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Chemical compositions and cytotoxic activity of fruits essential oil from *Xylopia aethiopica* (Dunal) A. Rich

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Abstract
The present study was designed to determine the composition of the essential oil from *Xylopia aethiopica* powder dried fruits using GC and MS The principal constituents identified in the fruits oil were β-pinene (15.06%), α- pinene (7.38%), β-phellandrene (8.24%), and Naphthalene (4.59%).
The cytotoxicity of the volatile oil was evaluated *in vitro* using brine shrimp lethality test and using 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by using Vero cell line with different concentration (500, 250 and 125 µg/ml) in comparison to triton-x100 (the reference control) which verified the safety of the examined extract with an IC_{50} less 100 µg/ml.

Keywords: Chemical composition, Cytotoxicit, *Xylopia aethiopica*, Friuts.

1. Introduction

Xylopia aethiopica (Dunal) A. Rich (Annonaceae) is a valuable medicinal plant widely distributed in the West African rainforest from Senegal and to Sudan in Eastern Africa, and down to Angola in Southern Africa (Irvine, 1961; Burkhill, 1985) [13, 5]. Essential oils or their constituents are odoriferous substances from plants and are extensively used as medicinal products, in the food industry as flavours and in the cosmetic industry as fragrances (Evans, 2003) [9].

Almost every morphological part of the plant *Xylopia* is used in traditional medicine for managing various ailments including skin infections, candidiasis, dyspepsia, cough, fever, dysentery and female sterility (Burkhill, 1985; Faulkner *et al*., 1985; Irvine, 1961; Mshana *et al.,* 2000; Ghana Herbal Pharmacopoeia, 1992) [13, 5, 10, 23, 11]. *Xylopia aethiopica* is medicinal plant of great repute in Africa which produces a variety of complex chemical compounds. It is commonly known as "African pepper", "Ethiopian pepper" or "Guinea pepper" and locally known as "Komba". (Burkill, 1985) [5]. In Sudan and Nigeria, the fruits are used in cough medicines as well as a carminative and as spice (EL-Kamali *et al*., 2007; Oliver-Bever, 1986) [7, 26]. In Cameroon, *X. aethiopica* fruits are used in the treatment of cough, bronchitis, dysentery and female fertility (Tatsadjieu *et al*., 2003) [31].

The essential oil of spice tree "African pepper" dried fruits from Cameroon contains more than 100 identified volatiles, and the main components are Beta-pinene (18%), terpinen-4-ol (8.9%), sabinene (7.2%), alpha-terpineol (4.1%), 1,8-cineole (2.5%), mytenol (2.4%) and kaurane derivatives (4.2%) Jirovetz *et al*. (1997) [17]. Tatsadjien *et al*. (2003) [31]. Obtained alpha-phellandrene (7.1%), and trans-Beta-ocimene (3.1%). Aseku and Adeniyi. (2004) [2], extracted from fruits oil *X.aethiopica* monoterpenoids 1, 8-cineole (15.15%) and terpinen-4-ol (6.6%) are the most abundant compounds. Ekundayo, (1989) [8] reported that *X. aethiopica* fruits consist mainly of mono- and sesquiterpenoids with typical constituents being alpha-and Beta-pinene, myrcene, p-cymene, limonene, linalool and 1,8-cineole. Sesquiterpenes, elemol and guaiol and some other terpenes like p-mentha3, 8-diene and p-mentha-3, 8-triene were found in the essential oil of the fruit from the Republic of Benin (Ayedoun *et al*., 1996) [3].

The essential oil obtained by hydrodistillation from the fruits of Sudanese native *Xylopia aethiopica* (Annonaceae) was analysed by Gas chromatography-Mass spectrometry (GC-MS). Forty five compounds which constitute 97.43 % of the total oil were identified. The oil was dominated by monoterpene fraction which accounted for 78.58 % of the oil. The most abundant components of monoterpene hydrocarbons are alphapinene (11.36%), alpha-phellandrene (10.50 %), Beta-phellandrene (8.94%) and gamma- terpinene (3.19%). 4-isopropylbenzyl alcohol (16.67 %), $C_{10}H_{16}O$ (8.12%), 1, 8-cineole (5.28%) and $C_{10}H_{14}O$ (2.57) are the main constituents of oxygenated monterpens. Sesquiterpene hydrocarbons contains gamma-cadinene (11.11%), and copaene (0.95%) as main constituents while alpha-eudesmol (1.08%) is the most abundant oxygenated sesquiterpene. EL-Kamali and Adam (2009) [7]. The main constituents detected by Itmad *et al* (2010) [15]. Were β- pinene (6.10%), 4-terpineol (11.30%), α-terpineol (6.02%), 1, 8-cineole (5.42%), o-cymene (2.82%). In Chat and Cameroon major constituents were α- pinene(5.56%) β- pinene (24.6%), β-phellandrene (12.36%), α- phellandrene (7.16%), Olonjsakin *et al*. (2007) [27], and α- pinene(5.56%), β- pinene (24.6, 28.2, and 35.7%). (Issakou *et al*., 2014) [14]. From Togo α--pinene (23.6%), β-pinene (11%), sabinene (9.8%), germacrene D (8.3%) and 1, 8 cineole (8.2%). were detected by Koba and Sanda *et al*. (2008) [20]. In Cameroon major constituents were β -phellandrene+1, 8, cineole (31%) β –pinene (8%), α- pinene (3.4%). (Noudjou *et al.*, 2007) [25]. From Mali major constituents were β -pinene (19.1%), γ-terpinene (14.7%), pinocarveol (8.6%), p-cymene (7.3%) and myrtenol (4.3%). (Keita *et al.*, 2003) [19]. From Guinea the major constituents were β –pinene (37-40.5%), α- pinene (13.6-18.4%). Sabinene (7.1-7.6%) and 1, 8, cineole(6.8- 8.4%). (Tomi and Casanova., 1996) [32]. From Ghana the major constituents were Germacrene (25.1), β –pinene (20.6%), α-pinene(8%), and 1,8, cineole (7.4%). (Karioti and Hadjipavlou,

2004) [18]. From Ivorycost major constituent was, β –pinene (16.06- 20.56%). (N dri Konan. 2009) [24].

Ekundayo, (1989) [8] reported that *X. aethiopica* consisted mainly of mono and sesquiterpenoids with typical constituents being α- and β-pinene, myrcene, β-cymene, limonene, linalool, and 1, 8-cineole. Two new sesquiterpenes, elemol and guaiol (among other terpenes) were found in the essential oil of the fruits of *X. aethiopica* from the Republic of Benin (Ayedoun *et al.*, 1996) [3]. A number of diterpenes from the bark, fruits, and pericarp of *X. aethiopica* have been reported by many authors Faulkner *et al*. (1985) [10]. Rabunmi and Pieeru, (1992) [29]. Harrigan *et al*., (1994) [12]. The essential oil from the dried fruits has been well characterized with linalool, β -Trans-ocimene, α -farnesene, α-pinene, β-pinene, myrtenol, β-phellandrene, and 3-ethylphenol as the major volatile constituents. Tairu *et al.,* (1999) [30]. The *X. aethiopica* contains anonaceine, an alkaloid, and rutin, volatile aromatic oil and a fixed oil. The plant contains high amounts of copper, manganese, and zinc. (Iwu *et al.,* 1993) [16].

The main objective of this study is to determine; the chemical composition, and cytotoxic activity of the essential oil X. *aethiopica* dried fruits

2. Materials and Methods

2.1 Plant material

The fruits of *Xylopia aethiopica* were purchased from Alyhya Mole in October 2013 and it was identified and authenticated by the taxonomist Dr. Haider Abd alGader, Medicinal and Aromatic Plants and Traditional Medicine Research Institute (MAP TMRI), Khartoum, Sudan.

Method of extraction:

The oil of the tested *Xylopia aethiopica* fruits was obtained by hydro- distillation technique using Clevenger's apparatus. Hundred grams from plant materials were placed in a two liters round bottom flask and distilled water was added and mixed thoroughly. The contents of the flask were boiled gently for four hours until the volatile oil has been distilled. The crude volatile oil was transferred by means of a pipette into a separate brown glass bottle. Anhydrous sodium sulphate was added, agitated gently to absorb the water and the clear oil was decanted into brown glass bottle and kept in the refrigerator until needed for analysis.

GC/MS analysis was conducted using Shimadzu Q P2010 GC/MS (Japan) instrument equipped with reference libraries... The flow rate of helium as carrying gas was (1 ml/min). The temperature program consisted of 50 – 280 ºC, at rate of 8 ºC /min. MS were taken at ionization voltage 70 eV. Library search was carried out using Wiley GC/MS library. The individual identifications were made by the comparison of fragmentation patterns with those found in the library of the Mass spectrometer and literature (Adam, 2001) [1].

Brine shrimp assay:

Bioactivity of the extract was monitored by the Brine Shrimp Lethality Test (Meyer *et al*., 1982) [21]. Brine Shrimp lethality Bioassay was carried out to investigate the cytoxicity of oil. 50 mg of *Artemia salina* (Leach) eggs were added to a hatching chamber containing artificial Sea water (75ml). The hatching chamber was kept under an inflorescent bulb for 48h for the eggs to hatch into shrimp larvae. 20mg of essential oil and 20mg (DMSO) were separately completed to 2ml of sea water, then 500, 50, and 5µl of each solution was transferred into vials corresponding to 1000, 100, and 10 µg/ml, respectively. Each dosage was tested in triplicate. 10 larvae of *A. Salina* Leach (taken 48 – 72h after the initiation of hatching) were added to each vial. The final volume of solution in each vial was adjusted to 5ml with Sea water immediately after adding the shrimps. One drop of dimethyl sulphoxide (DMSO) was added to the test and control vials before adding the shrimps to enhance the solubility of test materials. LD_{50} values were determined at 95% confidence intervals by analysing the data on a computer loaded with a "Finney Programme." The concentration at which it could kill 50% larvae (LD_{50}) was determined (McLaughlin *et al.,* 1998) [22].

2.2 Cytotoxicity Screening

Microculture tetrazolium MTT assay was utilized to evaluate the cytotoxicity of the fruits of *X. aethiopica*.

Preparation of *X. aethiopica* oil Solutions:

Using a sensitive balance 5 mg of each extract was weighed and put in eppendorf tubes. 50 µl of DMSO was added to the extract and the volume was completed to 1 ml with distilled water obtaining a concentration of 5 mg/ml. The mixture was vortexed and stirred by magnetic stirrer to obtain a homogenous solution.

2.3 Procedure

The monolayer cell culture formed in the culturing flasks was trypsinized and the cells were put in centrifuging tube and centrifuged for 5 minutes separating the cells from the supernatant that flicked out. 1 ml complete medium was added to the cells and all the cell suspension was contained in a basin. In a 96- well microtitre plate, serial dilutions of each extracts were prepared. 3 duplicated concentrations for each extracts i.e. 6 wells for each of 8 extracts. All wells in rows A, B and C were used in addition to first 4 wells from each rows D, E and F. The first 2 wells of row G were used for the negative control and the first 2 wells of row H were used for the positive control Triton X. 20 µl complete medium pipetted in all wells in rows B, C and mentioned wells of rows E and F. Then 20 µl from each extracts were pipetted in rows A and B and first 4 wells of rows E and F. 20 µl taken from row B were pipetted and mixed well in row C from which 20 µl were taken and flicked out. The same was done from E to F. After that 80 µl complete medium were added to all used wells. Then adjusting the cell account to 3000 cell/well, 100 µl of cell suspension were added completing all wells to the volume 200 µl. Now, we have duplicated three concentrations 500, 250, 125 µg/ml for each extract. Then the plate was covered and incubated at 37 ºC for 96 hours.

On the fourth day, the supernatant was removed from each well without detaching the cells. MTT suspension stock (5 mg/ml) prepared earlier in 100 ml phosphate buffer solution (PBS) was diluted (1:3.5) in a culture medium. To each well of the 96-well plate, 50 µl of diluted MTT were added. The plate was incubated for further 4 hours at 37 ºC. MTT was removed carefully without detaching cells, and 100 µl of DMSO were added to each well. The plate was agitated at room temperature for 10 minutes then read at 540 nm using microplate reader. The percentage growth inhibition was calculated using the formula below:

$$\% \text{ cell inhibition} = 100-\{(Ac-At)/Ac\} \times 100$$

Where, At = Absorbance value of test compound; Ac =Absorbance value of control.

Statistical analysis:

All data were presented as means ± S.D. Statistical analysis for all the assays results were done using Microsoft Excel program. Student t test was used to determine significant difference between control and plant extracts at level of $P < 0.05$.

3. Results and Discussion

3.1 Chemical compositions of the essential oil of *Xylopia aethiopica* Fruits:

The extracted yield of essential oil of the fruits of *X. aethiopica* was 6% (Table 1). The chemical composition of *X. aethiopica* was analysed by GC/MS and seventy two compounds representing 93.82% of the total oil were identified and ten compounds representing only1.43% were unidentified Table (1). The major constituents were β-pinene (15.06%), 3Cyclohexen-1-01-, 4methyl-1-1(13.22%),β–phellandrene (8.24%), α-pinene (7.38%), Bicyclo [3.1.1] hept-2-en-6-ol,2,7,7(7.66%). Table (1) and Figure (1).

β-pinene appears like an important compound in the essential oil of *X. aethiopica* since it is present in oils from all African countires like Sudan (Elkamali and Adam 2009; Itimad *et al*., 2010) [7, 15], Benin (Poitou *et al.*, 1996) [28] Cameroon (Jirovetz *et al.,* 1997; Tat sadjien *et al*., 2003) [17], Nigeria (Asekeun and Adeniyi 2004; Olonisakin *et al*., 2007) [2, 27], Chad and Cameroon (Issakan *et al.,* 2014), Tago (koba and Sanda, 2008) [20], Mali (Keita *et al*., 2003) [19], Ghana (Karioti *et al*., 2004) [18] and Cameroon, (Noudjoui *et al*., 2007) [25], Ivory Cost (N'dni Konan, 2009) [24].

Upon comparing the composition of Sudanese oil with that of some other African origins, some variation was noted. The variations may be attributed to the fact that the quantity and quality of secondary metabolites like essential oil are greatly influenced by genetic factors, climatic conditions, soil and cultivation techniques.

Table 1: GC/MS analysis of *Xylopia aethiopica* Essential oil

R-Time	Name	Area %
3.558	.alpha. –Phellandrene	2.93
3.692	alpha.-Pinene	7.38
4.108	Camphene	0.22
4.267	Benzene, butyl-	0.04
4.425	trans-Verbenol	0.09
4.608	.beta. –Phellandrene	8.24
4.675	Beta. –Pinene	15.06
5.183	3-Carene	0.29
5.392	1,3 Cyclohexadiene, 1- methyl-4(1-1)	1.81
5.600	D-Limonene	1.09
6.850	1,3,6- Qctatriene, 3,7-dimethyl-(E)-	0.61
6.042	Bicyclo[3.1.1] hept -2-en-6-ol,2,7,7—t	7.66
6.350	1,4-Cyclohexadiene, 1-methyl-4-(1	2.96
6.867	Unidentified	0.18
7.958	- Cyclohexene, 1-methyl-4-(1-methyl	0.80
7.958	1,6 Octadine-3-ol,3,7-dimethyl-	0.44
7.667	7-Oxabicyclo[2.2.1] heptane, 1-meth	0.16
8.783	Benezene,(2-methyl-1-1-propenyl)-	0.08
8.067	2-Cyclohexen-1-ol, 1-methyl-4-(1-m	0.54
8.250	Thujone	0.06
8.583	5-Caranol, (IS,3R,5S,6R)	0.53
8.842	trans-Pinocarveol	1.03
9.950	Unidentified	0.08
9.242	Artemiseole	0.22
9.361	3-Cyclohexen-1-01, 4-methyl-1-1(1-m	13.22
9.658	Unidentified	0.41
9.817	P-menth-1-en-8-01	3.35
10.90	-Bicyclo[3.1.1]hept-2-ene-2-carboxal	0.61
10.083	1-4-Cyclohexadiene-1-methanol, 4-	0.90
10.167	Bicyclo[3.1.0]hexan-2-one, 5-(1-met	0.39
10.417	Benezenemethanol, alpha.,.alpha.,4-	0.20
10.667	(IR)_(-)- Myrtenal	0.76
10.750	2-Cyclohexen-1-one, 4-(1-methyleth	0.28
11.908	Cyclohexene, 4-ethenyl-4-methyl-3-	2.23
11.083	.alpha.-Cubebene	0.64
11.242	Unidentified	0.39
11.308	Unidentified	0.05
11.433	Ledol	0.02
11.483	1,4-Methanoazulene, decahydro-4,8	0.02
11.592	Ylangene	0.30
11.675	Copaene	1.07
11.783	1-Acetoxy-p-menth-3-one	0.08
11.983	1-Cyclohexene-1- carboxaldehyde,4	0.35
12.100	1H-Cyclopenta[1,3] cyclopropa[1,2]	0.23
12.192	Cyclohexxane, 1-ethenyl-1-1-methyl-2,	0.65
12.442	2-Caren-10-al	0.08
12.558	Unidentified	0.10
12.667	Benzenemethanol,4-(1-methyl-2	0.12
12.767	Naphthalene, 1,2,3,4,4a,5,6,8a-octal	0.23
12.850	Caryophyllene	0.28
12.950	1HCyclopenta[1,3] cyclopropa[1,2]	0.18
13.075	.gamma.-Elemene	0.59
13.150	Unidentified	0.13
13.258	Aristolene	0.09
13.417	Azulene,1,2,3,4,5,6,7,8-octahydro-1	0.15
13.542	Unidentified	0.05
13.867	Isoledene	0.22
13.992	1,6Cyclodecadiene,1-methyl-5-mel	1.20
14.158	Naphthalene, 1,2,3,4,4a,5,6,8a0Octal	4.59
14.575	1,5 Cyclodecadiene, 1,5-dimethyl-1-8-	0.36
14.808	1H-Cyclopropa[a]naphthalene	0.60
14.925	Naphthalene,1,2,3,5,6,8a-hexahydr	1.46
15.150	.alpha,-Cubebene	0.07
15.208	alpha,-Muurolene	0.16
15.392	Naphthalene, 1,2,3,4-tetrahydro-1,6	0.27
15.508	Unidentified	0.07
15.825	Cyclohexanemethanol,4-ethenyl-a	0.54
15.942	Longipinocarveol, trans-	0.16
16.042	Naphthalne,1,2-dihydro-1,1,6-trin	0.16
16.333	Unidentified	0,03
16.417	1-Naphthalenol, 1,2,3,4,4a,7,8,8a,	o.o6
16.483	9-Methoxycalamenene	0.11
16.583	Glaucyl alcohol	0.12
16.658	1H-Cycloprop[e]azulen-7-ol, decahy	0.50
16.725	Caryophyllene oxide	0.23
16.958	Bioallethrin	0.28
17.542	1H-Cycloprop[e]azulen-7-ol, decahy	0.47
18.525	Diethyl Phthalate	2.23
18.908	Cyclooctasiloxane, hexadecamethyl	0.11
19.467	2,2,7,7-Tetramethyltricyclo[6.2.1.0	0.06
24.108	1H-Naphtho[2,1-b]pyran, 3-ethenyl	0.69
24.892	Kaurene	0.28

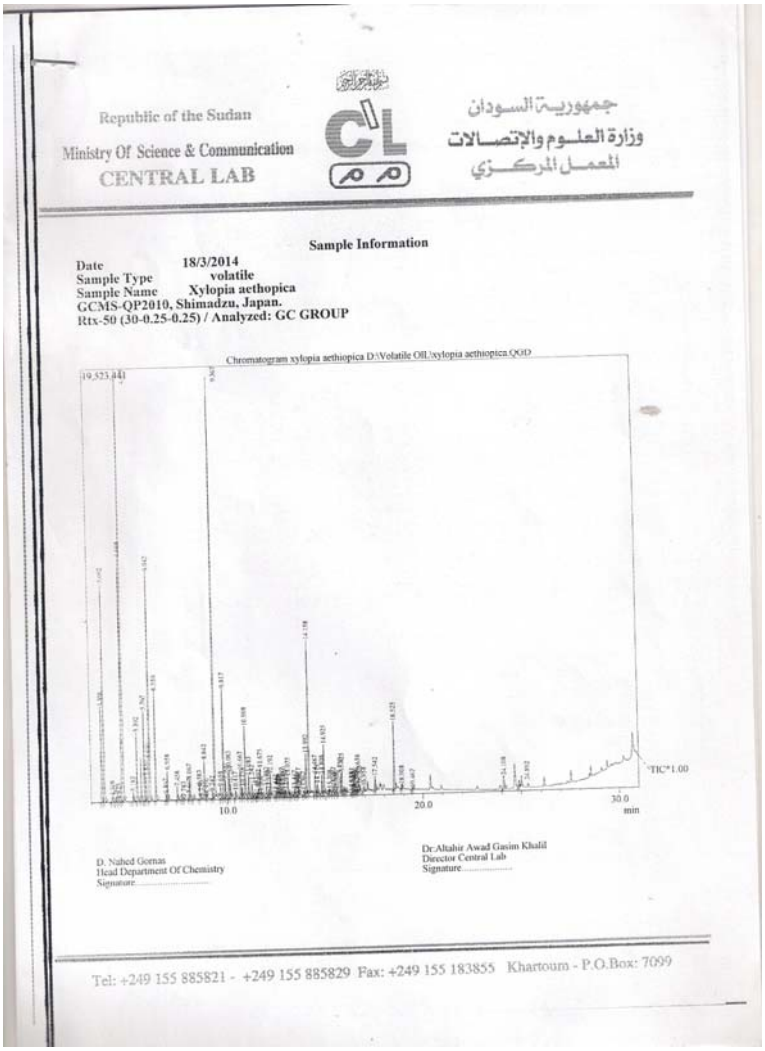

Fig 1: GC/MS analysis of Xylopia *aethiopica* Essential oil.

The cytotoxicity by Brine Shrimp Lethality Test:
LD_{50} of *X. aethiopica* was less than < 5.68 µg/ml and this is considerd as highly toxic according to Bussmann *et al*. (2011) [4]. They stated that LD_{50} values below 249 µg/ml are considered as highly toxic, 250– 499 µg/ml as medium toxicity and 500–1000 µg/ml as light toxicity. Values above 1000 µg/ml are regarded as non-toxic.

Table 2: The cytotoxicity of *Xylopia aethiopica* using Brine Shrimp Lethality tests

Name of Essential Oils	Total Number of Brine Shrimps	Concentrations (µg/ml.) Number of dead shrimps			Concentrations (µg/ml.) Number of survive shrimps			LD_{50}	The degree of toxicity
		1000	100	10	1000	100	10		
Xylopia aethiopica	30	24	25	24	6	5	6	<5.68µg/ml.	High toxic

Interpretation: LD_{50} <249 µg/ml: High toxic; 250-499 µg/ml: Medium toxicity; 500-1000 µg/ml Light toxicity; > 1000 µg/ml Non Toxic.
Cytotoxicity assay of *X. aethiopica*:
Oil of *X. aethiopica* a was found to be nontoxic against Vero cell line. They have IC_{50} > 100 mg/ml compared to the control "Triton-x100" which was highly toxic with IC_{50} < 30 mg/ml. The results from MTT-assay verified the safety of the examined oil. (Table3).

Table 3: The results of MTT-assay of Xylopia aethiopica

Name of Extract	Concentration (µg/ml)	Inhibition (%) ± SD	IC_{50} (µg/ml)	The degree of toxicity
Xylopia aethiopica	500	58.39±0.07	>100	Non-toxic
	250	50.80±0.11		
	125	36.84±0.32		
Ctrl + Ve		95.96±0.00	< 30	Highly Toxic

Key: IC_{50} <30 µg/ml: High toxic. *Control = Triton-x100 was used as control positive at 0.2 µg/ml. The maximum concentration used was 500 µg/ml. When this concentration produced less than 50% inhibition, the IC_{50} cannot be calculated. IC_{50} < 30 µg/ml: High toxic.

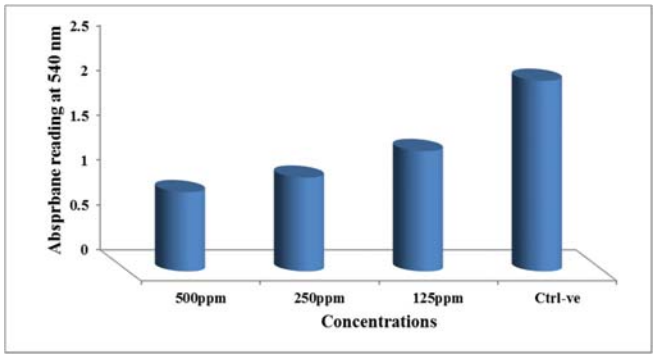

Fig 2: MTT reduction cytotoxicity assay for evaluation of *X. aethiopica* oil.

Conclusion

Although 72 compounds were identified by GC/MS but β-pinene seemed to be the most important compound in the essential oil of *X. aethiopica* as it is present in many African countries. The result of MTT assay verified the safety of the essential oil.

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