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Antitrypanosomal activity and toxicity of substituted citronellal semicarbazones and thiosemicarbazones hemi-synthesized *in situ* in the essential oil of *Eucalyptus citriodora*

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ABSTRACT

*In this work, the essential oil of Eucalyptus citriodora was extracted and analyzed by Gas Chromatography / Mass Spectrometry. From this analysis, it appears that citronellal is the major compound (68.2%) of this oil. This aldehyde was used in situ in the essential oil of Eucalyptus citriodora for the hemi-synthesis of four structures carbazones. That was citronellal semicarbazone (**1**), citronellal thiosemicarbazone (**2**), citronellal 4-phenyl semicarbazone (**3**) and citronellal 4-phenyl thiosemicarbazone (**4**). The structures of these compounds were confirmed by IR spectrometry, proton NMR and carbon-13 and by Mass Spectrometry. From the test againstTrypanosomabruceibrucei, it appears that citronellal semicarbazone (IC_{50} = 473.93 µM) and citronellal thiosemicarbazone (IC_{50} = 440.53 µM) had low activity. Citronellal 4-phenyl semicarbazone (IC_{50} = 57.26 µM) and 4-phenyl thiosemicarbazone citronellal (IC_{50} = 19.63 µM) had moderate activity. The activity of citronellal 4-phenyl thiosemicarbazonewas still pronounced. The study of the toxicity against Artemiasalinashowed that all compounds except the compound **1**were moderately toxic andmay have antitumor properties. But the most antitrypanosomal compound **4**was particularly selective on trypanosome cells (SI = 4.92) and could be a good candidate against this parasite.*

Key word: Essential oil, *Eucalyptus citriodora*, hemi-synthesis, citronellalthiosemicarbazone, *Trypanosoma bruceibrucei*, toxicity.

INTRODUCTION

Since prehistoric period, medicinal plants used in traditional medicine play significant role to heal human diseases and disorders [1-3]. *Eucalyptus citriodora*(Lemon scented gum) belongs to the family Myrtaceae [4,5] made from these plants. In traditional medicine, essential oil of *Eucalyptus* species has been applied for the treatment of respiratory tract disorders, cold, chest pain, coughs and infections [6, 7]. Its major component is citronellal [8]. The

main disadvantage of this molecule is their intrinsic instability and propensity to oxidation. This inconvenience, together with their high volatility, in the case of low molecular weight molecules used in the perfumery field, for instance, makes the use of aldehydes less appealing for some applications [9,10].Ouédraogo et al. (2009)found more interesting to realize from aldehyde, more stable compounds with pharmacological properties [11].

Many infectious diseases are caused by pathogenic microorganisms like bacteria, viruses, fungi, protozoa and multicellular parasites. These diseases are often communicable or transmissible diseases, because they can be transmitted from one person to another. Infectious diseases account for about half of the deaths in tropical countries [12].

Sleeping sickness is one example which threatens millions of people in 36 countries in sub-Saharan Africa [13]. The disease in domestic animals, particularly cattle, is also a major obstacle to the economic development of affected rural areas [14]. Displacement of populations, war and poverty are important factors leading to increased transmission and this alters the distribution of the disease due to weakened or non-existent health systems [15]. The parasites concerned are protozoa belonging to the *Trypanosoma* genus. They are transmitted to humans by tsetse fly (*Glossina* genus) [16]. The disease is fatal if untreated. Drugs used for the treatment of this disease are decades old [17]. In addition, parasites develop more resistance against these drugs [18].So it is essential to find new, effective and less toxic drugs ideally with all application to control the disease.

Thiosemicarbazones display a broad spectrum of pharmacological properties, including antitumor, antifungal, antibacterial, antiviral and antimalarial, antitrypanosomal [19-22] activities among others as well as parasiticidal activity against *Plasmodium falciparum, Plasmodium berghei*[23,24], *Trypanosoma bruceirhodesiense*[25], and *Toxoplasma gondii*[26], *Trypanosoma cruzi*[27].Biological studies of commercial citronellal thiosemicarbazone synthesized by Tarasconie et al. showed inhibitory properties on leukemia cells proliferation [28].

In this research, citronellal semicarbazones and thiosemicarbazones substituted were hemi-synthesized *in situ* in *Eucalyptus citriodora* essential oil. Their antiparasitic activity against *Trypanosoma bruceibrucei* and their toxicity against *Artemiasalina* were also evaluated.

We believe that the completion of this work in which there is local production of the substrate in the hemi-synthesis reactions could help to make it less expensive. By this work, a new research very interesting in the field of essential oils could be also developed.

EXPERIMENTAL SECTION

General techniques

Essential oil analysis

The analysis was performed on a FOCUS GC with a capillary column CP Wax 52 CB (J & W Scientific from Agilent Technologies Column, No. US1670726A, USA) of dimension 15 x 0.25 mm with 0.25 μm internal diameter.

In order to confirm the specificity and selectivity of the GC method, GC/MS analysis were performed on a TRACE GC 2000 series (Thermo Quest, Rodano, Italy), equipped with an AS2000 auto sampler (GC System Thermo Quest. coupled to a mass spectrometer type Thero Quest Trace MS) operating in electron impact mode [29].

The compounds were identified by comparing their retention time and mass spectra with those of reference compounds.

Hemi-synthesized compounds identification

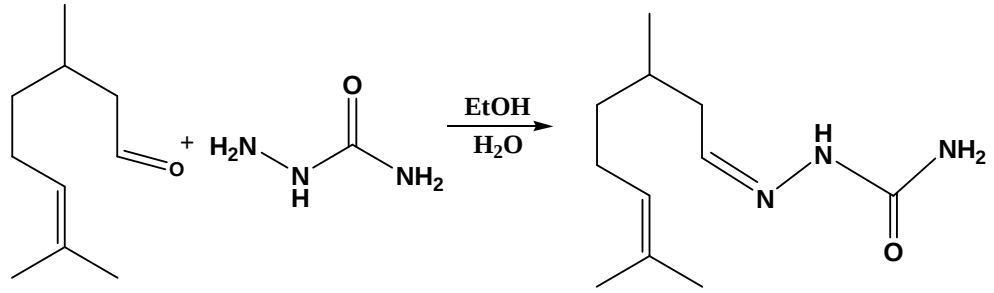
The melting points were taken on a fusionometer type *electrothermal 1A 9000* and are uncorrected. The IR spectra were recorded on a Perkin-Elmer FTIR 286. The frequencies of absorption bands are expressed in cm^{-1}. The NMR spectra were registered on a Brucker 500 in $CDCl_3$ (chloroform-d6) or DMSO-d6 (dimethylsulfoxide-d6) which frequencies for ^{1}H and ^{13}C are 400 MHz and 100 MHz respectively. Chemical shifts are given in parts per million (ppm) relative to tetra-methyl silane as a benchmark. Multiplicity is designated as singlet (s), triplet (t), doublet (d) and multiplet (m).MS spectrometrical data of compounds were reported in APCI mode.

Extraction of essential oil

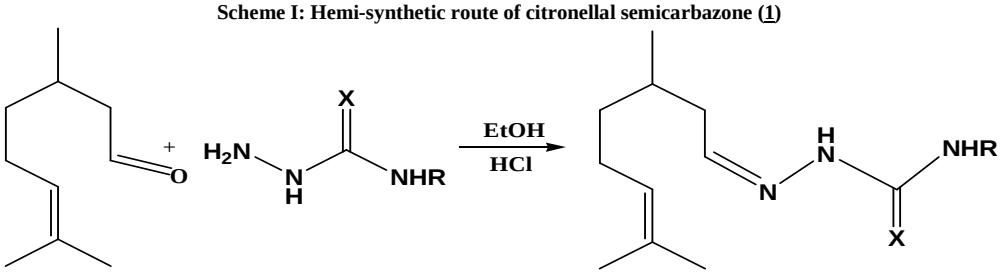
The fresh leaves of *Eucalyptus citriodora* harvested in the morning around Abomey-Calavi University (Benin) were used as material plant. The extraction took place immediately after harvest. The essential oil was obtained by hydrodistillation using a Clevenger type apparatus [30].

Hemi-synthesis

Semicarbazones and thiosemicarbazones were synthesized according to the methods used in our previous work [31]. All reagents (semicarbazide, thiosemicarbazide, 4-phenyl-3- semicarbazide and 4-phenyl-3- thiosemicarbazide), obtained from SIGMA-ALDRICH were used *in situ*on citronellal in *Eucalyptus citriodora* essential oil. Hydrochloric acid is also from SIGMA-ALDRICH.



Scheme I: Hemi-synthetic route of citronellal semicarbazone (1)



2: X=S, R=H
3: X=O, R=Ph
4: X=S, R=Ph

Scheme II: Hemi-synthetic routes of citronellal thiosemicarbazones and semicarbazonessubtitued (2, 3, 4)

Citronellalsemicarbazone (1)

1 mmol (111.5 mg) of semicarbazide hydrochloride dissolved in 2 ml of distilled water was added to a stirred mixture of 308 mg of essential oil of *Eucalyptus citriodora* dissolved in 3 ml of ethanol at 95 °. 5 drops of triethylamine were added to a mixture after a minute of stirring. The crystals appeared after 5 hours of agitation. The stirring was maintained for another hour. The resulting crystals were filtered, washed until neutral, dried, weighed and then recrystallized in ethanol at 95 °.

Citronellalthiosemicarbazonessemicarbazone substituted (2, 3, 4).

To a stirring of 308 mg of essential oil of *Eucalyptus citriodora* dissolved in 3 ml of ethanol at 95° was added 1 mmol of substituted semicarbazide or thiosemicarbazides appropriate dissolved in 3 ml of hydrochloric acid (1N). After the appearance of crystals between five to ten minutes, stirring was continued for one hour. The resulting crystals were filtered, washed until neutral, dried, weighed and recrystallized in ethanol at 95 °.

Anti-trypanosomal activity (LILIT, AlamarBlue^{TM})

The test was performed on the bloodstream form of the strain 427 of *Trypanosoma bruceibrucei* by the "Lilit Alamar Blue" method [32-34]. The stock solutions of thiosemicarbazones have been prepared from an initial concentration of 10 mg/ml in DMSO. The trypanosomes are grown in a medium containing 10% of heat-inactivated fetal calf serum and bloodstream form supporting factor. The trypanosome suspensions were adjusted to 5.10^{4}tryp/mL. In each well, 50 µl of different dilutions of the stock solution were added to 50 µl of suspension of trypanosomes. The plates were then incubated at 37 °C for 72 hours in an atmosphere with 5% CO_2. 10 µl of dye "AlamarBlue^{TM}" was added to each well and then incubated for 4 hours. The dye "AlamarBlue^{TM}" is a reagent for detecting enzymatic activity. The wells in which the concentration of compound was insufficient to inhibit the

proliferation of trypanosomes are stained. The CMI was the concentration of unstained wells in which there was the lowest amount of thiosemicarbazone. The plate reading was made in comparison with control wells on a fluorescence plate reader using an excitation wavelength of 530 nm and an emission wavelength 590 nm.

Toxicity Test against Artemiasalina

The test was performed against *Artemiasalina* LEACH by the method of Michael et al. [35] resumed by Vanhaecke et al [36] and by Sleet and Brendel [37]. The eggs of *Arténiasalina* were incubated in seawater until hatching of young larvae (48 hours). Then, series of solutions of test substance at varying and progressive concentrations were prepared. A defined number of larvae was introduced into each solution. All solutions and control solution containing no active substance were left stirring for 24 hours. Counting under a microscope the number of Death larvae in each solution was used to evaluate the toxicity of the solution. In the case where there was death in the control medium, the data was corrected by Abbott's formula: % Death = [(test - control) / control)] x 100. Data (dose-response) are transformed by logarithm and the $LC_{50}$was determined by linear regression [22].

RESULTS AND DISCUSSION

Extraction

The yield obtained after the extraction of the essential oil of *Eucalyptus citriodora* was 4.3%. The yield of essential oil of a plant depends heavily on fresh or dry plant material [38]. In this work, essential oil was obtained from fresh plant material. The analysis indicates that citronellal(67.5%) is the major compound of this oil. The percentages ofisopulegol(11.95%) and citronellol(8.68%) were also significant (table 1).

Table 1: compounds of the essential oil of *Eucalyptus citriodora*

Components	IK	percentage
isobutyrate d isobutyle	925,3	0,12%
α-pinène	940,9	0,18%
β-pinène	979,6	0,41%
δ-2-carène	998,1	0,57%
limonène	1029	0,24%
1,8-cinéole	1033	0,37%
bergamal	1054	0,21%
linalol	1100	0,57%
isopulégol	1151	11,95%
citonellal	1158	67,50%
néo-isopulégol	1181	0,78%
α-terpinéol	1196	0,15%
citronellol	1228	8,68%
pipéritone	1256	0,59%
géranial	1268	0,10%
acétate de citronellyle	1348	1,56%
eugénol	1351	0,53%
β-élémène	1389	0,13%
acétate de phenylethyle	1392	0,17%
β-caryophyllène	1421	1,82%
α-humulène	1457	0,10%
germacrène-D	1482	0,12%
oxyde de caryophyllène	1583	0,17%
Total		97,02%

The table also showed other compounds present in the oil. These percentages are consistent with those found in the literature [39]. The slight differences are often related to soil types, climates and seasons of harvest and even the hours of harvest [38]. The major component is the one that interests us most in this oil.

Synthesis

Citronellal was used *in situ* in essential oil of *Eucalyptus citriodora* for the hemi-synthesis of citronellal semicarbazone, citronellal thiosemicarabzone, citronella l4-phenylsemicarbazoneand4-phenylthiosemicarbazone citronellal.

Apart from citronellal semicarabzone, all compounds hemi-synthesized were obtained with yields all above 50%. Spectrometric analyzes in SM showed very little difference between the theoretical masses and practices masses.

In IR spectrometry, the vibration frequencies corresponding to-NH_2 groups were around 3500 cm^{-1}. These of =NH group are around 3100 cm^{-1}. Also we noted the presence of the respective groups C = O and C = N at about 1600 and 1500 cm^{-1}. No band corresponding to the SH group whose frequencies lie between 2600 and 2500 cm^{-1} was observed at citronellal thiosemicarbazones. We note the presence of bands related to C = S groups with frequencies of misrepresentation between 979 and strain 878 cm^{-1}.

Spectrometric analyzes by proton NMR clearly mentioned the different types of protons. In all compounds, the proton of = NH group was more deshielded. Its chemical shift was between 9.7 and 8.1 ppm. That was certainly because it was between the two withdrawing groups C = N and C = O or C = S. Protons of-CH = N- were also deshielded and their chemical shifts were between 7.9 and 7.1ppm. Then, came the protons of the nitrogen terminal group. The vinyl groups were also present. Spectra also provide information on the presence of methyl groups. NMR carbon 13 chemical shifts of the C = O groups are presents very remarkable level of semicarbazones and are around 158ppm. Those corresponding to C = S groups of thosemicarbazones are also present around 180 ppm. These results are consistent with those obtained by Pieralberto et al. in 2000 about citronellal thiosemicarbazone [28].

The spectrometric proprieties of hemi-synthesized compounds are mentioned below.
Citronellal semicarbazone (**1)** Yield: 48%; m.p: 83-85°C; **IR** (NaCl, cm-1): 3594; 3481 ν(NH2); 3257, 3182 ν(NH); 1668, 1638 ν(C=O); 1578 ν(C=N); 1518, 1449 ν(C=C). **13C NMR** ($CDCl_3$, 100MHz) δ(ppm): 158 ; 144 ; 132 ; 120 ; 36 ; 34 ; 27 ; 26 ; 22 ; 19 ; **1H NMR** ($CDCl_3$, 400MHz), δ(ppm): 0.9 (d, 3H, -$CHCH_3$); 1.3, and 1.6 (s, 6H, -$C(CH_3)_2$); 1.7 (m, 2H, -CH_2CH); 1.9 (m, 1H, -$CHCH_2$); 2.8 (m, 2H, -CH_2CH=); 3.4 (t, 2H, -CH_2CH=N); 4.3 (t, 1H, -CH=C); 6.9 and 6.5 (s, 2H, -NH_2); 7.1 (t, 1H, -CH=N); 8.1 (s, 1H=NNH-). **MS** (m/z): [MH+] 211.17; [MH+] found 211.17.

Citronellal thiosemicarbazone (**2)** Yield: 67%; m.p: 178-180°C; **IR** (NaCl, cm-1): 3606, 3534, 3445 ν(NH2); 3234, 3177 ν(NH); 1576 ν(C=N); 1483, 1459, 1448 ν(C=C); 979 ν(C=S). **13C NMR** ($CDCl_3$, 100MHz) δ(ppm): 177 ; 145 ; 131 ; 122 ; 33; 32; 26; 25; 23; 22 ; **1H NMR** ($CDCl_3$, 400MHz), 1.1 (d, 3H, -$CHCH_3$); 1.4, and 1.6 (s, 6H, -$C(CH_3)_2$); 1.8 (m, 2H, -CH_2CH); 1.9 (m, 1H, -$CHCH_2$); 2.6 (m, 2H, -CH_2CH=); 3.5 (t, 2H, -CH_2CH=N); 5.2 (t, 1H, -CH=C); 7.1 and 6.8 (s, 2H, -NH_2); 7.3 (t, 1H, -CH=N); 9.6 (s, 1H=NNH-). **MS** (m/z): [MH+] 227.15; [MH+] found 227.00.

4-phenyl Citronellal semicarbazone (**3)** Yield: 58%; m.p: 120-122°C; **IR** (NaCl, cm-1): 3639, 3529, 3441 ν(NH2); 3200 ν(NH); 1684 ν(C=O); 1594 ν(C=N); 1500, 1446 ν(C=C). **13C NMR** ($CDCl_3$, 100MHz) δ(ppm): 158 ; 141 ; 136 ; 134 ;129; 124 ; 119 ; 36; 34 ; 29 ; 27 ; 22 ; 21 ; 19; **1H NMR** ($CDCl_3$, 400MHz), 0.9 (d, 3H, -$CHCH_3$); 1.6, and 1.4 (s, 6H, -$C(CH_3)_2$); 1.6 (m, 2H, -CH_2CH); 1.8 (m, 1H, -$CHCH_2$); 2.5 (m, 2H, -CH_2CH=); 3.5 (t, 2H, -CH_2CH=N); 4.1 (t, 1H, -CH=C); 7.2-6.9 (H aromatics) 7.6 (s, 2H, -NH_2); 7.9 (t, 1H, -CH=N); 8.4 (s, 1H=NNH-). **MS** (m/z): [MH+] 287.2; [MH+] found 287.27.

4-phenyl Citronellal thiosemicarbazone (**4)** Yield: 77%; m.p: 143-145°C; **IR** (NaCl, cm-1): 3621, 3528, 3442 ν(NH2); 3181 ν(NH); 1600, 1590 ν(C=N); 1498, 1458, 1448 ν(C=C); 878 ν(C=S). **13C NMR** ($CDCl_3$, 100MHz) δ(ppm): 183 ; 142 ; 128 ; 125 ;124; 123 ; 37; 34 ; 27 ; 25 ; **1H NMR** ($CDCl_3$, 400MHz), 1.0 (d, 3H, -$CHCH_3$); 1.8, and 1.7 (d, 6H, -$C(CH_3)_2$); 2.05 (m, 2H, -CH_2CH); 2.2 (m, 1H, -$CHCH_2$); 3 (m, 2H, -CH_2CH=); 3.8 (t, 2H, -CH_2CH=N); 4.6 (t, 1H, -CH=C); 7.6-7.1 (H aromatic) 7.1 and 6.9 (s, 2H, -NH_2); 7.7 (t, 1H, -CH=N); 9.7 (s, 1H, =NNH-). **MS** (m/z): [MH+] 303.18; [MH+] found 303.15.

Synthesis and evaluation of some properties of citronellalsemicarbazone and thiosemicarbazone and their metal complexes have been reported in the literature[28]. As against, this is the first time that the semicarbazone and thiosemicarbazone substituted are realized. More antitrypanosomal activities and toxicity of all molecules have never been studied. Also the synthesis route of semicarbazones and thiosemicarbazones in the essential oil of *Eucalyptus citriodora*is new.

Linpinsky rule
Lipinsky rule allows having an idea on the possible biological properties for component hemi-synthesized. Lipinski described desired ranges for certain properties thought to be important for pharmacokinetics and drug development. They are *C* log *P* < 5, number of hydrogen bond donors < 5, number of hydrogen bond acceptors < 10, and

molecular weight <500 [40]. A compound that fulfills at least three out of the four criteria adheres to Lipinski's rule [40]. Table 2 lists such properties of the four compounds.

Table 2: Linpinsky rule

Compounds	Molecular weight	*Clog P*	No. of H bond donors	No. of H bond acceptors	No. of criteria met
Rule	< 500	< 5	< 5	< 10	at least 3
1	211	3,70	3	4	All
2	227	3,44	3	3	All
3	287	5,16	2	3	3
4	303	6,28	2	3	3

All compounds obtained in this work respect the rule of Lipinski. They could therefore consist of good drugs. In this way, compounds hemi-synthesized are tested on *Trypanosoma bruceibruce*ito assess their antitrypanosomal property.

antitrypanosomal activity of compounds

According to the work of Du et al. and Fujii et al., thiosemicarbazones are the trypanocidal when their IC_{50} values are less than 10 µM, are regarded as moderate agents antitrypanosomal if these values are between 10 and 100 µM, and have little or no activity when their IC_{50} are higher than 100 µM [25,26].

In crescent order of activity against trypanosome cells, we have respectively citronellal semicarbazone(IC_{50} =473,93 µM), citronellal thiosemicarbazone(IC_{50} = 440,53 µM), citronellal 4-phényl semicarbazone (IC_{50} =57,26 µM) and citronellal 4-phényl thiosemicarbazone (IC_{50} =19,63 µM) (table5).According to this scale, it is clear that the compounds **1** and **2** have a low activity on trypanosome cells. Compounds **3** and **4** have moderate activity. But component **4**activity against *Trypanosomabruceibruce*iis very interesting.

We observe that replacement one of the hydrogens of the terminal nitrogen atom significantly augment the activity of the compounds **3** and **4**. Also we note that the substitution of oxygen by sulfur also augments thiosemicarbazones activities.

As pointed out Kasuga et al. in 2001and many other authors, we come to realize that thiosemicarbazones are much more active than semicarbazones [41,42].

Toxicity of compounds

This test indicates the sensitivity of shrimp larvae facing compounds and can give an idea about potential antitumor activities. According to Santos et al., there is a good correlation Between toxicity of thiosemicarbazones on shrimp larvae and cytotoxicity activity on cells 9KB and 9PS (human carcinomanasopharygien) apart, cellsA-549lungcarcinomaandHT-29cellsofcarcinomaof the colonon theother [43,44,45]. Theyhave come tothe conclusion thata compound having aLC_{50}less than 280µMcould haveantitumor activity.The comparison was madewith lapachol;areference compoundwhich LC_{50}value was 281µM [43].

LC_{50} values of compounds**1**, **2**, **3** and **4** are respectively: 462,08 µM; 121,15µM; 45,05 µM, 96,53µM (table 5). Thus only compound **1**was not toxic. Particularly the compound**3**,in addition tohis activityagainst trypanosome cellscouldalso be a goodantitumor agent.

Selectivity index

Theselectivity index (SI) wasby definition, the ratio ofthe value of thetoxicity test onthe value of theantitrypanosomaltest.If the SI value obtained was greater than unity, the test compound was considered to be selective on the parasites. However, if IS was less than unity, the test compound was more toxic than anti-parasitic [46].

SI of compounds 1, 2, 3 are less than unity, so there selectivity on the parasite wasalmost negligible. There was aremarkable selectivityof compound **4** (SI = 4.92) intrypanosomecells (table 5).

Table 5: Selectivity index values

Compounds	LC_{50}µM	IC_{50} µM	Selectivity index (SI = LC_{50}/IC_{50})
1	462,08	473,93	0,97
2	121,15	440,53	0,27
3	45,05	57,26	0,79
4	96,53	19,63	4,92

CONCLUSION

At the end ofthis work, citronellal contentin the essential oilof*Eucalyptuscitriodora*,can be used insynthesis reactions for production ofpure moleculeswhichcanbe usedas an active ingredientdrugsagainst parasitic diseases.

Apart fromreducing thecost ofwork due tolocal productionof the substrate,this work offersgreat potentialin the field ofessential oils.

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