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First Report on Chemical Composition and Antimicrobial Activity of *Artabotrys velutinus* Scott-Elliot Extracts Against Some Clinical Strains in Benin

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Abstract: This work aims to study the chemical composition and to evaluate the antimicrobial activity, for the first time, of essential oil and non-volatile extracts of *Artabotrys velutinus* against *Klebsiella pneumoniae* 818E, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25933, three clinical strains of reference. The chemical analysis of essential oil of *Artabotrys velutinus* by GC and GC-MS showed that this oil rich in aromatic components (62.6%) and sesquiterpene hydrocarbons (29.9%) contains 30 compounds representing 98.9% of the oil. The major components of the essential oil were benzyl benzoate (61.2%) also called ascabiol with and E-β-caryophyllene (9.1%). The phytochemical screening of leaves powder of *Artabotrys velutinus* revealed the presence of saponins, catechin tannins, mucilages, flavonoids, alkaloid, anthocyanins, leuco-anthocyanins, reducing compounds, sterols and terpenes. The *in vitro* antibacterial activity of the extracts by agar diffusion method showed that only the ethanolic extract of the plant was more effective against *E. coli* with the highest inhibition zone of 13 mm at 100mg/mL and Minimal Inhibitory Concentration equal to 50 mg/mL. However, the activity of ethanolic extract of this plant was less active than those of reference antibiotics chloramphenicol and gentamycin which were very effective against the strains tested. In sum, essential oil of *Artabotrys velutinus* and its hydroethanolic extract present weakness antimicrobial activity contrary to its ethanolic extract which possesses moderate activity against clinical strains tested. This study suggests the used of ethanolic extract of *Artabotrys velutinus* in combination with others active extracts to fight against *E. coli*.

Keywords: *Artabotrys velutinus*, Essential Oil, Non-volatile Extract, Clinical Strains

1. Introduction

The genus *Artabotrys* was originally composed of 100 species of which 44 were described. *Artabotrys velutinus* Scott-Elliot, syn. *Artabotrys djalonis* A. Chev.; *Artabotrys nigericus* Hutch. (Annonaceae), is a useful plant of West Tropical Africa (Guinee, Sierra Leone, Ghana and Nigeria) [1]. It's a climbing shrub or vine persistent, the nascent branches pubescent. Leaves up to 15cm long and 6cm broad, its outer petals linear or lanceolate are not sublets [2].

Artabotrys velutinus is used as an aphrodisiac. Traditionally, in tropical Africa and Benin in particular, people with low income, very often use plants collected locally for treatment of infections due pathogenic isolates such as *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*. *Escherichia coli* is responsible of affections manifested mainly by diarrhea whereas *Staphylococcus aureus* is mainly responsible of post-operative wound infections, endocarditis, osteomyelitis and food poisoning [3]. The most recent data of the International System of Monitoring of the Nosocomiales Infections arrange *Klebsiella pneumoniae* among the seventh most common agents implied in the nosocomiales infections of the urinary tract, infections of blood physiology, and the cardiovascular infections, the infections of the nose, the ears and the throat. It is also classified as fourth cause of acquired hospital pneumonias [4].

Only a very small number of species belonging to *Artabotrys* have so far been studied chemically. As far as we know, no investigations have been made on the chemical study and biological activity of the volatile oil obtained from *Artabotrys velutinus*. Therefore, as part of our continuous search for beneficial effects of plants extracts from Benin, we aimed to evaluate the antimicrobial activity of the essential oil hydrodistilled from leaves and ethanolic and hydroethanolic extrcats of *Artabotrys velutinus* after undertaken chemical investigation of these extracts in order to establish the composition–activity relationship.

2. Materials and Methods

2.1. Plants Material

Leaves of Artabotrys velutinus were collected in Bonou in the department of Oueme-Plateau in Benin. They were dried naturally on laboratory benches at room temperature until constant weight. Identification of the plant was made by Professor Akouègninou of National Herbarium of Benin in University of Abomey-Calavi where a voucher specimen was deposited under N°AA6656/HNB.

2.2. Methods

2.2.1. Phytochemical Screening

The plant extracts were screened for the presence of mucilages, alkaloids, flavonoids, steroids and terpenes, anthraquinones, tannins, saponins and heterosides based on the colouring and/or precipitation reactions of the chemical compounds contained in the plant following standards methods.

i. Alkaloids

Alkaloids were identified by Mayer's reagent and confirmed by Bouchardat test. Formation of cream or brown precipitate respectively indicated the presence of alkaloids [5].

ii. Flavonoids

A portion (1g) of the extract was added to 1mL of 10% NaOH. Formation of a yellow coloration indicated the presence of flavonoids [6].

iii. Sterols and terpens

They have been demonstrated by Liebermann-Burchard test [7].

iv. Saponins

A portion (1g) of each extract was added to 5mL of distilled water and vigorously shaken for 2 min. Formation of froth indicated the presence of saponins [8].

v. Tannins

10 mL of distilled water was added to 2g of each extract, stirred and filtered. 1 mL of ferric chloride was then added to the filtrate. Formation of a blue-green precipitate indicated the presence of tannins [9].

vi. Leuco-anthocyanins

0.5 mL of 12 N HCl was poured into 3 mL of extract. The acidified solution was brought to boiling water bath for 30 minutes. After cooling, the appearance of a purplish red color indicated the presence of leuco-anthocyanins [10].

vii. Anthocyanins

To an infusion, 5 mL of 10% H_2SO_4 and 5ml of 50% NH_4OH were added. The appearance of a red color that turned purplish blue in basic medium indicates the presence of anthocyanins [11].

viii. Mucilages

1 mL of decoction 10% of plant powder was introduced in a test tube and 5 ml of ethyl ether were added. After ten minutes, a flocculent precipitate indicates the presence of mucilages [10].

ix. Carotenoids and quinones

Free anthraquinones and combined quinones (O-heteroside and C-heteroside) were characterized respectively by the reaction of Bornträger [12] and standard method reported by [13].

2.2.2. Quantitative Analysis of Phenolic Compounds

Total polyphenols: The method of determination of total polyphenols consisted to use a mixture of phosphotungstic and phosphomolybdic acid which was reduced during the oxidation of phenols in the mixture of tungsten blue oxide and molybden [14, 15]. The absorbance was measured by a spectrophotometer (JENWAY 50/60 Hz) at 765 nm. Gallic acid was used as reference and the total polyphenol content in the extract was expressed in mg of gallic acid equivalents per gram of dry matter.

Total flavonoids: The method of aluminum trichloride ($AlCl_3$) was used to quantify the total flavonoids. This technique was based on the formation of the aluminum-flavonoids complex that had a maximum absorption at 500 nm [14, 16].

2.2.3. Extraction of the Essential Oil

The dry leaves (100 g) of *Artabotrys velutinus* were separately ground and subjected to hydrodistillation with a Clevenger-type apparatus using 750 ml of deionised water for 4 h. The oil collected was dried over anhydrous sodium sulfate and stored at -20°C until used.

2.2.4. Chemical Analysis of Essential Oil

i. Gas chromatography

GC analyses were performed on a Varian gas

chromatograph, model CP-3380, with a flame ionization detector equipped with a silica capillary column: HP5 J&W Agilent (5%-phenyl-methylpolysiloxane) (30 m x 0.25 mm i.d. x 0.25 µm film); N2 was the carrier gas at 0.8 mL/min; injection of 1 µL 1:10 CH_2Cl_2 solution, split ratio 1:100; injector temperature 220°C, detector temperature 250°C; temperature program 60-220°C at 3°C/min, then kept at 220°C during 20 min. The linear retention indices of the components were determined relative to the retention times of a series of n-alkanes with linear interpolation. The percentage composition of the essential oil was computed by the normalization method from the GC/FID peak areas on the HP5 capillary column, response factors being taken as one for all compounds.

ii. Gas chromatography-mass spectrometry

GC/MS analyses were performed using a Hewlett–Packard GC 5890 series II equipped with a HP5 (5%-phenyl-methylpolysiloxane) fused silica column (30 m x 0.25 mm; film thickness 0.25 µm) and a DB-Wax fused silica column (30 m x 0.25 mm; film thickness 0.25 µm) interfaced with a quadrupole detector (Model 5972) applying the same temperature program as for the GC/FID analyses with the apolar column; the temperature program was 70°C for 2 min, 70-220°C at 5°C/min, then kept at 220°C during 38 min using the polar column (calculation of the linear retention indices on this column by coinjection with a series of n-alkanes); injector temperature, 220°C; MS transfer line temperature, 250°C; carrier gas, helium at a flow rate of 0.6 mL/min; injection type, split, 1:10 (1µL 10:100 CH_2Cl_2 solution); ionization voltage, 70 eV; electron multiplier 1460 eV; scan range 35-300 amu; scan rate, 2.96 scan/s. The identification of the constituents was based on comparison of their relative retention indices with either those of authentic samples or with published data in the literature [17] and by matching their mass spectra with those obtained with authentic samples and/or the NBS75K, Wiley 7th NIST 98 EPA/NIH, and FFNSC 2 libraries spectra.

2.2.5. Ethanolic and Hydroethanolic Extracts Preparation

50g of leaves powder of *A. Velutinus* were mixed with 500 cm^3 of concerned solvent. Ethanol 96° and ethanol-water (50/50) were used respectively for ethanolic extract and hydroethanolic extract. The mixture of leaves powder and solvent was filtered and evaporated to dryness at 40°C using rotary evaporator. Each residue was then allowed to cool, weighed and stored in refrigerator until needed.

2.2.6. Antibacterial Assay

i. Agar disc diffusion method

The agar disc diffusion method described by National Committee for Clinical Laboratory Standards reported by [18] was performed to determine the antibacterial activities of hydroethanolic, ethanolic extracts and essential oil of A. velutinus against *E. coli* ATCC 25922, *S. aureus* 25923 and *K. pneumoniae* 818E, three pathogenic clinical bacteria of reference provided by the Laboratory of Bacteriology-Parasitology of Centre National Hospitalo-Universitaire (CNHU), the first and biggest hospital of Benin. The bacterial cultures were first grown on Nutrient agar plates at 37°C for 18 to 24 h. One or several colonies of the respective bacteria were transferred into normal saline and adjusted to 0.5 McFarland turbidity standards. The inoculum of the respective bacteria were streaked into Muller Hinton agar plates using a sterile swab and were then dried at 37°C during 15 min. Sterile filter discs having 6 mm of diameter soaked with 25 µL of 100 mg/mL of each non-volatile extract, 5 and 10 µL of pure essential oil extract separately were placed at the surface of Muller Hinton agar plates. The plates were incubated for 24 hrs at 370°C. The antibacterial activity was evaluated by measuring the clear zone surrounding the whatman paper. Standard discs of antibiotics (Gentamicin 10µg and Chloramphenicol 30µg) were applied as positive antibacterial controls. Each assay was performed in triplicates [12, 19].

ii. Minimal Inhibitory Concentration Determination

The method used is that reported by [3] using the microplates with 96 wells and Muller Hinton Broth (MHB). A negative control made of a mixing of tested ethanolic extract and the medium MHB and a positive control carried out with a mixing of tested microorganism and the MHB without the extract standardize the method. Serial dilutions have been made well by well, beginning by the first well to the twelfth one; the remaining aliquot is rejected at the end. The cultured microplates are incubated at 37 ± 1°C for 24 hours, covered with a parafilm paper. For the reading, the wells corresponding to the smallest concentration of extract of essential oil for which we do not observe turbidity or visible growth to the naked eye is taken as the minimum inhibitory concentration (MIC) of the extract on the strain tested.

2.2.7. Statistical Analysis

All assays were conducted at least three times with three different sample preparations. All data were expressed as mean ± standard deviation (SD). Analysis of variance was performed using In Stat (Graph Pad software, San Diego CA). A one-way ANOVA and unpaired Student's t-test were used for these analyses, and $p < 0.05$ was considered to be statistically significant.

3. Results and Discussion

3.1. Chemical Composition of the Leaves Powder of Artabotrys Velutinus

The table 1 presents the results of chemical characterization of the leaves of *Artabotrys velutinus*. Various secondary metabolites have been identified in the leaves by a series of color reactions and precipitation more or less specific to each class of active ingredients. Among these secondary metabolites we have saponins, catechin tannins, mucilages, flavonoids, alkaloid, anthocyanins, leuco-anthocyanins, reducing compounds, sterols and terpenes.

Table 1. *Main Metabolites of A. velutinus leaves powder.*

Secondary metabolites		*A. velutinus*
Tannins	Cathechic	+
	Galliques	-
Flavonoids		+
Anthocyans		+
Leuco-anthocyans		+
Sterols et terpenes		+
Alkaloïds		+
Mucilages		+
Reducing compounds		+
Combined anthraquinones	o-heterosides	-
	o-heterosides with reduced genines	+
	c-heterosides	-

-: absence; +: presence

3.2. Phenolic Compounds Contents of Ethanolic and Hydroethanolic Extracts

The contents of total polyphenols, flavonoïds and condensed tanins of semi and ethanolic extracts of the leaves *Artabotrys velutinus* expressed out of equivalent mg of gallic acid per gram (mgEAg/g) and out of equivalent mg of catechin per gram (mgEC/g) of dry matter (MS) are indicated by the figure 1. The total phenols contents of the ethanolic and semi extracts studied are respectively of (256.622±10.797) mgEAG/g MS and (340±7.932) mgEAG/g MS and then (210.420±3.998) mgEC/g and (284.733±7.425) mgEC/g for the contents of total flavonoïds. Regarding the condensed tanins, the highest content is obtained with the semi-ethanolic extract (135.813±1.973) mgEC/g followed by ethanolic extract (108.604±3.617) mgEC/g. These contents increase with the polarity of extraction solvent.

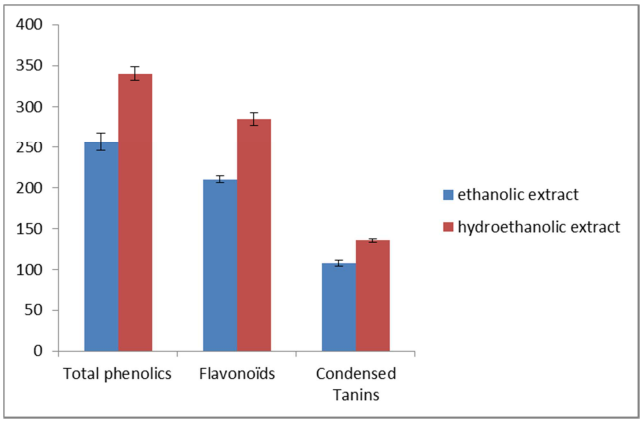

Figure 1. *Phenolic compounds contents of volatile extracts studied.*

3.3. Chemical Composition of the Essential oil Hydrodistilled from Leaves of A. Velutinus

The chemical composition of the essential oil obtained from the leaves of *Artabotrys velutinus* is reported in table 2. Results showed that the major fraction of the oil was aromatic compounds (62.6%) followed by (29.9%) of sesquiterpens hydrocarbons and oxygenated compounds (5.4%). As shown by their proportions given in table 2, the most abundant components in *Artabotrys velutinus* leaves essential oil are benzyl benzoate (61.2%) also called ascabiol and E-(β)-caryophyllene 9.1% while the minor components are α-(E,E)-farnesene (4.2%), caryophyllene oxide (3.4%), germacrene-D (2.6%), α-humulene (2.5%), valencene (2.4%), α-copaene (1.9), methoxy benzyl benzoate (1.3%), β-elemene (1.2%), γ-amorphene (1.2%), α-selinene (1.0%) and bicyclogermacrene (1.0%). The other minor compounds contents are less than 1.0% each one. An extensive review of literature on the biological activities of the major constituents of this oil shows that benzyl benzoate, the major compound has potent antiparasitic properties on mites including *Sarcoptes scabei* [20, 21, 22, 23, 24] whereas (E)-β-caryophyllene, a sesquiterpene widely distributed in essential oils of various plants possesses several biological properties such as anti-inflammatory, antibiotic, antioxidant, anticarcinogenic and local anaesthetic activities [25]. In sum, essential of *Artabotrys velutinus* contains major chemical compounds which could allow it use as acaricidal agent.

Table 2. *Chemical composition of the essential oil hydrodistilled from leaves of Artabotrys velutinus.*

N° Pic	RI[a]	RI[b]	Components	%
1	844	1023	(2E)-hexenal	0.1
2	937	1034	α-pinene	0.1
3	982	1139	sabinene	0.3
4	1027	1243	p-cymene	0.3
5	1384	1478	α-copaene	1.9
6	1394	1502	β-bourbonene	0.4
7	1398	1580	β-elemene	1.2
8	1431	1583	(E)-β-caryophyllene	9.1
9	1441	1585	β-copaene	0.3
10	1454	1589	*trans*-α-bergamotene	0.3
11	1465	1650	α-humulene	2.5
12	1472	1656	allo-aromadendrene	0.5
13	1491	1659	germacrene-D	2.6
14	1494	1662	cis-β-guaiene	0.2
15a	1498	1696	γ-amorphene	1.2
15b	1498	1698	valencene	2.4
16a	1505	1702	α-selinene	1.0
16b	1505	1703	bicyclogermacrene	1.0
17	1509	1730	α-(E,E)-farnesene	4.2
18	1530	1734	δ-cadinene	0.7
19	1563	1802	germacrene B	0.4
20	1568	2006	M= 220	0.3
21	1577	2022	(E)-nerolidol	0.3
22	1587	2100	spathulenol	0.8
23	1594	2109	caryophyllene oxide	3.4
24	1622	2171	humulene epoxide II	0.4
26	1664	2198	epi-α-muurolol	0.4
27	1781	2610	benzyl benzoate	61.2
28	2000	2616	methoxy benzyl benzoate	1.3
30	2136	2642	phytol	0.1
Monoterpene hydrocarbons				0.7
Sesquiterpene hydrocarbons				29.9
Oxygenated sesquiterpenes				5.4
Aromatic compounds				62.6
Non identified				0.3
Total identified				98.9

RI[a], Retention index relative to n-alkanes (C_9-C_{20}) on a DB_1 capillary column (5%-phenyl-methylpolysiloxane);
RI[b], Retention index on Supelcowax 10 (polyethylene glycol);

3.4. Antibacterial Activity of Artabotrys Velutinus Extracts

The results for antibacterial screening of extracts investigated are presented in Table 3. The non-volatile extracts were tested at a concentration of 100 $mg.mL^{-1}$ in hydroethanolic solvent whereas 5 microliters and 10 microliters per disc of essential oil of the plant were tested. These results showed that only the ethanolic extract showed larger zones of inhibition against *E. coli* equal to 13mm. The Minimal Inhibitory Concentration determinated for ethanolic extract of this plant on *E. coli* was 50 mg/mL. In contrast, the essential oil of this plant possessed weakness activity on *Staphylococcus aureus* with diameter of inhibition zone varying between 8 and 9 mm. All the extracts investigated present a zero or a null activity on *Klebsiella pneumoniae*. The oil as well as the hydroethanolic extract possessed a null activity on *E. coli* whereas the non-volatile extracts don't affect the growth of *S. aureus* strains. In contrast, the standards antibiotics used as reference such as Gentamycin and chloramphenicol to compare their activity with that of the extracts were very actives on all the strains with the pronounced activity demonstrated by the chloramphenicol. The effect of ethanolic extract on *E. coli* can be related to the phenols contained in this extract with synergic to other compounds associated that the hydroethanolic extract would not contain. Globally, the volatile extracts investigated as well as the essential oils are not active on the clinical strains studied. These extracts could be qualified of not suitable extracts for fighting against bacteria.

Table 3. *Diameters (mm) of inhibition zone produced by the extracts on strains investigated.*

Samples			**Averages of the diameter of the inhibition zones**		
			E. coli	***S. aureus***	***K. pneumoniae***
A. velutinus	Essential oil	5µL	0.00d	8±00d	0.00c
		10µL	0.00d	9±00c	0.00c
	Ethanolic extract		13±00c	0.00e	0.00c
	Hydroethanolic extract		0.00d	0.00e	0.00c
Gentamycin			23±1.5b	29±00b	25±00b
Chloramphenicol			30±00a	32±00a	28±00a
Ethanol/Eau (4/6)			0.00d	0.00e	0.00c

The values in column followed by different letters are significantly different at $p < 0.05$

4. Conclusion

In this study, the activity of two crude extracts and essential oil from the leaves of *Artabotrys velutinus* of Benin was evaluated in vitro against *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Escherichia coli*. Of these extracts, only ethanolic extract exhibited moderate activity against *E. coli*. This is the first report of the activity of these plants which is rich in phenolic compounds, sesquiternes components and aromatic compounds such as benzyl benzoate with acaricidal activity. The oil of this plant could be useful for the fight against mites especially *Sarcoptes scabei.*

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