SELECTIVE TRYPANOCIDE ACTIVITY OF SOME SUBSTITUTED THIOSEMICARBAZONES OF CITRAL FROM BENIN *CYMBOPOGON CITRATUS* ESSENTIAL OIL AND THEIR TOXICITY AGAINST *ARTEMIA SALINA* LEACH.

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ABSTRACT

Extraction and analysis GC/FID and GC/MS showed that citral (neral + geranial) is the major compound of the essential oil of Benin Cymbopogoncitratus. This aldehyde was used as target for the hemi-synthesis in situ of the semicarbazone and substituted thiosemicarbazones. Their structures were confirmed by spectrometric analysis IR, 1H and 13C NMR. Their antiparasitic activities have been evaluated on Trypanosomabruceibrucei by determining their half-inhibitory concentrations (IC50). Among them, citral 4-phenyl-3-thiosemicarbazone (IC50 = 1.96 µM) and citralthiosemicarbazone (IC50 = 7.6 µM) showed a strong trypanocidal activity. Citral 2-methyl-3-thiosemicarbazone (IC50 = 60.87 µM) showed a moderate activity. Citral 4-methyl-3-thiosemicarbazone (IC50 = 172.84 µM) and citralsemicarbazone (IC50 = 234.64 µM) were less actives. Toxicity test against Artemiasalina indicated that citral 4-phenyl-3-thiosemicarbazone is the most toxic compound (LC50 = 70.70 µM). The toxicities of other compounds are low. Citral 4-phenyl-3-thiosemicarbazone could have excellent anti-cancer properties. The selectivity index calculated from these data showed that all the molecules obtained are selective about the parasites Trypanosomabruceibrucei.

Keywords:*Cymbopogoncitratus, essential oil, citral, thiosemicarbazones, spectrometrical analysis, antiparasitic activities, Trypanosomabruceibrucei, Artemiasalina, selectivity index.*

1. INTRODUCTION

Plant essential oils and their components have been known to exhibit biological activities, especially antimicrobial [1], antifungal [2,3], Antibacterial [4-6], Antimycotic [7] and antioxidant activities [8]. *Cymbopogoncitratus*is aromatic spice, that belong to the family poaceae and is cultivated almost in all tropical and subtropical countries [9]. Some studies proved that its essential oil have antimalarial [10],anti-Leishmaniase [11],Antifungal [12], Antinociceptive[13], insecticidal [14,15] and antimicrobial [16] activities. Its major component is citral which can be used in hemi-synthesis reaction to give else components more active [17].

Infectious diseases caused by protozoan parasites remain chronic problems for humanity. *Trypanosomabruceigambiense, and* T. bruceirhodesiense are the major species of African trypanosomiasis that primarily cause disease in domestic livestock [18-20]. There are transmitted by the tsetse fly (*Glossina* spp.) [21]. The economic impact of African trypanosomiasis is enormous [22]. The disease is fatal if untreated so it is essential to find new, effective and less toxic drugs ideally with all application to control the disease [23-24].

Thiosemicarbazones, an important class of synthetic compounds, have a variety of applications due to their wide spectrum of biological activities [25,26], which include antiviral [27], anticonvulsant [28],antitumoral [29,30], antytrypanosomal [31-33] activities among others as well as parasiticidal activity against *Plasmodiumfalciparum, Plasmodium berghei*[34,35], *Trypanosomabruceirhodesiense*, and *Trypanosomacruzi*[36].

Recently, the work of Fujii et al.[37] has reported that thiosemicarbazone derivatives were found to be potent inhibitors of cruzain and rhodesain, essential proteases in the life cycles of *Trypanosomacruzi* and *Trypanosomabruceirhodesiense*.

The several neurological activities of citral4-aryl substituted semicarbazones of have been reported by Aggarwal et al. [38]. Biological studies of citralthiosemicarbazones also showed inhibitory properties on leukemia cells proliferation [39]. Antitrypanosomal activity against *Trypanosomabruceibrucei*of citral substituted thiosemicarbazones in *Cymbopogoncitratus* essential oil was also known [40].

In present work, the toxicity test against *artemiasalina* of citralsemicarbazone and thiosemicarbazoneshemi-synthesized *in situ*in *Cymbopogoncitratus* essential oil will be done and allow to appreciate the selectivity index on *Trypanosomabruceibrucei.*

2. MATERIAL AND METHODS

2.1. General techniques

I.Essential oil analysis by gas chromatography flame ionization detection (GC/FID)

The analysis is 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.

II.Analysis-GC coupled with mass spectrometry (GC/MS)

In order to confirm the specificity and selectivity of the GC method, GC/MS analysis were performed on a TRACE GC 2000 series (ThermoQuest, Rodano, Italy), equipped with an AS2000 autosampler (GC System ThermoQuest. coupled to a mass spectrometer type TheroQuest Trace MS) operating in electron impact mode [41].

II.Identification of compounds

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

IV.Synthesis and identification of compounds

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 1H and 13C 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.

2.2. Methods

I.Extraction of essential oil

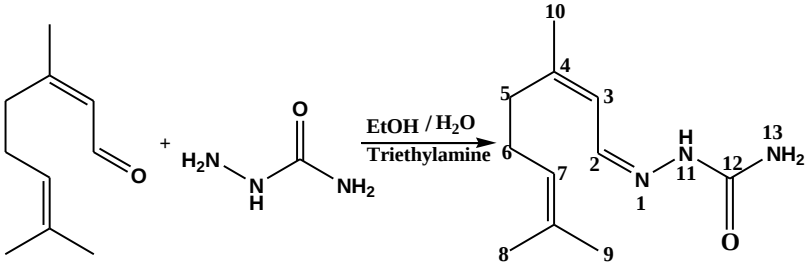
The fresh leaves of *Cymbopogoncitratus*harvested in the morning at Abomey-Calavi (Benin) on the shores of Nokoué Lake are used as material plant. The extraction took place immediately after harvest. The essential oil is obtained by hydrodistillation using a Clevenger type apparatus.

II.Protocol synthesis of citralsemicarbazone and citralthiosemicarbazones

The semicarbazone and thiosemicarbazones have been synthesized in one step at room temperature. The oil is regarded as 100% of citral. The reaction is equimolar. But this oil contains only 70.13% of citral, so there is a slight excess of reagent (semicarbazide or thiosemicarbazides).

a. Citralsemicarbazone**1**

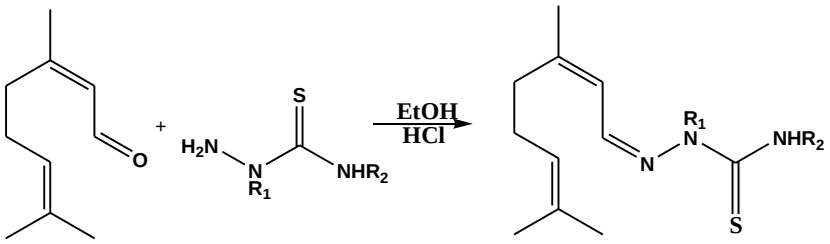
To a stirring mixture of 0.001 mol of essential oil of *Cymbopogoncitratus* (152 mg) dissolved in 1.5 ml of ethanol at 95 ° and 0.001 mol of semicarbazide hydrochloride (111,5 mg) dissolved in 1 ml of distilled water, we added two drops of triethylamine after one minute of stirring. Stirring continued for one hour. The precipitate obtained was then filtered, washed until neutral, dried, weighed and then recrystallized in ethanol at 95°C.



*Scheme 1.Synthetic route of semicarbazone**1.***

b. Citralthiosemicarbazone**2**, Citral 2-methyl-3-thiosemicarbazone **3**, citral 4-methyl-3-thiosemicarbazone **4**, citral 4-methyl -3-thiosemicarbazone **5**.

To a stirring mixture of 0.001 mol of essential oil of *Cymbopogoncitratus* (152 mg) dissolved in 1.5 ml of ethanol at 95 ° was added 0.001 mol of thiosemicarbazide or substituted thiosemicarbazide dissolved in 2 ml of 1N hydrochloric acid. This mixture was stirred until thiosemicarbazone or substituted thiosemicarbazone crystals were observed after three minutes. Stirring continued for one hour. The precipitate is filtered, washed until neutral, dried, weighed and then recrystallized in ethanol.



2: R_1 = H ; R_2 = H

3: R_1 = CH_3 ; R_2 = H

4: R_1 = H ; R_2 = CH_3

5: R_1 = H ; R_2 = Ph

*Scheme2.Synthetic routes of thiosemicarbazones.**2**, **3**, **4**, **5**.*

III.Pharmacology

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

The test is performed on the bloodstream form of the strain 427 of *Trypanosomabruceibrucei* by the "LilitAlamar Blue" method [42-44]. 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}" is 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 is insufficient to inhibit the proliferation of trypanosomes are stained. The CMI is the concentration of unstained wells in which there is the lowest amount of thiosemicarbazone. The plate reading is made in comparison with control wells on a fluorescence plate reader using an excitation wavelength of 530 nm and an emission wavelength 590 nm.

b) Toxicity Test against *Artemiasalina*

The test is performed against *Artemiasalina* LEACH by the method of Michael et al. [45] resumed by Vanhaecke et al [46] and by Sleet and Brendel [47]. The eggs of *Arténiasalina* are 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 are 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 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} is determined by linear regression.

3. RESULTS AND DISCUSSION

3.1. Extraction

From 500 g of fresh plant material was extracted 2.3 g of essential oil of *Cymbopogoncitratus* to yield 1.3%. The GC analysis revealed the presence of (Z) - 3,7-dimethyl-2, 6-Octadiènal (neral) to 33.49% and (E) -3,7-dimethyl-2 ,6-Octadiènal (geranial) to 36.64%. There are also minor compounds such as β-pinene, 6-methyl-5-heptene-2-one, nerol, acetate of geranyl or neryl, 3-methyl-3-(4-methyl-3-pentenyl) - Oxiranecarboxaldéhyde etc.

The yield of essential oil depends on the fresh or dry material plant. The fresh material containing more water, its mass is higher and thus leads to a lower yield. Some authors have reported yields of 1.02 to 1.5% from the dry material by location. The percentage of citral (neral + geranial) is estimated at 70.13%. These results are confirmed by those of literature [14,16].

Citral (neral + geranial) in this oil was used as substrate for the hemi-synthesis of the semicarbazone and thiosemicarbazones.

This achievement of the essential oil of *Cymbopogoncitratus* is made possible thanks to its availability in large quantities in Benin and its essential oil yield substantial. In addition to reducing the cost of synthesis, this work will develop a new line of research in the field of essential oils.

3.2. Hemi-synthesis

Fives compounds were obtained with the good yields. There are: citralsemicarbazone**1** **(**79 %), citralthiosemicarbazone**2** **(**83 %), citral 2-methyl-3-thiosemicarbazone **3**(73 %), citral 4-methyl-3-thiosemicarbazone **4** **(**80 %), citral 4-phenyl-3-thiosemicarbazone **5** **(**91%). The formulas of the various products are shown in Figure 1. We performed the hemi-synthesis of five carbazones which have been prepared by this method for the first time. The spectroscopic analysis showed the presence of the semicarbazone and thiosemicarbazones of the two isomers (neral and geranial) of citral.

3.3. Physical properties

The semicarbazone is white and the thiosemicarbazones are yellow. The color of the 4-phenyl-3-thiosemicarbazone tends towards orange. The molecular formulas and melting points are given in Table 1.

Table 1.Physical properties of compounds.

compounds	Forms Raw	Molecular weight(g/mol)	Melting point (°C)	Yields (%)
1	$C_{11}H_{19}ON_3$	209	120	79
2	$C_{11}H_{19}SN_3$	225	105	83
3	$C_{12}H_{21}SN_3$	239	79	73
4	$C_{12}H_{21}SN_3$	239	102	80
5	$C_{17}H_{23}SN_3$	301	82	91

Through several washes and recrystallizations secondary synthetic products have been eliminated such as: semicarbazones and thiosemicarbazones of minor carbonyl compounds and other compounds in the essential oil of departure.

3.4. Spectrometric analysis

I. IR spectrum

The values of the vibrational frequencies of the products are grouped in Table 2. The values of the vibrational frequencies of the NH_2 group are between 3251- 3429 cm^{-1}. Those of the secondary NH group are between 3280 cm^{-1} and 3165 cm^{-1}. The CH_3 group frequencies of vibration are between 3028 cm^{-1} and 3158 cm^{-1}. The carbonyl (C = O) of the semicarbazone indicates a vibration frequency of 1661 cm^{-1}. The C = S frequencies of deformation ofthioamides are between 836 cm^{-1} and 857 cm^{-1}.

Table 2.Frequencies of vibration of citralsemicarbazone and thiosemicarbazones.

Attributions	Frequencies of vibration of the semicarbazone and thiosemicarbazones (cm^{-1})				
	semicarbazone	Thiosemicarbazones			
	1	**2**	**3**	**4**	**5**
($-NH_2$)	3429	3373; 3271	3427 ; 3251	3424	3418
(NH)	3310	3165	-	3275	3280
(CH_3)	3177	3028	3129	3151	3158
(CH_2)	2969 ; 2931	2968 ; 2925	2962 ; 2912	3003 ; 2910	2965 ; 2912
(CN)	1598	1643	1642	1639	1644
(C=C)	1598	1609	1586	1544	1595
(C=S) (deformation)	-	836	857	855	855
(C=O)	1661	-	-	-	-

This table shows in most cases bands with shoulders. This may suggest the presence of two products from the two isomers of citral (neral and geranial).

The values of the vibrational frequencies of the products are similar to those found in the literature Agrawal et al. [39]. In general, the vibrational frequencies of NH bonds of the NH_2 group are the highest. For each group we have two NH_2 vibrational frequencies because the environment of each NH bond is different from each other. In the case of 4-methyl-3-thiosemicarbazone and 4-phenyl-3-thiosemicarbazone, the second value NH_2 disappears because the

hydrogen is replaced by methyl or phenyl. The vibrational frequencies of the following are the secondary bond NH. They are between 3280 cm^{-1} and 3165 cm^{-1}.

The substitution of hydrogen by methyl N (11) leads to the absence of vibration frequency in the 2-methyl-3-thiosemicarbazone. The frequencies of vibration of the CH_3 groups are also present and are between 3028 cm^{-1} and 3158 cm^{-1}. Those of the methylene present each time the value of the either groups. The carbonyl (C = O) of the semicarbazone indicates a vibration frequency of 1661 cm^{-1} which is absent in thiosemicarbazones having rather different at the C = S frequency of deformation of thioamides between 836 cm^{-1} and 857 cm^{-1} [18].

II.^{1}H-NMR spectrum

The chemical shifts of ethylenic protons are between 5.9 ppm and 4.8 ppm. The radical methyls displacements are between 1.8 ppm and 2.3 ppm. Protons of the group N-NH displacements are between 9.7 ppm and 10.4 ppm. Protons of the group NH_2 displacements are between 5.9 ppm and 9.1 ppm. Table 3 gives the different chemical shifts in ^{1}H NMR of the citralsemicarbazone and the citralthiosemicarbazones.

Table 3.^{1}H NMR Chemical Shifts of the citralsemicarbazone and citralthiosemicarbazones.

Attributions	^{1}H NMR Chemical Shifts of the semicarbazone and thiosemicarbazones				
	1	**2**[16]	**3**	**4**	**5**
N-NH(11) (1H) (s)	9,9	10,4		9,7	10,4
CH(1)=N (1H) (d)	7,7	7,9	7,6	7,8	8
NH_2(13) (2ou1H) (s)	5,9	7,1 ; 7,2	7,2 ; 7,6	7,2	9,1
CH(2)=C (1H) (d)	5,9	5,8	5,9	5,8	6,0
CH(6)=(1H) (t)	5,2	5,2	5,2	5,1	5,1
CH_2(4) (2H) (m)	2,3	2,2	2,2	2,1	2,2
CH_2(5) (2H) (t)		1,9	1,9	1,8	1,8
CH_3(10) (3H) (d)	1,8	1,6	1,6	1,6	1,6
CH_3 (8 ; 9) (6H) (s)	1,7	1,5	1,5	1,5	1,5
N-CH_3 N(11)(s)			3,7	3,2	

H(n) = hydrogen number n, (s) =singlet, (d) =doublet, (m) = multiplet

The chemical proton shift of HN (11) of the secondary amine is higher in all cases. This value is justified by the fact that the nitrogen bearing the hydrogen is found between two electron-withdrawing groups (C = O, C = S). As might be expected 2-methyl-3-thiosemicarbazone has no chemical shift in this area since H is replaced by CH_3. The two hydrogens of the terminal nitrogen (NH_2 group) indicate two different chemical shifts. These protons are different because of their different environments. The chemical shifts of ethylenic protons are between 5.9 ppm and 4.8 ppm. The radical methyls displacements are between 1.8 ppm and 2.3 ppm. The methyl groups which substitute the hydrogens on nitrogen atoms are strongly deshielded and are found at 3.2 ppm and 3.7. ppm [18].

III.*^{13}C-NMR spectrum*

The chemical shifts of ethylenic carbons are found between 141 and 120 ppm, the methylene allylic 40-30 ppm and methyl esters 26-17 ppm. The chemical shifts of the semicarbazone and thiosemicarbazones in ^{13}C NMR spectra are shown in Table 4.

Table 4. Chemical Shifts of the semicarbazone and thiosemicarbazones.

Attributions	^{13}C NMR chemical Shifts of the semicarbazone and thiosemicarbazones				
	1	**2**	**3**	**4**	**5**
C(12)=O ou C(12)=S	158,34 ; 158,23	177,53	181,06	177,85	175,09 ; 174,97
C(1)=N	147,17 ; 147,05	151,30 ; 151,23	149,96 ; 149,86	150,17 ; 150,02	151,33 ; 151,22

The peak corresponding to each carbon atom is often split. This confirms the existence of the semicarbazone and thiosemicarbazones of the two isomers of citral.

3.5. Pharmacology

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 <500.26 [48]. A compound that fulfills at least three out of the four criteria adheres to Lipinski's rule. Table 5 lists such properties of the nine trypanocidal compounds.

Table5. Compounds Physical Properties Compatible with Reasonable Pharmacokinetics and Drug Availability.

	Molecular weight	*C*log *P*	No. of H bond donors	No. of H bond acceptors	No. of criteria met
Rule	< 500	< 5	< 5	< 10	at least 3
1	209	3.62	3	4	all
2	225	3.36	3	3	All
3	239	3.72	2	3	All
4	239	4.286	2	3	All
5	301	5.455	2	3	3

All the molecules obey the rule of Lipinsky. So they may have excellent pharmacological properties. Reason why it was planned to test them on Trypanosomabruceibrucei and shrimp larvae.

a)Anti-trypanosomal test

The IC_{50} values determined in this work are in Table 6.

Table 6.Antitrypanosomal activities of the compounds.

Compounds	IC_{50} (µM)	Activities
Semicarbazonecitral**1**	234,64	Little
Thiosemicarbazonecitral**2**	7,61	Trypanocidal
2-méthyl-3-thiosemicarbazone citral **3**	60,87	Moderate
4-méthyl-3-thiosecarbazone citral **4**	172,84	Little
4-phényl-3-thiosemicarbazone citral **5**	1,96	Trypanocidal

To our knowledge, this study has never taken place with substituted citralthiosemicarbazones we synthesized. In descending order of activity, there are the compound **5** (1.96 µM), **2** (7.61 µM), **3** (60.87 µM), **4** (172.84 µM) and **1** (234.64 µM).

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 [37,38].

This work allows us to classify the compounds **5** and **2** as trypanocidal, compound **3** as moderate and trypanosomal agent compounds **4** and **1** as having a low or no activity on *Trypanosomabruceibrucei* (Table 6).

The thiosemicarbazones are generally more active than the semicarbazones [26-28]. This is confirmed by the results of our work: the semicarbazone of citral (IC_{50} = 234.64 µM) is significantly less active than all the thiosemicarbazones studied (Table 6).

a) Toxicity test

LC_{50} of different compounds are giving in table 6.

Table 7. Toxicity Test of compounds.

Compounds	LC_{50} (µM)	Toxicity Activities
Semicarbazonecitral1	420.39	No toxic
Thiosemicarbazonecitral2	390.22	No toxic
2-méthyl-3-thiosemicarbazone citral 3	‘’	‘’
4-méthyl-3-thiosecarbazone citral 4	275.36	No toxic
4-phényl-3-thiosemicarbazone citral **5**	70.70	toxic

The toxicity test on larval shrimp (*Artemiasalina*) was performed. In ascending order of toxicity was: the citralsemicarbazone (LC_{50} = 373.68 µM), citralthiosemicarbazone (LC_{50} = 347.68 µM), citral 4-methyl-3-thiosemicarbazone (LC_{50} = 326.77 µM) and finally citral 4-phenyl-3-thiosemicarbazone (LC_{50} = 70.70 µM). Compared to lapachol (LC_{50} = 281µM) known as a reference compound [49], 4-phenyl-3-thiosemicarbazone is the only compound toxic synthesized in this work. The most trypanocidal product is also the most toxic. Moreover, the literature shows a good correlation between the toxicity on the larvae of shrimp and anticancer activity. It indicates that the most toxic is also cytotoxic to some human tumors [49,50]. 4-phenyl-3-thiosemicarbazone could have a good anticancer activity.

From the valuesof the twopharmacological tests, the selectivity of those compounds can be determined by calculating of their selectivity index (SI = LC50 larvae / IC50 parasite). If the IS value obtained is greater than unity, the test compound is considered to be selective on the parasites. However, if IS is less than unity, the test compound is more toxic than anti-parasitic [51]. Therefore, the index of selectivity of all synthesized compounds was calculated (Table 8).

Table 8.Selectivity index of synthesized compounds.

Compounds	LC_{50}µM	IC_{50} µM	Selectivity index ($SI = LC_{50}/IC_{50}$)
1	420.39	234,64	1.79
2	390.22	7,61	51.27
3			
4	275.36	172,84	1.59
5	70.70	1,96	36.07

Productshemi-synthesizedin particular products**2** and **5**are very selectiveon theparasitesof *Trypanosomabruceibrucei*,sincetheirselectivity indexaregreater than unity.

4. CONCLUSION

Extraction and analysis of leaves of *Cymbopogoncitratus* showed that citral is the major component. This citral without firstly being isolated is used *in situ* for hemi-synthesis of citralsemicarbazone**1**, thiosemicarbazone**2**, 2-methyl-3-thiosemicarbazone **3**, 4-methyl-3-thiosemicarbazone **4** and 4-phenyl-3-thiosemicarbazone **5**. The structures of these molecules are confirmed by IR, ^{1}H and ^{13}C NMR spectrometers.

These molecules were tested *in vitro* on *Trypanosomabruceibrucei*. The inhibitory effect is stronger with 4-phenyl-3-thiosemicarbazone **5** (IC_{50} = 1.96 µM) and thiosemicarbazone**2** (IC_{50} = 7.6 µM). 2-methyl-3-thiosemicarbazone **3** showed a moderate inhibition (IC_{50} = 60.87 µM). Unlikely, semicarbazone (IC_{50} = 234.64 µM) and 4-methyl-3-thiosemicarbazone (IC_{50} = 172.84 µM) indicate a low activity. Toxicity tests on larvae shrimp indicated that only 4-phenyl-3-thiosemicarbazone is the most toxic compound LC_{50} = 70.70 µM. So, it could have a good anticancer activity. Citralthiosemicarbazone is the best product because it is trypanocidal and little toxic.

The pharmacological properties of the essential oil of *Cymbopogoncitratus* are already largely developed in the literature. The hemi-synthesis performed through our research work; without doubt, contributes more to value this oil in the field of therapy.

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