any visible microbial growth.

Several concentrations of the extracts were prepared (0.25, 0.20, 0.15, 0.10, 0.05, 0.005ml/ ml). The solutions were agitated vigorously. 20µl of each concentration was transferred into the disc. Then the discs were transferred in the Petri dishes containing the testing microorganisms. The plates were incubated at 30°C (72h), 36°C (48h) and 37°C (24h) for the fungal, the yeast and the bacterial respectively. After incubation the numbers of colonies in each plate were counted. Each assay replicated tree times.

Preparation of essential oil: The essential oil was prepared according to a previously reported method (Muanda, et al., 2011). Dried powered plant materials (250g were placed in a 2L round-bottom flask and mixed with 500 ml of deionised water. The solution was distilled for 5h. The essential oil obtained was dried over anhydrous sodium sulphat. This was subsequently removed by filtration.

Phenolic compounds analysis

Extraction and isolation: The dried powdered leaves (500g) of *D. adscendens* were exhaustively extracted with methanol (70%) for 72h, and concentrated under reduced pressure; the concentrated MeOH-water solution was delipidated by n-hexane solvent. The methanol solution was separated and concentrated.

The splitting process requires the combination of several techniques including: the column chromatography and thin layer. The splitting is done using a column of silica gel, the composition of each fraction was determined by TLC (plate: silica 60 F254 support send (Merk, Darmstadt, Germany)) and is revealed under UV at 254 nm.

Fractionation, isolation and purification of phenolic compounds were performed on methanol-water extracts of leaves of *D. adscendens*.

***Protocol*:** 3g of the residue from methanol-water extracts are chromatographed on a column 3cm in diameter and 50cm long containing 100g of silica (60-200µ). A gradient elution (DCM / MeOH) was established, (n-Hexane, DCM, MeOH / DCM, which varies from 1% to 50% by volume) to separate the different phenolic compounds according to their polarity. The scheme of separation of phenolic compounds contained in *D. adscendens* leaves is reported below.

Identification of isolated compounds: From the fractions, the isolated compounds were identified by HPLC according to the method previously described by Muanda, et al. 2009.

Extracted sample was filtered through a 0.45µm PTFE syringe tip filter. The flow rate was set at 1ml / minute at room temperature. A gradient of three mobile phases

was used in the study, solvent A: 50 mM ammonium phosphate ($NH_4H_2PO_4$) pH 2.6 (Adjusted with phosphoric acid); solvent B: Which was constituted of 80: 20 (v/v) acetonitrile / solvent A, and solvent C, constituted of 200 mM phosphoric acid pH 1.5 (pH adjusted with ammonium hydroxide). The solvents were filtered through a Whatman Maidstone England paper N° 113 and putted in an ultrasonic apparatus for 25 minutes. The gradient profile for 60 minutes was : 100% solvent A at zero minutes, 92 % A / 8 % B at 4 minutes, 14 % B / 86% C at10 minutes, 16 %B / 84 % C at 22.5 minutes, 25% B /75% C at 27.5 minutes, 80% B / 20% C at 50 minutes, 100% A at 55 minutes, 100% A at 60 minutes. After each run, the system was reconditioned for 10 minutes before analysis of next sample. Under these conditions, 20 µl of sample were injected. All sample analysis was done in triplicate.

Phenolic standards prepared by dissolving 1mg/ ml were used to generate characteristic UV spectra and calibration curves. Individual phenols in the sample were identified by comparison of their UV-Vis spectra and retention times with spiked in put of the corresponding phenolic standards.

The detection was carried out at 280 and 320 nm and their quantification was obtained by the comparison of the peaks area with the corresponding standards calibration curves. Collected results were reported as equivalent amount of commercial standard.

The retention times of the isolated phenolic compounds were comparing to the corresponding standards. Some isolated compounds were not identified by lack of standard equivalents.

Characterizations of compounds identified*:* The solvents in the mixtures of compounds isolated and identified were evaporated under vacuum. Part of the residue obtained was immediately dissolved in deuterated methanol (CD_3OD) for structural analysis by NMR and MS. The NMR and MS have provided useful information for determining the basic structure of isolated compounds.

Statistical analysis: Results are presented as Mean±Standard Error; statistical analysis of experimental result was based on analysis of variance one way ANOVA. Significant difference was statistically considered at the level of $P \leq 0.001$.

RESULTS

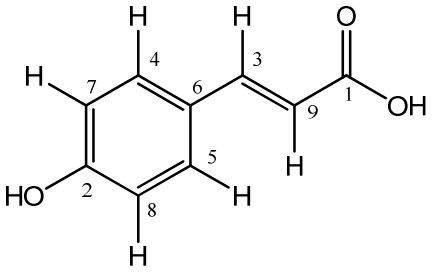
Biological analysis: Antimicrobial properties are reported in tables 1 and 2.

GC-MS analysis: Yields of essential oils from the leaves of *D. adscendens* were 0.25± 0.02g/10g of dry leaves (w/w). The value is the mean of standard deviation (n = 3) ($P<0.05$). Quantitative and qualitative analytical results are shown in table 3.

Phenolic compounds analysis: With fraction (F9), about 20 phenolic compounds were isolated and 5 were identified by HPLC. Because of the lake of suitable standards, all compounds were not identified. The result is shown in table 4.

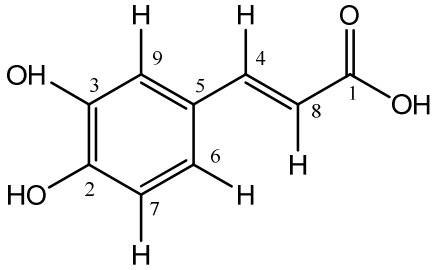
NMR and SM characterization

Compound (X_1): The NMR results (1D (^1H, ^{13}C)) are reported in table 5.



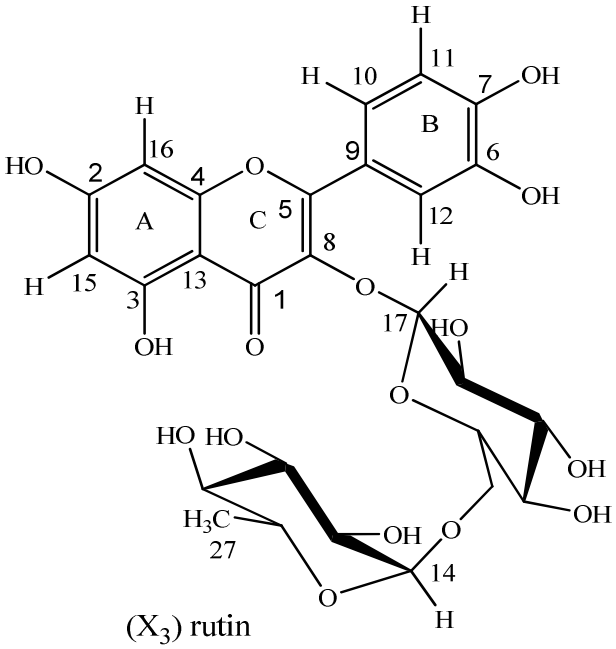
(X_1**)** acide *p*-coumarique

Compound (X₂): The NMR results (1D (^{1}H, ^{13}C)) of ***X₂*** compound are reported in table 6.



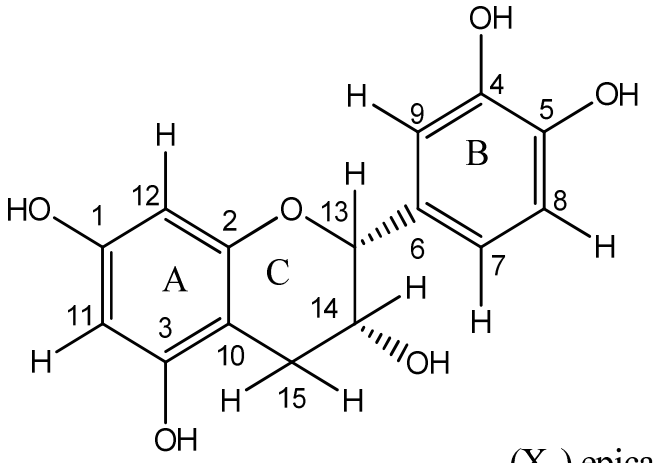
(X_2) caffeic acid

Compound (X₃): The NMR results (1D (^{1}H, ^{13}C)) and the HMQC experiences in NMR 2D of X_3 compound are reported in table 7.



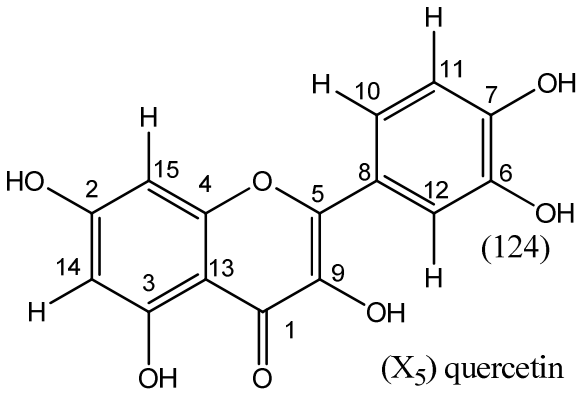
(X_3) rutin

Compound (X_4): The NMR results (1D (^{1}H, ^{13}C)) of X_4 are reported in table 8.



(X_4) epicatechine

Compound (X_5): The NMR results (1D (^{1}H, ^{13}C)) and the HMQC 2D NMR experiences are reported in table 9.



(X_5) quercetin

DISCUSION

The extracts had varying degree of antimicrobial activities against the organism tested. The MWE has the highest activity with inhibition zone varying between (10-13 mm). The WE showed a moderate activity.

About 200 compounds are identified in *D. adscendens* leaves essentials oil, as the results demonstrate the majors identified compounds are phytone (14,72 %), caryophyllene oxide (11,32%), esdesma (7,41%), geraniol (5,42 %), linalool (5,33%), palmitic acid (5,06 %), α-caryophyllene (4,76 %), scytalone (3,83%), ß-ionone (3,47%), 2,2-dimethyl-hexanale (3,37 %), pelargonaldehyde (3,26 %), hyperforine (3,27%), 2-pentyl furan (2,71), oleic acid (2,68 %), and 4- azidoheptane (2,02 %). Most of these compounds are important in the aromas of essential oils, for example caryophyllene possess a woody-spicy odour with a somewhat bitter taste and has been used particularly for chewing gum as well as in spice blends and flavor compositions (El-Massy, et al., 2009). But, it must be noted that, several authors showed that the relative composition of some compounds change significantly because of the dry of leaves [9]. For the isolated compounds, the analysis of HMQC in 2D NMR have

permitted to establish H-4/ C-4, H-5/ C-5, H-7/ C-7, H-8/ C-8) correlations and the δ 7,46-7,43 *ppm* (2H, m) et 6,82-6,81 *ppm* (2H, m) signals show the presence of a aromatic ring substituted on *para*. The δ 7, 63-7,57 *ppm* (1H, d, J=15Hz) et δ 6,31-6,25 *ppm* (1H, d, J=15Hz) signals indicate that the compounds is a ethylene compound with trans configuration.

All so, the SM spectral analysis revealed that the compound (X_1) have a molecular pic with $[M-H]^-$, m/z = [164, 04 – 1]. This corresponds to *p*-coumaric acid molar mass.

The NMR (^1H, ^{13}C) and SM spectral analyses show that this compound is similar to *p*-coumaric acid. Theses results are in accord with those found by Gerothanassis, et al. (1998).

The HMQC experiences in 2D NMR have permitted to establish H-4/ C-4, H-6/ C-6, H-7/ C-7, H-8/ C-8 and H-9/ C-9 correlations. The δ 7,04-6,75 *ppm* (3H, m) signals indicate the presence of a aromatic ring substituted on *para* similar to *p*-coumaric acid but with 3H. All so, δ 7,53-7,50 *ppm* (2H, m) signals indicate that the compounds is a ethylene compound with trans configuration similar to *p*-coumaric compounds, the SM spectral analysis revealed that the compound (X_2**)** have a molecular pic with $[M-H]^-$, *m/z* = [180,12 - 1] corresponding to the mass molar of caffeic acid.

The NMR (^1H, ^{13}C) and SM spectral analyses show that this compound is similar to the caffeic acid. Theses results are in accord with those found by Gerothanassis, et al. (1998).

The analysis of these results has permitted to conclude that X_3 compound is a glycosyl flavonoid. The ^1H spectral analysis showed that these protons signal are characteristic of 2 aromatic rings (A and B). The signal protons δ 6,21 *ppm* (1H, d, J = 1,3 Hz) and 6,20 *ppm* (1H, d, J = 1,3 Hz) coupling at AX and the protons signals δ 7,67-6,85 *ppm* (3H, m) are respectively the characteristic signals of the aromatic rings A and B of flavonoid.

The ^1H and ^{13}C signals showed that X_3 compound is a di- glucosyl compound similar to those found by Niassy, et al. (2004).

The SM spectral analysis revealed that the compound have a molecular pic with $[M-H]^-$, *m/z* = [610, 52 – 1], corresponding to the mass molar of rutin.

The NMR (^1H, ^{13}C) and SM spectral analyses show that this compound is similar to the rutin. Theses results are in accord with those found by Niassy, et al. (2004).

The HMQC experiences in 2D NMR analysis indicate that X_4 is a flavonoid. The ^1H spectral characteristics in CD_3OD solvent have shown some aromatic proton signal characteristics with δ 5,94 *ppm* (1H, m) and 5,90 *ppm* (1H, m) of A cycle of flavonoid. Then the protons with δ 6, 98-6, 97 *ppm* (1H, d, J= 2, 5 Hz) and δ 6, 82-6, 74 *ppm* (m) are characteristics of B ring.

The SM specter analysis revealed that the compound have a molecular pic with $[M-H]^-$, *m/z* = $[M-H]^-$, *m/z* = [290, 27-1] corresponding to the mass molar of epicatechin.

The NMR (^1H, ^{13}C) and SM spectral analyses show that this compound is similar to the epicatechin. Theses results are in accord with those found by Saucier, et al. (1997).

The analyses of these results indicate that X_5 compound is a flavonoid.

The characteristics of the ^1H spectral in CD3OD solvent are similar to proton aromatic signals. The signals δ 6,39 *ppm* (1H, d, J= 2,5 Hz) and δ 6,38 *ppm* (1H, d, J= 2,5Hz) are coupled in AX, characteristic of A cycle of flavonoid. The spectral ^1H analyses with δ 6,98-6,97 (d) *ppm* (1H, d, J= 2,5 Hz) and δ 6,82-6,74 *ppm* (2H, m) correspond to B cycle.

The SM specter analysis revealed that the compound have a molecular pic with $[M-H]^-$, m/z = [201,02 – 1]. This corresponds to quercetin molar mass.
The NMR (^1H, ^{13}C) and SM spectral analyses show that this compound is similar to quercetin.

CONCLUSION

For the first time in this study, biological properties and phenolic compounds contents in *D. adscendens* leaves were examined. The results indicated that caffeic acid, p-coumaric acid, epicatechin, rutin and quercetin are some of the compounds of the leaves of *D. adscendens*. The biological properties evaluation showed that MWE has the best antimicrobial activities than WE.

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