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Wound Healing and Antibacterial Properties of Leaf Essential Oil of *Vitex simplicifolia* Oliv. from Burkina Faso

Magid Abdel Ouoba[1], Jean Koudou[2*] , Noya Some[3], Sylvin Ouedraogo[2,3] and Innocent Pierre Guissou[1,2]
[1]*Unit of Teaching and Research (UFR) of Health Sciences, University of Ouagadougou,*
[2]*Laboratory of Chemistry of Natural Products, Faculty of Sciences, University of Bangui, Bangui,*
[3]*Institute of Research in Health Sciences, CNRST, Ouagadougou,*
[1,3]*Burkina Faso*
[2]*Central African Republic*

1. Introduction

Vitex simplicifolia Oliv. (Verbenaceae) is a perennial shrub or small tree which grows to a height of aproximatively 8 m and is widely distributed from Egypt to Guinea. In Burkina Faso, the plant is used for internal or external use to treat various diseases like skin diseases, dermatitis, bilharzia, migraines, fever, aches, amoebiasis, sore teeth, colic, infant tetanus(Nacoulma,1996). Our ethnobotanical investigations have revealed that this plant is also used in the treatment of skin infections and wounds healing. In Burkina Faso, infectious diseases are the leading cause of infant mortality (2.37%) and maternal (14.6%), therefore they constitute public health problems. The treatment of skin diseases dates back to ancient times, and many treatments were using medicinal plants. About 30% of traditional remedies are used to treat wounds and skin lesions, compared to only 1-3% of modern drugs (Mantle et al., 2001). The healing process is an immune response that begins after injury and takes place in three stages: vascular and inflammatory stage, phase of tissue repair and phase of maturation. A drug having simultaneously the potential antioxidant and antimicrobial activities may be a good therapeutic agent to accelerate cicatrization and wound healing [Houghton et al., 2005; Phillips et al., 1991; Heike et al., 1999]. Aromatherapy is now considered to be another alternative way in healing people, and therapeutic values of aromatic plants lie in their volatile constituents such as monoterpenoids, sesquiterpenoids and phenolic compounds that produce a definite physiological action on the human body [Bruneton, 1993].To the best of our knowledge, there is no report on pharmacological studies of this plant. The present work reported results of a detailed investigation of

* Corresponding Author

cicatrization and antibacterial activities of the leaf essential oil with the aim to contributing to the search for beneficial uses of this plant.

2. Materials and methods

2.1 Animals

The experiments were conducted using six male and female rabbits aged 7 months and weighing between 1±0.05 and 2±0.06 kg, housed alone under 12h light-dark cycle, controlled humidity (75%), and temperature (20-25°C) conditions with free access to food and water [Draize et al., 1944]. In addition, three other rabbits were taken as controls. The experiments were carried out according to the method as described previously [Draize et al., 1944] in accordance with the guidelines for the care of laboratory animals and ethical guidelines for the investigation of experimental pain in conscious animals and revised by Official Journal of France (1971/04/21).

2.2 Plant material and extraction

The leaves of *V. simplicifolia* Oliv were collected in January 2007 from the Kadiogo region, village of Balgui, 10 km near Ouagadougou, Burkina Faso. A voucher specimen was identified by Pr. Jeanne Millogo, botanist (University of Ouagadougou) and deposited at the herbarium of IRSS of Ouagadougou.

Dried and powdered leaves (500g) were subjected to hydrodistillation for 4h with a clavenger-type apparatus. The essential oil was collected and dried, after decantation, over anhydrous sodium sulfate and stored in refrigerator at 4°C for further use [Ouoba et al., 2009].

2.3 Reference bacteria strains

Microorganisms used in this study were:

Bacillus cereus LMG13569BHI, *Listeria innocua* LMG13568BHI, *Staphylococcus aureus* ATCC 25293BHI, *Staphylococcus camorum* LMG 13567BHI, *Staphylococcus aureus* ATCC9144BHI, *Enterococcus faecalis* CIP103907BHI, *Proteus mirabilis* CIP104588, *Shigella dysenteria* CIP5451, *Salmonella enterica* CIP105150, *Escherichia coli* CIP105182. These strains were identified by the conventional methods and tested. Bacteria were obtained from stock cultures of the laboratory of pharmacology and clinic biochemistry of CRSBAN, University of Ouagadougou. The bacteria stock cultures were maintained on Müller-Hinton agar and which were stored at 4°C.

2.4 Wound healing activity

Assessment of the healing power of the oil was performed using the method [Draize et al., 1944], on 6 male and female rabbits housed in individual cages. Both flanks of each rabbit were shaved, deeply incised prior to application of the essential oil. Rabbits were fixed horizontally from their ears and legs. One flank was covered with a compress soaked with 0.44 mg (0.50 ml) of the pure oil and held by a sticking-plaster, the other untreated flank serving as control. The same operation was repeated with Cicatryl as a reference standard, with a dose of 1 g per flank. The rabbits were returned to their cages after treatment.

Observation of the evolution of wound healing versus time was carried out at 48h and 96h after treatment. All of the tests were made in duplicate.

2.5 Antibacterial activity

Determination of the strain sensitivity

The test was performed using Müller-Hinton medium for bacteria using disk diffusion method following the National Committee for Clinical Laboratory Standards methods [Kiehlbauch et al., 2000]. Overnight broth cultures of each strain were prepared in nutriment Broth (Diagnostic Pasteur, France). The final concentration of each inoculums was got making dilution of each strain in 9 % NaCl solution. The turbidity of each inoculum was compared with McFarland 0.5 solution. The final concentration of each inoculum (approximatively 5.10^5 CFU / ml) was confirmed by viable count on Plate Count Agar (Merck, Germany). 3µl of essential oil was put on every disk (8 mm diameter).

Positive and negative growth controls were performed for every test. The plates were incubated aerobically at 30°C or 37 °C for 24 hours. The bacterial sensitivity to the essential oil was assessed by measuring the diameter of inhibition zone and recorded if the zone of inhibition is greater than 9 mm. The inhibition zones were compared with that of ampicilline (BIO-RAD Marne- la- coquette, France) and tetracycline (BIO-RAD Marne- la-coquette, France). All of the tests were made in triplicate.

Determination of MIC and MBC values

A broth microdilution method was used to determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) [Bassolé et al., 2003] All tests were performed in Mueller-Hinton Broth (Becton Dickinson, USA). A serial of double of each essential oil was prepared in 96 well-plates over the range 0.03-8% (v/v). The broth was supplemented with tween 80 at a concentration of 0.1% in order to enhance essential oil solubility. The tween 80 was at the final concentration of 0.001% (v/v). Overnight broth cultures of each strain were prepared in Nutrient Broth (Diagnostic Pasteur, France) and the final concentration in each well was adjusted to 5×10^5 CFU/ml following inoculation. The concentration of each inoculum was confirmed by viable count on Plate Count Agar (Merck, Germany).

Positive and negative growth controls were included in every test. The tray was incubated aerobically at 30 °C (Reference Gram-negative strain) or 37 °C (Reference and isolated Gram-positive) and MICs were determined. The MIC defined as the lowest concentration of the essential oil at which the microorganism tested does not demonstrate visible growth. To determine MBCs, 10µl suspension were taken from each well and inoculated in Mueller–Hinton Agar (Becton Dickinson, USA) for 24 h at 30 or 37 °C. The MBC is defined as the lowest concentration of the essential oil killing 99.9% of bacteria inocula [Michel Briand, 1986]. All tests were performed in triplicate.

Statistical analysis

Data were expressed as mean±SEM. A one way variance was use to analyse data. $p<0.01$ represented significant difference between means (Duncans multiple range test).

3. Results

3.1 Analyses

GC and GC/MS analyses of the essential oil composition of *Vitex simplicifolia* were as previously described [Ouoba et al., 2009] The oil contained monoterpenoids as predominant (71.02%). Among monoterpene hydrocarbon, myrcene (53.50%) had been found as the major component and four components were detected as predominant: α-pinene (5.13%), β-pinene (2.48%) and β-phellandrene (1.38%). In the oxygenated fraction, 10 monoterpenes (6.32%) and 12 sesquiterpenes (5.58%) were present with linalool (4.70%) and humulen-1,2- epoxyde (1.15%) as the major constituents. Among mono and sesquiterpenes three ketones are detected as minor compounds piperitone (0.05%) cis-jasmone (0.11%) and salvia-4(14)en-1-one (0.07%). No phenolic compound has been detected in the oil.

3.2 Wound healing activity

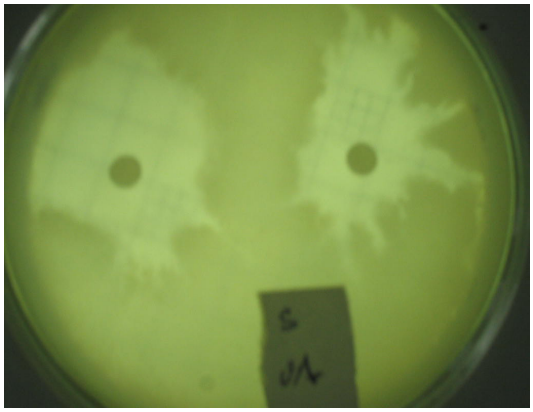
Experiments on rabbits showed that the oil has a healing effect. As shown in Table1, the different stages of evolution of healing capacity of essential oil in comparison with those of cicatryl and natural immunity of rabbit. The different stages of evolution of healing were characterized by changes in the color of the wound over time, the closure of lacerations and the absence of erythema and edema of the wounds.

rabbits treated	wounds at 48h	wounds at 96h	wounds from 6 to 10 days
Essential oil	vascular and inflammatory stage tissue répair stage maturation stage (start)	maturation stage (end of cicatrization) **complete healing**	
Cicatryl	vascular and inflammatory stage tissue répair stage maturation stage (start)	tissue repair stage (end) maturation stage (start)	maturation stage (end of cicatrization) **complete healing**
Rabbits untreated	vascular and inflammatory stage tissue répair stage maturation stage (start)	tissue repair stage (end) maturation stage (start)	maturation stage (end of cicatrization) **complete healing**

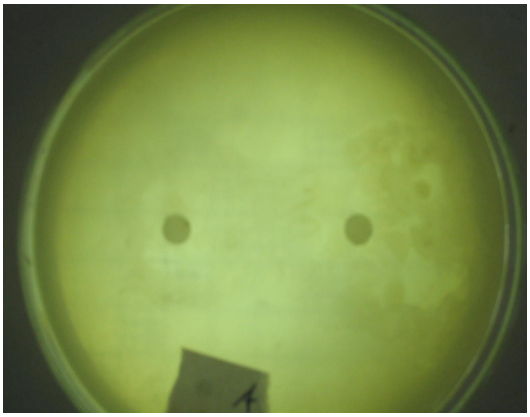
Table 1. Different stages of evolution of wounds healing.

3.3 Determination of the strain sensitivity

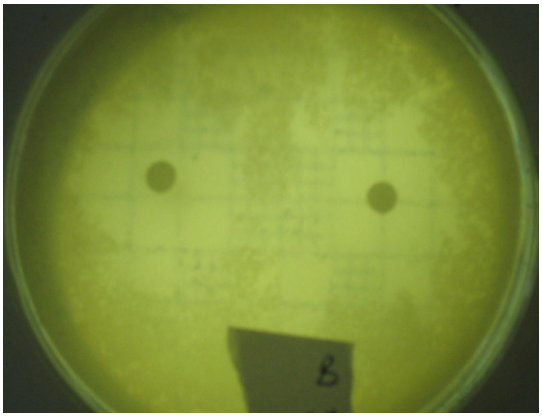
The results showed that almost of the bacterial strains were sensitive to *Vitex simplicifolia* essential oil (Table2). *Staphylococcus aureus* ATCC9144 BHI (zone of inhibition 34.5mm) was the most sensitive bacteria tested. Only *Bacillus cereus* LMG 13569 BHI was not sensible to *Vitex simplicifolia* (zone of inhibition 8.5mm) (Fig1).



(1a) *Salmonella enterica*



(1b) *Shigella dysenteria*



(1c) *Staphylococcus aureus*

Fig. 1. Inhibition zones for some bacterial strains.

Reference strains	Origin	V. s	Amp	Te
Enterococcus faecalis CIP 103907 BHI	CIP	21	35	20
Bacillus cereus LMG 13569 BHI	LMG	8.5	31	22
Listeria innocua LMG 13568 BHI	LMG	16.5	45	12
Staphylococcus aureus ATCC 25293 BHI	ATCC	24.5	56	27
Staphylococcus camorum LMG 13567 BHI	LMG	19.5	54	21
Staphylococcus aureus ATCC 9144 BHI	ATCC	34.5	40	32
Escherichia coli CIP 105182	CIP	10.5	43	21
Proteus mirabilis CIP 104588	CIP	11.5	21	18
Shigella dysenteria CIP 5451	CIP	24.5	11	22
Salmonella enterica *CIP 105150*	CIP	28	36	24

V.s.: *Vitex simplicifolia* Amp: ampicilline Te: tetracycline

Table 2. Diameter of inhibition zone (mm) of bacteria growth.

3.4 Determination of antibacterial activity

The MICs and MBCs of *Vitex simplicifolia* essential oil were consigned in Table3. Five bacterial strains were selected and tested because of their highest sensitivity to essential oil. The oil inhibited the growth of these bacteria with MIC of 0.50% except for *Staphylococcus aureus* ATCC 9144BHI that was more sensitive with MIC of 0.25%. The results of MBC demonstrated a bacteriostatic effect.

Reference strains	Origin	MIC	MBC	MBC/MIC
Enterococcus faecalis CIP 103907	CIP	0,5	8	16
Escherichia coli CIP 105182	CIP	0,5	4	8
Listeria innocua LMG 135668	LMG	0,5	4	8
Staphylococcus aureus ATCC 25293	ATCC	0,5	8	16
Staphylococcus camorum LMG 13567	LMG	0,5	8	16
Staphylococcus aureus ATCC 9144 BHI	ATCC	0.25	1	4

Table 3. Minimum inhibitory concentration, minimum bactericidal concentration data (%v/v) obtained by microdilution method.

4. Discussion

Wound healing is very complex, it involves a sequence of multifactorial events including several cellular and biochemical processes. These processes aim to ensure the regeneration

and reconstruction of anatomical and functional disturbances of the skin[Chattopadhyay et al., 2002]. The repair of damaged tissues occurs as a sequence of events that included inflammation, proliferation and migration of different cell types [Sidhu et al., 1999]. At the dose of 0.44 mg used only once the essential oil healed wounds for 96h. Essential oil of *Vitex simplicifolia* accelerated the three stages of cicatrization process: vascular and inflammatory, tissue repair and maturation. While at the dose of 1000 mg used only once, cicatryl exhibited a complete healing with for 7 days against 10 days for the effect of natural immunity of rabbit. In the phase of maturation a renewal of the skin was seen, the old skin started to fall and made way for the new. The essential oil of *Vitex simplicifolia* exhibited a stronger healing effect than cicatryl and natural immunity. This effect could be due to the presence of ketones in the oil that activated the healing process with stimulating of new cell growth, reducing old scare tissue in wound and were highly immunostimulatory [Willem, 2004]. The presence of minor compounds as aldehydes and sesquiterpenes activated anti inflammatory, calming and sedative effects. Thus, their low proportion allowed to consider possible synergistic effects of these compounds in the oil. The significant presence of monoterpenoids in the oil might cause analgesic, antioxidative, antiseptic effects and stimulating the immune system [Mertz et al., 1993].

In other hand, the skin infections are in most cases due to staphylococci with the pathogenic species is Staphylococcus aureus. It is responsible for suppurative infections, widespread and food poisoning. Thus, wound infections are most common in developing countries because of poor sanitation. *Staphylococcus aureus, Shigella dysenteria*, *Salmonella enterica*, *Escherichia coli* are important microorganisms causing an infection of the wound [Mansouri et al., 2011]. The best sensitivity to *Vitex simplicifolia* essential oil was, respectively, obtained on *Staphylococcus aureus* ATCC 9144 BHI (34.5mm), *Salmonella enterica* CIP 105150 (28mm), *Shigella dysenteria* CIP 5451(24.5mm), *Staphylococcus aureus* ATCC 25293 BHI (24.5mm), *Enterococcus faecalis* CIP 103907 BHI(21mm). Following the results in Table 2 the different strains were less sensitive to V.s than ampicilline, while *shigella dysenteria* CIP 5451 was sensitive to V.s. The most important information was that essential oil exhibited more activity on *Staphylococcus aureus* ATCC 9144 BHI (34.5mm), *Salmonella enterica* CIP 105150 (28mm) *Shigella dysenteria* CIP5451(24.5mm) than tetracycline and ampicilline (*Shigella dysenteria* CIP 5451, 11mm). The essential oil failed to inhibit *Staphylococcus aureus* ATCC 9144 BHI at the lowest MIC 0.25%. The essential oil was bacteriostatic for *Staphylococcus aureus* ATCC 9144 BHI, *Escherichia coli* CIP 105182 and *Listeria innocua* LMG 135668. The most resistant strains with highest MBC (8%) were *Enterococcus faecalis* CIP 103907, *Staphylococcus aureus* ATCC 25293 and *Staphylococcus camorum* LMG 13567. Considering MICs and MBCs no significant difference could be seen between Gram-positive and Gram-negative bacteria. The chemical composition of the oil consisted of various constituents. Therefore, the determination of the component responsible for activity was very difficult. Furthermore the essential oil consists of complex mixture of numerous constituents. Major or minor compounds might cause the bacteriostatic and cicatrization activities exhibited, terpinene-4-ol and other monoterpenes in essential oil may act as antiseptic, anti-inflammatory and antimicrobial: myrcene, sabinene, terpinene, cadinene and limonene [Sinan Dayisoylu et al. 2009]. In addition, the presence of α-pinene, β-pinene [Houghton Peter, 2004] terpinen-4-ol [Lee et al., 2001] and

γ-terpinene[Sonboli et al., 2005] were responsible of antioxidative and antiseptic activities of essential oils studied. However, caryophyllene oxide, E-nerolidol humulene epoxide-1,2 possessed antiinflammatory activity [Chavan et al., 2010; Yu-Tang et al., 2008; Wanjohi Mwangi et al., 2009] Possible synergistic and antagonistic effects compounds in *V. simplicifolia* essential oil should also be taken into consideration. These reports are compatible with our results in the present study.

5. Conclusion

This study shows in *vivo* wound healing activity and in *vitro* bacteriostatic effect of *Vitex simplicifolia* essential oil. The oil demonstrates the strongest wound cicatrization activity than cicatryl and natural immunity. In addition the oil may help to prevent wound infections and others such diarrhoea, dysentery and skin diseases. These results indicate that the plant could be use as a natural potential remedy for healing wounds and antiseptic agent. Further investigations will be performed by determination of analgesic, antioxidant and anti inflammatory activities of the essential oil and to expand to other *Vitex* species.

6. Remarks

1. Choice of rabbits: we have chosen rabbits because of they were available in the laboratory and cheaper. They were also very easy to be used in the cicatrization effect than mice and rats
2. The resolution of photographs depend of the quality of the apparatus, we deleted them because we have not a best quality. We are sorry for the bad quality of photos. Thank you for your understanding.

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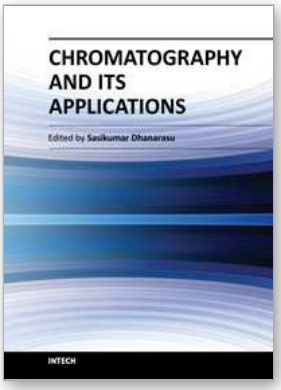
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Chromatography is a powerful separation tool that is used in all branches of science, and is often the only means of separating components from complex mixtures. The Russian botanist Mikhail Tswett coined the term chromatography in 1906. The first analytical use of chromatography was described by James and Martin in 1952, for the use of gas chromatography for the analysis of fatty acid mixtures. A wide range of chromatographic procedures makes use of differences in size, binding affinities, charge, and other properties. Many types of chromatography have been developed. These include Column chromatography, High performance liquid chromatography (HPLC), Gas chromatography, Size exclusion chromatography, Ion exchange chromatography etc. In this book contains more details about the applications of chromatography by various research findings. Each and every topics of this book have included lists of references at the end to provide students and researchers with starting points for independent chromatography explorations. I welcome comments, criticisms, and suggestions from students, faculty and researchers.

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Phone: +86-21-62489820
Fax: +86-21-62489821

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