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RESEARCH ARTICLE

Chemical composition and antiproliferative activity of leaves and stems essential oils of *Ozoroa pulcherrima* upon breast cancer cells MCF-7

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Abstract

The present work reported the results of the chemical study and the in vitro antiproliferative activity evaluation of the essential oils of *Ozoroa pulcherrima* (OP) on breast cancer cells MCF-7. The phytochemical screening carried out prior to the evaluation of the biological activities revealed the presence of several metabolites, mainly phenolic compounds (tannins, flavonoids) mucillages, saponins, sterols and terpens. The extraction yields of essential oils were very low (0.2 and traces respectively for OP1 and OP2). Essential oils extracted from stems and leaves of *Ozoroa pulcherrima* harvested at different times (OP1 and OP2), were analyzed by gas chromatography (GC) and gas chromatography coupled to mass spectrometry (GC/MS). The investigated essential oil samples, obtained in very low yields, are potentially rich in hydrogenated monoterpenes (89.3 to 90.3%). The main identified compounds, regardless of the harvest period, were α-pinene (30.70%, 42.68%), β-pinene (25.23%, 36.23%) and myrcene (28.51%, 6.34%) respectively for OP1 and OP2. Fractionnations made upon the methanolic extract of *Ozoroa pulcherrima* lead to the isolation of lanosterol. Antiproliferative studies performed on cancer cell lines MCF-7 had shown essential oils of *Ozoroa pulcherrima* were active. Inhibitory concentration (IC_{50}) determined were low 35 µg/mL for OP2 and may be related to the presence of this sample α-pinene, β-pinene and exceptionally limonene content.

Introduction:-

Plants play a vital role in sustaining of human life on the earth. For thousands of years, they have been used for various purposes including food, spices, medicines, cosmetics etc. In recent years, people's interest in medicinal plants in particular has experienced a significant rebound in the treatment of many diseases among which is the breast cancer (Heinrich and Bremner, 2006; Cordell *et al.* 1991 and Heinrich and Gibbons, 2001). Breast cancer is the most commonly diagnosed cancer in women: pregnant or not, young or old. According to the International Agency for Research on Cancer, the number of women with breast cancer worldwide in 2008 was estimated at 1,384,155 with 458,503 deaths recorded. These figures represent 39% of all counted cancer diseases and 12.9% of

all cancer deaths worldwide (World Cancer Report, 2008). To date, several methods have been used with varying success to treat this deadly disease: radiotherapy, chemotherapy, hormonal therapy, immunotherapy and surgery (Zelek and Khayat 2002; Chauvergne and Hoerni (1991); Thyss and Pivot 1998). Due to the lack of these modern treatments in some cases, cancer pathologies continue to wreak havoc in human being. Therefore, it is crucial to find other treatments. Among these, it seems pertinent to study the activity of aromatic plants, rich in essential oils and whose impact on human health could be significant (Jelnar *et al.*, 2010; Cole *et al.* 2007; Sigurdsson *et al.*, 2005 and Cavalieri *et al.*, 2004).

Ozoroa pulcherrima is a plant species of tree savannas, widespread from Senegal to Cameroon (Adjanohoun *et al.*, 1988; Akoègninou *et al.*, 1998 and de Souza, 1988). The aqueous decoction of stems together with leaves of this shrub, up 1 to 1.5 m, is used by oral route to treat dystocia, hyperthermia and purulent conjunctivitis (Adjanohoun *et al.*, 1989). The powder produced from the bark in combination with fresh milk, is taken as infusion to relieve abdominal pain and diarrhea in humans and in young cattle. In West Africa and Benin in particular, the root is used in infusions to treat gastrointestinal problems. At the present stage of investigations, no manuscript in the literature mentions a chemical composition of the essential oil extracted from *Ozoroa pulcherrima*, nor its cytotoxicity. Nevertheless, it is important to note that recently Tsague Dongmo *et al.*. have isolated three compounds from the roots of this variety of *Ozoroa*: the ozocardic A, a new alkylanacardic acid, 6-tridecylanacardic acid and β-sitosterol (Tsague Dongmo *et al.*, 2011a and Tsague Dongmo *et al.*, 2011b). However, the literature reports the chemical composition of essential oil of *Ozoroa insignis*, a species very close to *O. pulcherrima*, with which a distinction was subjected to large ambiguities (Hedberg *et al.*, 1982 and Akoègninou *et al.*, 2006b). In 1998, Ayedoun *et al.*. revealed in the essential oil extracted from the leaves of *O. insignis* harvested at Matoukou in Benin, the presence of significant proportions of α-pinene (27.4%), β-pinene (27.9%) and myrcene (30.6%). Also the chromatographic investigations carried out on the same volatile oil extracted from flowers by the same authors, showed a proportion of myrcene (69.4%) two times greater than that detected in leaves. Indeed, the two isomers of pinene were identified with equal rates (11.5%, 11.6% respectively) in the chemical analysis of the same extract (Ayédoun *et al.*, 1998). Also, Noudogbessi (2009) had showed that α-pinene (13.7%), β-pinene (13.7%) and myrcene (58.9%) were the major compounds of the oil essential extracted from the leaves of *O. insignis* collected at Natitingou (Benin) (Noudogbessi, 2009).

Some non volatile extracts of *O. insignis* have been investigated by several authors and compounds have been isolated and identified like ozoranone, ozoralide 1 and nine tirucallanes (Ng'ang'a *et al.*, 2009; Abreu and Liu 2007; Liu and Abreu, 2006). The ethanolic extract of *O. insignis* has been also tested on KB cancer cell lines, A549 and MDA-MB. These cells were sensitive to the effects of ethanolic extract and IC_{50} values generated from the data measured were respectively 30.5, 22.0 and 15.5 µg/mL (Abreu *et al.*, 1999).

In the present work, investigations are made on the chemical study and the in vitro antiproliferative activity evaluation of the essential oils of *Ozoroa pulcherrima* (OP) on breast cancer cells MCF-7.

Material and Methods:-

Plant Material:-

The aerial parts of *O. pulcherrima* were harvested at Cotiakou (Benin) in January (OP1) and October (OP2) 2010. In the laboratory, plant material was stored at 20°C and protected from sunlight during the extractions. The identification and authentication were made at the National Herbarium of the University of Abomey-Calavi (Benin). Voucher number was assigned to the specimen.

Animals:-

The cell line under investigation was human breast adenocarcinoma (MCF-7). MCF-7 stands for Michigan Cancer Foundation-7, in reference to the Detroit Institute, where the line was established (Lacroix, 2004 and Soule, 1973).

Phytochemical screening :-

The main non volatile secondary metabolites contained in the leaves and stems of *Ozoroa pulcherrima* have been determined by colorimetric and precipitation test tube according to the method of Houghton (Houghton and Raman , 1998). Polyphenols are characterized by the reaction with ferric chloride (FeCl3, 2%) while flavonoids were detected by reaction with cyanidin. Catechic tannins identification was carried out using reagent of Stiasny. For gallic tannins, the solution of catechic tannins was filtered and the filtrate was collected and saturated with sodium acetate. The addition of 3 drops of $FeCl_3$ (1%) allowed to identify the presence of gallic tannins by the appearance of

intense blue-black coloration. Reagents of Mayer (iodo-iodized reagent) and Dragendorff (iodo-reactive potassium bismuthate) allowed to the characterization of alkaloids. In order to identify the saponins, we based on their aphrogène power. Sterols and triterpenes were identified by Liebermann-Burchard test. The presence of mucillages was determined by the treatment of a decoction realized previously with absolute ethanol, and the presence of mucillages was noticed by the appearance of a flaky precipitate. An alcoholic extract was examined under UV light (365 nm) to reveal the presence of coumarins. The appearance of a bluish fluorescence indicated a positive reaction.

Extraction of essential oils:-

Essential oils were obtained by hydrodistillation using a Clevenger-type apparatus for a period of three hours. The volatile oil collected after decantation, was dried over anhydrous sodium sulfate and then stored at 4°C in amber glass vials until analysis.

Gas chromatography-mass spectrometry analysis of the volatile oils:-

The essential oils were analysed on a AGILENT gas chromatograph Model 7890, coupled to a AGILENT mass spectrometer model 5975, equipped with a DB5 MS column (20m X 0,20mm, 0,20µm), programming from 50°C (5 min) to 300°C at 8°C/min, with a 5 min hold. Helium was used as the carrier gas (1.0 mL/min); injection in split mode (1: 250); injector and detector temperature, 250 and 280°C respectively. The mass spectrometer worked in electron impact mode at 70 eV ; electron multiplier, 1500 V ; ion source temperature, 230°C ; mass spectra data were acquired in the scan mode in *m/z* range 33-450. The essential oil was diluted in hexane: 1/30.

Isolation of lanosterol:-

1 Kg of *Ozoroa pulcherrima* powder was subjected to maceration in cyclohexan for 24 hours. The solvent was evaporated. The recovered marc was dried and macerated in methanol for 48 hours. The methanolic extract was evaporated to provide 56.41 g of a brown residue (OP). 14.5 g of this extract were chromatographed on silica gel column in normal phase and provided 22 fractions.

Fraction F2 (55.1 mg) was subjected to silica gel column in normal phase and gave 8 sub-fractions. The sub-fraction 2 is subjected to flash chromatography in isocratic mode cyclohexane / ethyl acetate (90/10) to result in a pure compound A (4.9 mg) and impure six subfractions. The F3 fraction (83.1 mg), after a silica gel column in normal phase, resulted in 6 sub-fractions with pure compound A (6 mg). Compound A was injected in GC / MS and submitted to Liebermann-Burchard test to check its belonging to the family of triterpenes or that of sterols.

Evaluation of anti-proliferative activity:-

The biological investigations were performed on cancer cells of human breast adenocarcinoma MCF-7. According to the cells growth profile, MCF-7 cells were seeded into the wells of microplates at a concentration of 50,000 cells/mL. The microplates were kept in an incubator at 37 ° C, in an humidified atmosphere containing 5% carbon dioxide. After 24 hours, the cells were treated with volatile oils initially dissolved in dimethylsulfoxide and sonicated.

In parallel, for each tested essential oil, a control of dimethylsulfoxide DMSO (solvent for dissolving the EO) is produced and the plates were returned into the incubator for 72 hours under the above conditions. After this final incubation period, the culture medium was replaced with a solution of resazurin (25 mg / mL), which in the presence of metabolically active cells is oxidized into resofurin, florescent at 590 nm. The fluorescence intensity was proportional to the number of viable cancer cells. Fluorescence was then measured using a plate reader (Fluoroskan Ascent ® FL, Thermo Electron Corporation, France) at 590 nm.

The fluorescence intensity obtained after reading the Fluoroskan Ascent (expressed in arbitrary units) was converted into percentage inhibition of proliferation relative to proliferation of cells control (DMSO). It was established that DMSO got no influence on cell proliferation, in comparison with a control performed without DMSO under the same conditions. The raw data were analyzed statistically using the t test in paired samples (n = 5) and (n = 6). To assess the antiproliferative activity of samples OP1 and OP2, an IC_{50} values were calculated. The results were presented as: Mean ± SEM (Standard deviation from the mean).

Results and Discussion :-

Phytochemical screening:-

The powder of *Ozoroa pulcherrima* (OP) leaves and stems was subjected to preliminary phytochemical analysis using standard procedures to find out the phytoconstituents present in the sample. Table II shows the different metabolites identified in the plant material studied.

Table I: Metabolites identified in the leaves of *Ozoroa pulcherrima*

Familles de composés	OP
Catechic tannins	+++
Gallic tannins	+++
Alkaloids	++
Sterols and terpens	+++
Saponins	+++
Mucillages	+++
Coumarins	+
Flavonoids	+++

Yield and Chemical Composition of essential oils:-

Two harvests of stem and leaves of *O. pulcherrima* have been made in january 2010 for OP1 and in october 2010 for OP2. The yield and chemical composition of each EO are mentioned in the table below.

Table II: Yield and Chemical Composition of essential oils

Plants Samples		**OP1**	**OP2**
Yields (%)		0,2	traces
Compounds	**KI**	**(%)**	**(%)**
Tricyclene	926	0.1	0.1
α-Pinene	**939**	**30.7**	**42.7**
Camphene	953	0.4	0.4
Sabinene	976	0.3	0.3
β-Pinene	**980**	**25.2**	**36.2**
Myrcene	**991**	**28.5**	**2.4**
δ-2-Carene	1001	0.2	-
α-Phellandrene	1005	0.1	0.1
α-Terpinene	1018	0.1	-
Para-cymene	1026	0.1	0.3
Limonene	**1031**	**2.8**	**6.3**
β-Phellandrene	1031	1.2	-
(E)-β-Ocimene	1050	0.2	0.2
γ-Terpinene	1062	0.1	0.1
Linalool cis oxyde	1071	0.1	-
Terpinolene	1088	0.2	0.2
6,7-epoxy-myrcene	1093	0.1	-
Perillene	1099	0.1	-
Linalool	1101	0.2	0.2
Nonanal	1105	0.1	t
Exo-fenchol	1120	0.1	0.1
Trans-Pinocarveol	1139	0.1	0.2
Trans-Verbenol	1144	-	0.1
1(7),5-Mentha dien-2-ol	1148	-	0.1
Camphene hydrate	1150	0.1	-
2,10-epoxypinane	1159	t	-
Pinocarvone	1162	0.1	0.
Borneol	1165	0.1	0.1
Terpinen-4-ol	1182	0.3	0.4
α-Terpineol	**1195**	**1.6**	**2.6**
Verbenone	1205	t	0.1
Undecanal	1307	t	0.1
Eugenol	1359	0.1	-
α-Copaene	1379	0.4	0.3
β-Bourbonene	1384	t	t
β-Cubebene	1388	0.1	0.2
β-Elemene	1389	0.1	-
β-Caryophyllene	1418	2.4	1.9
β-Copaene	1430	t	-
Trans-α-Bergamotene	1435	0.1	0.1
Trans -β-Bergamotene	1435	0.1	-
Neryl acetone	1436	0.1	-
α-Humulene	1454	t	0.2
γ-Patchoulene	1471	-	0.1
γ-Muurolene	1477	t	0.1
Germacrene-D	1480	1.4	0.8
γ-Cadinene	1510	t	-
δ-Cadinene	1520	0.3	0.2
Trans calamenene	1529	t	-
(+) Spanthulenol	1578	0.1	0.1

Caryophyllene oxide	1581	0.4	0.4
Guaiol	1595	-	0.1
Humulene epoxyde II	1606	0.1	t
Zizanyl acetate	1618	-	t
1-Epi-Cubenol	1629	0.1	0.1
Epi-α-Cadinol	1640	0.1	t
Epi-α-Muurolol	1642	0.1	0.1
α-Cadinol	1654	0.1	0.1
Hydrogenated monoterpenes		**90.3**	**89.3**
Oxygenated monoterpenes		**2.9**	**4.0**
Hydrogenated sesquiterpenes		**4.9**	**3.9**
Oxygenated sesquiterpenes		**1.1**	**0.9**
Aldehydes		**0.1**	**0.1**
Total identified		**99.3**	**98.2**
Unidentified		**0.7**	**1.8**

t (traces) < 0,05%
KI = Kovats Indice

The investigation work carried out showed that the organs of *O. pulcherrima* were very poor in EO. Indeed, the EO content, obtained after hydrodistillation of the sample OP1 was 0.20% whereas in OP2, only traces of EO were collected (Table). The chromatographic analyses, performed by GC/MS have revealed the presence of 53 compounds in OP1 and 43 in OP2 corresponding respectively to 99.3 and 98.2% of the total compounds identified in the studied samples. Both volatile extracts are marked by high proportions of hydrogenated monoterpenes (90.3% for OP1 and 89.3% for OP2, Table). The other components represent less than 5%. Those appeared in traces (< 0.05%) were especially constituted by sesquiterpenes. Overall, there is the presence of the same major constituents for both extracts OP1 and OP2: main compounds were: α-pinene (30.7 and 42.7%), β-pinene (25.2 and 36.2%), limonene (2.8 and 6.3%), and α-terpineol (1.6 and 2.6%). However, a large decrease in the content of myrcene (28.51% for OP1 to 2.41% for OP2) was observed while the rate of α- and β-pinenes sample OP2 rose more than 10% compared to the values of the same pinene isomers in OP1. The chemical composition of volatile constituents of this plant being innovative, a comparison was performed with those of *O. insignis* collected in Benin. Indeed Noudogbessi (2009), showed that α-pinene (13.7%), β-pinene (13.7%) and myrcene (58.9%) were the major constituents of essential oil extracted from *O. insignis* collected at Natitingou Noudogbessi (2009). Previously in 1998, Ayedoun *et al*.., have established the chemical composition of the essential oil extracted from the leaves and flowers of *O. insignis* collected at Matoukou (Benin). They have indicated, from the chemical profile, obtained as that α-pinene (27.4%), β-pinene (27.9%) and myrcene (30.6%) were the major compounds of essential oil extracted from the leaves. The essential oil extracted from the flowers contained the same major compounds in the proportions of 11.5%, 11.6% and 69.4% respectively Noudogbessi (2009). All samples showed high proportions of hydrogenated monoterpenes. From this work results, it appeared that the essential oil of *O. pulcherrima* studied showed considerable similarities with samples of *O. insignis* from Benin; confirming closer taxonomic bringing of these two plant species (Hedberg *et al.*, 1982 and Akoègninou *et al.*, 2006b).

Isolation and caracterization of lanosterol :-

GC MS Spectrum (400 MHz, $CDCl_3$)

The mass spectrum showed a molecular peak corresponding to the mass of 426. Fragmentations observed on this mass spectrum were identical to those described by Zhukovich *et al.* on lanosterol.

^{1}H NMR Spectrum

^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound A showed a concentration of signals between 0.75 and 2.2 ppm with the exception of two types of protons at 3.26 ppm and 5.11 ppm corresponding to an hydroxylic proton and an ethylenic proton. The signal at 5.11 ppm indicated a triplet (J = 8.0 Hz) corresponding to the olefinic proton H-24 and the signal at 3.26 ppm was assimilated to the hydroxylic proton H-3. The concentration of other signals between 0.75 and 2.2 ppm indicated the absence of hydroxylic and ethylenic proton. All this observations suggested a sterol skeleton as secondary metabolite. Moreover, the spectrum showed eight signals, each integrating for three protons, six methyl groups resonating between 0.73 and 1.02 ppm and two others resonating between 1.71 and 1.74 ppm. Both chemical shifts supposed the presence of the double bond (Me-26 and Me-27). Among the 6 remaining signals, only one appeared as a doublet, the other 5 appeared as singlet corresponding to methyl bridged confirming that

compound A belong to the family of sterols. The resonant doublet at 0.83 ppm (J = 6.4 Hz) probably corresponded to the Me-21. Compound A was identified to lanosterol, previously described in a Saharan Euphorbiaceae (Teresa *et al.*, 1987 and Ohtsu *et al*., 1998).

^{13}C NMR Spectrum

The ^{13}C NMR spectrum showed signals corresponding to 30 carbon atoms including eight methyl, ten methylene, five methyne and seven quaternary carbon, confirming the triterpenoid nature of compound A. The ^{13}C NMR spectrum and HSQC spectrum revealed the double bond C-24 = C-25 respectively at 125.4 and 133.7ppm. The signal at 134.1 ppm was attributed to a quaternary carbon.

Anti-proliferative activities upon the MCF-7 cells:-

The cytotoxicity of the two EO (OP1 and OP2) was evaluated on breast cancer cells MCF-7. Both samples tested caused a marked inhibition of cancer cell growth.

The results showed that the essential oil of *O. pulcherrima* have presented different activities upon the MCF-7 cells depending to the harvesting period. The sample OP2 (IC_{50} = 35 µg/mL) was very active compared to OP1 (IC_{50} = 319 µg/mL). These results were better than those obtained by Noudogbessi whose work has shown that the IC_{50} of the essential oil of *O. insignis* on the MCF-7 cells was greater than 327.6 µg/mL (Noudogbessi, 2009).

The explanation for the difference of activities observed for OP1 and OP2 might be due to their chemical compositions. The responsible compounds for the cytotoxicity of both EO studied might be α-pinene, β-pinene, limonene and α-terpineol. The proportion of myrcene was significantly higher in OP1 than in OP2 whereas OP2 was more active than OP1. In consequence, this component should not be responsible of the inhibition of the cells growth. Moreover, the EO of *O. insignis* investigated by Noudogbessi 2009, which also contained myrcene as major constituent in a proportion of 58.9%, had shown no effect on the same cells tested. These results were in complete agreement with the work of Sylvestre *et al.* who had observed that EO containing a significant proportion of myrcene have been ineffective on cancer cell lines tested (Sylvestre *et al.*, 2005 and Sylvestre *et al.*, 2007). Another explanation for the different activities of these two essential oils must reside in the significant increase in limonene and α-terpineol proportions. These two compounds would be most involved in the activity observed in OP2 with a probable synergy with the two isomers of pinene. Previous works have already shown the efficacy of limonene and α-terpineol against the MCF-7 cells growth. (Bicas *et al.*, 2011; Bardon *et al.*, 1998; and Crowell, 1999). The EO activity could be in relation to the presence of these two compounds. Synergy with two pinene isomers should not been set aside.

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