African Journal of Pharmacy and Pharmacology Vol. 7(7), pp. 360-367, 22 February, 2013
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.600
ISSN 1996-0816 ©2013 Academic Journals

Full Length Research Paper

Chemical composition, antioxidant activity and cytotoxicity of the essential oils of the leaves and stem of *Tarchonanthus camphoratus*

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Accepted 4 January, 2013

The aim of this study was to investigate the chemical composition, antioxidant potential and cytotoxicity of the essential oil of the fresh leaf, dry leaf and dry stem of *Tarchonanathus campharatus.* The antioxidant activity of the oils were examined by the 1,1-Diphenyl-2-picryl-hydrazil (DPPH), 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$), nitric oxide radical scavenging and reducing power methods. The brine shrimp lethality test and the MTT cytotoxicity test were used to investigate the cytotoxicity of the oils. Sesquiterpene hydrocarbons are the major components in the essential oil of the fresh leaves (36.46%) and of the dry leaves (59.18) whereas an aldehyde, butanal (35.77%) is the major component in the essential oil of the dry stem. The oils did not show significant antioxidant activity as evidenced by their high LC_{50} values in all the antioxidant assays. The cytotoxicity results indicated that the oils had low toxicity with LC_{50} values ranging from 400 to 900 µg/ml and 400 to 1100 µg/ml for the brine shrimp lethality test and MTT cytotoxicity assay respectively.

Key words: *Tarchonanthus camphoratus*, essential oil, antioxidant activity, cytotoxicity.

INTRODUCTION

In living systems, free radicals are constantly generated and when in excess they can cause extensive damage to tissues and biomolecules leading to various disease conditions, especially degenerative diseases, and extensive lysis (Halliwell and Gutteridge, 1998). The interest in antioxidants has been increasing because of their high capacity in scavenging free radicals which are related to various diseases (Silva et al., 2007). The most commonly used synthetic antioxidants; butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propylgallate (PG) and test butylatedhydroquinone have been reported to cause liver damage and carcinogenesis (Sherwin et al., 1990). There is growing interest in natural antioxidants present in medicinal plants that might help attenuate oxidative damage (Silva et al., 2005; Muhammad et al., 2012). The health promoting effects of plants were found to be due to bioactive substances such as essential oils, flavonoids and phenolic compounds which have antioxidant activity (Liu, 2003; Komal et al., 2012).

Tarchonanathus campharatus L., (family Asteraceae) is a shrub of rarely more than six meters in height with a greyish appearance and occurs in a wide range of habitats (van Wyk et al., 1997). The strongly scented tree of *T. campharatus* has many medicinal applications in

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traditional healing mainly by smoking from burning leaves or by drinking infusions or decoctions. Traditionally, infusions and tinctures of the leaves are used for stomach trouble, headache, toothache, asthma, bronchitis, inflammation, rheumatism, venereal diseases, indigestion, heartburn, coughs, paralysis and cerebral haemorrhage (Hutchings et al., 1994; Anthony, 1999). The plant also shows powerful insect repellent action (Omolo et al., 2004; Essential oil newsletter, 2005).

In this study, the chemical composition, antioxidant potential and the cytotoxicity of the essential oils of the leaves and stem of *T. camphoratus* were investigated in order to find out their suitability as raw materials in food, pharmaceutical and industrial products.

MATERIALS AND METHODS

Plant material

Fresh materials of *T. camphoratus* were collected from Sangoyana in the northern part of Kwa-Zulu Natal province, South Africa during the month of March, 2010. The plant was identified by the local people during the time of collection and further identified by Mrs N.R Ntuli in the Department of Botany, University of Zululand. A Voucher specimen, (NSKN 1), was deposited at the University of Zululand herbarium. The fresh plant material was separated into leaves and the other part with leaves still attached to the stem was dried at room temperature.

Extraction of the essential oil

The fresh leaves, dry leaves and the dry stem were subjected to hydro-distillation using a Clevenger-type apparatus. The essential oils were collected 4 h after boiling, weighed and kept at 4^{o}C in sealed glass vials before analysis and bioassay.

Gas chromatography-mass spectrometry analysis

The GC-MS analysis was carried out using an Agilent 6890 GC with an Agilent 5973 mass selective detector [MSD, operated in the EI mode (electron energy = 70 eV), scan range = 45 to 400 amu, and scan rate = 3.99 scans/sec], and an Agilent ChemStation data system. The GC was equipped with a fused silica capillary HP-5 MS column of an internal diameter of 0.25 mm, film thickness 0.25 µm and a length of 30 m. The initial temperature of the column was 70°C and was heated to 240°C at a rate of 5°C/min. Helium was used as the carrier gas at a flow rate of 1 ml/min. The split ratio was 1:25. Scan time was 50 min with a scanning range of 35 to 450 amu. A 1%, w/v, solution of the samples in hexane was prepared and 1 µL was injected using a splitless injection technique.

Identification of components

The identification of the oil constituents was based on their retention indices determined by reference to a homologous series of *n*-alkanes (C_8-C_{30}), and by comparison of their mass spectral fragmentation patterns with those reported in the literature (Joulain and Koenig, 1998; Adams, 2007) and stored in the MS library [NIST database (G1036A, revision D.01.00)/ChemStation data system (G1701CA version C.00.01.080)]. The percentages of each component are reported as raw percentages based on the total ion current without standardization.

DPPH radical scavenging assay

50 µL of various dilutions of the essential oil (50 to 250, µg/ml) were mixed with 5 mL of a 0.004% methanol solution of DPPH and incubated for 30 min. The absorbance was measured against a corresponding blank at 517 nm. BHT (Sigma) was used as a positive control. Inhibition percentage of free radical DPPH was calculated in the following way:

% DPPH radical scavenging = [(control absorbance – sample absorbance)/control absorbance)] × 100

ABTS [2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation scavenging assay

The ABTS radical cation was generated by mixing 7 mM of ABTS solution with 2.45 mM. Potassium persulfate was left to stand in the dark for 16 h at room temperature. 1 ml of ABTS was added to 1 ml of different essential oil concentrations (25 to 250 µg/ml) and absorbance of the mixture was measured at 734 nm after 6 min.

% inhibition = [(Control absorbance – Sample absorbance)/Control absorbance)] × 100

Nitric oxide radical inhibition assay

The reaction mixture containing 2 ml sodium nitroprusside (10 mM), 0.5 ml phosphate buffer saline (pH 7.4) and 0.5 ml of different concentrations of the essential oil (50 to 250, µg/ml) or standard solution (ascorbic acid, 0.5 ml) was incubated at 25ºC for 150 min. Then 1 ml of sulfanilic acid reagent (0.33% sulfanilic acid in 20% glacial acetic acid) was added to 0.5 ml of reaction mixture for 5 min to complete diazotization and 1 ml naphthyl ethylene diamine dihydrochloride was added and allowed to stand for 30 min at 25ºC. The absorbances of these solutions were measured at 540 nm (Badami et al., 2005).

% Nitric oxide scavenged = [(Control absorbance – Sample absorbance)/Control absorbance)] × 100

Total reducing power

The reducing power was determined according to the method of Oyaizu (1986). Different concentrations, (25 to 250 µg/ml), of the essential oils in methanol (2.5 ml) were mixed with 2.5 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min followed by addition of 2.5 ml of 10% trichloroacetic acid (TCA) and centrifuged at 1000 rpm for 10 min. 2.5 ml of the mixture was mixed with 2.5 ml of deionised water and 0.5 ml of 0.1% ferric chloride and its absorbance measured at 700 nm against a blank. Ascorbic acid was used as the reference standard. To determine reducing power, 2.5 ml of the mixture was mixed with 2.5 ml of deionised water and 0.5 ml of 0.1% ferric chloride and its absorbance measured at 700 nm. Ascorbic acid was used as the reference standard. Higher absorbance of the reaction mixture indicates greater reducing power.

Brine shrimp cytotoxicity assay

The brine shrimps were hatched in sea water for 48 h at room

temperature. The nauplii (harvested shrimps) were attracted to one side of the vessel with a light source. The essential oil were prepared at 1000, 500, 100 and 10 µg/ml (each test in triplicates) in 0.02% Tween 80. The essential oil (0.5 ml) was introduced in a test-tube and sea water (4 ml) added. Ten shrimps per test tube were added for each concentration and made up to 5 ml with sea water.

Potassium dichromate was used as positive control. The negative control was 0.02% Tween 80 (5 ml). The surviving larvae were counted after 24 h and the percent deaths at each dose and positive control were determined.

Cytotoxicity analysis by the MTT assay

The MTT assay was done using two cell lines, human embryonic kidney cells and human hepatocellular carcinoma cells. The cells were grown to confluenecy in 25 cm^3 flasks. This was then trypsinised and plated into 48 well plates at specific seeding densities. Cells were incubated overnight at 37°C. The medium was removed and fresh medium (MEM + Glutmax + antibiotics) was added. Extracts (50 to 100 µg) were added in triplicate and incubated for 4 h. The medium was again removed and replaced by a complete medium (MEM + Glutmax + antibiotics + 10% Fetal bovine serum). After 48 h the cells were subjected to MTT [3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide] assay. Briefly, the medium was removed from the cells and 200 µl of 5 mg/ml MTT in phosphate buffered saline (PBS) as well as 200 µl of medium were added to each well containing cells. The multiwell plate was incubated for 4 h and thereafter the medium and MTT were removed and 200 µl of DMSO was added to each well and incubated at 37°C for 10 min. Absorbance of the dissolved solutions were read using a Mindray Plate Reader at 570 nm. The cytotoxicity was calculated after comparing with the control. The control consisted of cells without the extract.

Statistical analysis

Results of antioxidant activity are presented as means ± SD of three measurements. Data were evaluated through regression analysis using QED statistics program and IC_{50} values, where applicable, were determined by linear regression. Means between treatments were compared by Tukey's Studentized Range Test using one way ANOVA.

RESULTS AND DISCUSSION

Chemical composition

Yellowish green oils with yields of 0.14, 0.09 and 0.03% were obtained from the fresh leaves, dry leaves and dry stem of the plant respectively. The oils gave a total of 33, 27 and 25 identified compounds representing 85.9, 73.01 and 72.66% of the total oil composition from the fresh leaves, dry leaves and dry stem respectively (Table 1). The major compounds in the oil of the fresh leaves were; α-cadinol (9.40%), 1,8 cineole (9.19%), δ-cadinene (6.89%), butanal (6.10%) and caryophyllene oxide (4.21%) while in the dry leaf oil were; β-guaiene (10.7%), γ-cadinene (9.09%), δ-cadinene (6.80%), aromandrene (6.12%), β-caryophyllene (5.48%) and γ-muurolene (5.13%). In the dry stem oil, butanal (35.77%), T-muurolol (10.33%) and δ-cadinene (5.54%) featured as the major compounds. Sesquiterpene hydrocarbons dominated the oils of the fresh and dry leaves.

DPPH radical scavenging assay

The radical scavenging activity of the essential oils of *T. camphoratus* was determined from the reduction in absorbance at 517 nm due to scavenging of the stable DPPH radical. DPPH is a stable nitrogen-centred free radical the colour of which changes from violet to yellow upon reduction by either the process of hydrogen or electron donation. The oils showed a relatively weak dose dependent inhibition of DPPH activity, with high LC_{50} of 12578.89, 9942.08 and 7010.03 µg/mL for fresh leaves, dry leaves and dry stem respectively (Table 2). The LC_{50} values of the oils were not comparable to that of the standard BHT at $p \leq 0.05$.

$ABTS^{\bullet+}$ radical scavenging assay

The ABTS radical cation is reactive towards most antioxidants and the decolorization of the $ABTS^{\bullet+}$ radical reflects the capacity of an antioxidant species to donate electrons or hydrogen atoms to inactivate this radical species (Re et al., 1999). The results for percent scavenging at different concentrations and LC_{50} values for the oils are shown in Table 3. There was a significant difference in the means of the oils and that of BHT at $p \leq 0.05$. The high LC_{50} values of the oils suggest poor $ABTS^{\bullet+}$ radical scavenging activity.

Nitric oxide (NO) assay

In this assay, the ability of the essential oils to counteract the oxidation of nitric oxide with oxygen and reduce the production of nitrite ions which act as free radicals was investigated.

Table 4 shows the %inhibition of nitric oxide generation by the essential oils and of the standard ascorbic acid. The activity of the standard ascorbic acid was more pronounced with LC_{50} value of 210.50 µg/ml when compared to LC_{50} values of the essential oils at $p \leq 0.05$.

Total reducing power

In the reducing power assay, the presence of antioxidants in the samples results in the reduction of Fe^{3+} to Fe^{2+} by donating an electron. The method evaluates the ability of plant extracts to reduce potassium ferricyanide solution which is monitored by measuring the formation of Perl's Prussian blue at 700 nm. Increasing absorbance at 700 nm indicates an increase in reductive ability. Figure 1 shows dose-response curves for the reducing powers of the essential oil. It was found that the reducing power

Table 1. Chemical constituents of the essential oils of the fresh leaves, dry leaves and dry stem of *T. camphoratus*.

Name of compound	Percentage composition			
	KI	FL	DL	DS
Monoterpene hydrocarbons		**9.57**	**1.61**	**0.00**
α- Pinene	938	2.52	0.45	
Camphene	952	2.15	0.33	
β- Pinene	978	0.65		
α-Terpinene	1017	2.61	0.65	
p-Cymene	1026	1.64	0.18	
Oxygenated monoterpenes		**11.79**	**6.26**	**0.79**
1,8-Cineole	1033	**9.19**	1.94	0.15
Linalool	1098	1.42	1.77	0.33
Camphor	1145		0.62	
(-)-Borneol	1169			0.07
Terpinene-4-ol	1180	0.56	0.43	0.08
(-)-α-Terpineol	1190	0.62	0.82	0.16
Carvacrol	1299		0.68	
Sesquiterpene hydrocarbons		**36.46**	**59.18**	**18.54**
α-Copaene	1378	1.45	2.33	1.21
Isocomene	1392			1.35
α-Elemene	1393		2.98	
Calarene	1403	2.02	3.60	
δ-gurjunene	1410	1.25		
(-)-Isoledene	1419	1.22	2.72	0.63
Beta-caryophyllene	1427	2.73	**5.48**	1.05
α-gurjunene	1436			0.13
α-Guaiene	1439		2.73	0.86
α-humulene	1461	0.64	0.97	0.29
γ-gurjunene	1472		0.43	
Aromandrene	1475	2.05	**6.12**	0.16
α-Muurolene	1476	3.50		1.35
γ-Muurolene	1480	3.19	**5.13**	2.11
Ledene	1482	0.93		
Germacrene D	1484			0.69
Eremophilene	1486	1.25	0.10	1.04
α-Selinene	1488	1.08		
Valencene	1491	2.39		
β-Guaiene	1500	1.20	**10.70**	
γ-Cadinene	1513	1.50	**9.09**	2.13
cis-calamenene	1520	3.17		
δ-Cadinene	1526	**6.89**	**6.80**	**5.54**
Oxygenated sesquiterpenes		**19.5**	**3.19**	**14.26**
Elemol	1549	**3.5**	2.76	
Spathulenol	1578		0.43	
Caryophyllene oxide	1580	**4.21**		2.90
Hinesol	1632	2.39		
t-Cadinol	1640			1.03
t-Muurolol	1642			**10.33**
α-cadinol	1650	**9.40**		

Table 1. Contd.

Others		**8.58**	**2.77**	**39.07**
Butanal	620	**6.10**	2.77	**35.77**
Isoaromadendrene epoxide	1579	1.5		
α-Costol	1801	0.98		
Hexadecanoic acid	2117			**3.30**

Table 2. DPPH radical scavenging assay of the essential oils from the fresh leaves, dry leaves and dry stem of *Tarchonanthus camphoratus.*

Concentration (µg/ml)	%scavenging activity			[d]BHT
	[a]Fresh leaf oil	[b]Dry leaf oil	[c]Dry stem oil	
25	0.00	0.00	0.00	57.47
50	0.00	3.13	4.87	82.03
100	7.73	9.00	7.50	92.87
150	11.13	13.47	10.85	94.80
200	15.90	16.67	14.20	96.80
250	18.47	19.75	26.55	99.10

a. Linear equation: y = 19.57X – 30.23 LC_{50} = 12578.89[a]
b. Linear equation: y = 19.82X – 29.23 LC_{50} = 9942.08[b]
c. Linear equation: y = 21.26X – 31.76 LC_{50} = 7010.03[c]
d. Linear equation: y = 38.77X + 9.81 LC_{50} = 10.88[d]

Table 3. $ABTS^{\bullet+}$ radical scavenging assay of the essential oils from the fresh leaves, dry leaves and dry stem of *Tarchonanthus camphoratus.*

Concentration (µg/ml)	%scavenging activity			[d]BHT
	[a]Fresh leaf oil	[b]Dry leaf oil	[c]Dry stem oil	
25	0.00	3.10	5.60	38.30
50	3.80	14.90	18.70	65.00
100	7.60	20.20	23.70	80.00
150	10.30	23.60	25.40	82.00
200	13.80	27.10	29.40	85.00
250	17.60	29.60	32.70	88.20

a. Linear equation: y = 18.81X – 29.16 LC_{50} = 16158.45[a]
b. Linear equation: y = 20.87X – 21.05 LC50 = 2539.42[b]
c. Linear equation: y = 19.01X – 14.23 LC50 = 2391.93[c]
d. Linear equation: y = 31.55X + 13.32 LC50 = 14.54[d]

of the essential oil of the dry stem, fresh and dry leaves of *T. camphoratus* was much lower than that of the standards, ascorbic acid and BHT.

Cytotoxicity assay

The LC_{50} values of the essential oil of the fresh leaves, dry leaves and dry stem were 889.0, 676.8, 442.9 µg/ml respectively and for the standard, potassium dichromate, 3.44 µg/ml (Table 5). There was a significant difference in the means of percent mortality of the essential oils and of the standard, potassium dichromate ($p \leq 0.05$). There was no significant difference in the activity of the essential oils against the brine shrimps ($p \leq 0.05$). The MTT assay is a well established method to assess mitochondrial competence (Freshney, 2000). Using this assay, we assessed the ability of the essential oils of the fresh leaf, dry leaf and dry stem to suppress mitochondrial respiration in human embryonic kidney cells and human hepatocellular carcinoma cells. The LC_{50} values of the essential oils used in this study in both cell lines were above 100 µg/ml (Table 6). The results revealed that there was no significant difference, ($p \geq 0.05$), in the

Table 4. Nitric oxide scavenging activity of the essential oils from the fresh leaves, dry leaves and dry stem of *Tarchonanthus camphoratus.*

Concentration (µg/ml)	% scavenging activity			[d]BHT
	[a]Fresh leaf oil	[b]Dry leaf oil	[c]Dry stem oil	
125	6.88	5.76	11.58	41.56
250	11.48	5.94	24.15	50.49
500	13.07	6.71	26.68	68.40
750	19.17	6.88	27.14	73.80
1000	24.74	27.51	28.95	89.30
1250	32.46	39.42	36.52	89.80

a. Linear equation: y = 22.71X – 43.22 LC_{50} = 12729.16[a]
b. Linear equation: y = 19.79X – 27.49 LC_{50} = 8735.82[b]
c. Linear equation: y = 27.77X – 59.45 LC_{50} = 8234.05[c]
d. Linear equation: y = 50.92X – 68.30 LC_{50} = 210.50[d]

Table 5. Inhibitory effects of the essential oils from the fresh leaves, dry leaves and dry stem of *Tarchonanthus camphoratus* on brine shrimps.

Concentration (µg/ml)	% inhibition			[d]Potassium dichromate
	[a]Fresh leaf oil	[b]Dry leaf oil	[c]Dry stem oil	
10	0.00	0.00	0.00	93.33
100	6.67	10.00	13.33	100.00
500	33.33	36.67	43.33	100.00
1000	66.67	70.00	76.67	100.00

a. Linear equation: y = 30.14x – 38.88 LC50 = 889.0[a]
b. Linear equation: y = 31.79x – 39.98 LC50 = 676.78[b]
c. Linear equation: y = 35.35x – 43.55 LC50 = 442.99[c]
d. Linear equation: y = 3.31x + 91.13 LC50 = 3.44[d]

Table 6. Cytotoxicity of the essential oils from the fresh leaves, dry leaves and dry stem of *Tarchonanthus camphoratus* on human embryonic kidney cells and hepatocellular carcinoma cells.

Plant parts	LC_{50}	Concentration (µg/ml)
	Hepatocellular carcinoma cells	Embryonic kidney cells
Fresh leaves	774.80	1042.15
Dry leaves	438.39	708.28
Dry stem	356.07	438.44

The LC_{50} values were expressed as the mean ±S.D determined from the results of MTT assay in triplicate experiments.

action of the essential oils of the dry stem, fresh and dry leaves on each of the human cells used.

DISCUSSION

Antioxidant

In all the antioxidant assays carried out in this study, the essential oils of the dry leaves, fresh leaves and dry stem of *T. camphoratus* showed poor antioxidant activity. A good correlation between the phenolic content in plants and their antioxidant activity has been reported (Tawaha et al., 2007; Othman et al., 2007; Nadeem et al., 2012). Essential oils rich in monoterpene hydrocarbons have also been reported to have high antioxidant activity (Tepe et al., 2005). The poor antioxidant activity of these essential oils, probably, is due to their lack of phenolic contents and low concentrations of monoterpene hydrocarbons. However, the low values of antioxidant and reducing power may not imply low medicinal value.

Emerging trends in antioxidant research point to the fact

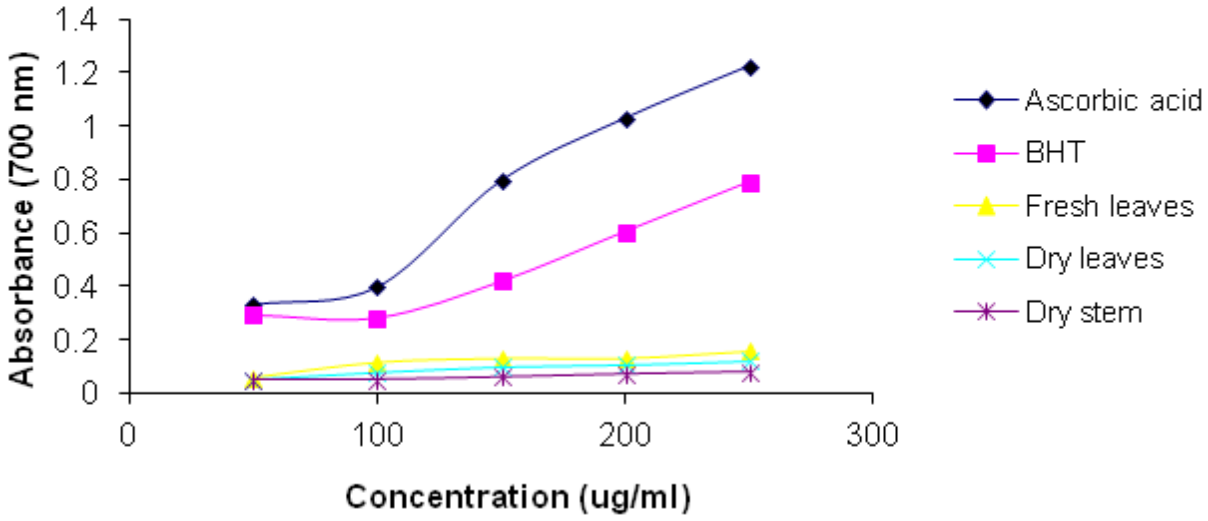

Figure 1. Total reducing power of the essential oils of the fresh leaves, dry leaves and dry stem of *Tarchonanthus camphoratus.* Ascorbic acid and BHT were used as the positive control.

that low levels of phenolics and other phytochemicals plus low value of antioxidant indices in plants do not translate to poor medical properties (Makari et al., 2008; Nasir et al., 2011).

Cytotoxicity

One indicator of a toxicity of a substance is LC_{50} which is the amount of a substance that kills 50% of the test organisms. All the essential oils investigated in this study, were found to have LC_{50} values > 30 µg/ml. According to the American National Cancer Institute, the LC_{50} limit to consider for a crude extract promising for further purification to isolate biologically active (toxic) compounds should be lower than 30 µg/ml (Suffness and Pezzuto, 1990). Other authors suggest that oils and extracts from plants presenting LC_{50} values below 1000 µg/ml are known to contain physiologically active principles (Meyer et al., 1982). The essential oils investigated showed low + antioxidant activities but do have some physiologically active principles.

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