

## Full Length Research Paper

# Evaluation of anti-oxidant properties in essential oil and solvent extracts from *Tagetes minuta*

Kyarimpa Christine<sup>1\*</sup>, Isaiah Ndiege Omolo<sup>1</sup>, John David Kabasa<sup>2</sup>, Christine Betty Nagawa<sup>3</sup>, John Wasswa<sup>4</sup> and Cliff Richard Kikawa<sup>5</sup>

<sup>1</sup> Department of Chemistry, Faculty of science, Kyambogo University Uganda.

<sup>2</sup> College of Veterinary medicine, Makerere University Uganda

<sup>3</sup> Department of Forestry, Biodiversity and Tourism School of Forestry, Environment and Geographical Sciences College of Agricultural and environmental Sciences, Makerere University Uganda.

<sup>4</sup> Department of Chemistry, College of Natural Sciences, Makerere University, Uganda.

<sup>5</sup> Tshwane University of Technology(SA), Faculty of Science, Dept of Mathematics and Statistics. Arcadia Campus- Pretoria.

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**Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are toxic and carcinogenic, thus they induce DNA damage. This calls for the need to find alternative antioxidants from natural products. *Tagetes minuta* (Asteraceae) is an annual herb that belongs to the Asteraceae family. It is used in common medicine and grows in temperate regions of South America, some parts of Africa and Asia. Essential oil from *T. minuta* was obtained by hydrodistillation while solvent extracts were obtained using ethyl acetate and methanol. Antioxidative compounds of *T. minuta* were isolated both from the Ethyl acetate extract and the essential oil. It was done by determining the scavenging activity using 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) using thin layer chromatography (TLC). The active compounds were tested quantitatively for their radical scavenging activity using the U-1100 UV-VIS Spectrophotometer. The active fractions were isolated using TLC and High performance liquid chromatography (HPLC) and later detected using both Gas chromatography mass spectrometry (GC-MS) and Nuclear magnetic resonance (NMR). One pure active compound was obtained from the ethylacetate extract (neophytadene) by a combination of GC-MS and NMR. The essential oil contained a number of compounds among which are trans-ocimen 15.90%, I-verbanone 15% of limonene 8.02%, tagetone 3.56%, and 2-pinen-4-one 7.84%.**

**Key words:** Antioxidant activity, essential oil, 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH), *Tagetes minuta*.

## INTRODUCTION

An antioxidant is any substance, if present at low concentrations in combination with an oxidisable substrate, significantly delays or prevents oxidation of the substrate. Based on the historical success of natural

products, a number of medicinal plants have been evaluated for their antioxidant potential (Argolo et al., 2004; Burits et al., 2001; Helle et al., 2004).

Free radical mediated damage is connected with

\*Corresponding author. E-mail: xtineky@gmail.com, Tel: +256772561171.

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several diseases, and therefore its prevention can play an important role in the cure of those diseases (Kanwal et al., 2011; Kulisica et al., 2004; Sharma and Trivedi, 2002; Smith et al., 2007). For example, oxidative stress has been widely postulated to be involved in the development and progression of some chronic diseases such as cardiovascular disease, neuronal disease, cataracts, and several types of cancer (Gua et al., 2009). There is increasing search for antioxidants that remove occurring naturally in vegetables, fruits and functional herbs to replace synthetic antioxidants. It has been found out that some synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been revealed to be potentially toxic and carcinogenic, they have been found to induce DNA damage (Helle et al., 2004).

*Tagetes minuta* (Asteraceae) is an annual herb that belongs to the Asteraceae family. Its leaves are slightly glossy, green and pinnately dissected into 4–6 pairs of pinnae (Cerruti et al., 2010; Daizy et al., 2007). It is used in common medicine and grows in temperate regions of South America, some parts of Africa and Asia (Hamill et al., 2000; Kamatenesi-Mugisha and Oryem-Origa, 2007; Tabuti et al., 2003; Vasudevan et al., 1997a). Infusions of leaves from different species of *Tagetes* have been used to treat stomach and intestinal diseases (Gakuya et al., 2013; Harris et al., 2002; Paul and Kasenene, 2007; Tabuti et al., 2003; Vasudevan et al., 1997a), and other species have been found to possess different biological activities, such as, antimicrobial, antiinflammatory, antioxidant and antiviral (Andreotti et al., 2013; Dharmagadda et al., 2005; Hamil et al., 2000; Paul and Kasenene, 2007).

*T. minuta* is a wild shrub in Uganda that thrives mostly in the rainy season (Tabuti et al., 2003). *Tagetes* species, commonly known as marigold are also grown as ornamental plants and thrive in varied agro-climates (Vasudevan et al., 1997b). Bioactive extracts of different *Tagetes* parts exhibit nematocidal, fungicidal and insecticidal activity (Vasudevan et al., 1997b). *T. minuta* has been used by the local people in Uganda to relieve a number of ailments (Hamil et al., 2000; Paul and Kasenene, 2007).

Previous work on *Tagetes* species, *Tagetes maxima* revealed strong antioxidant properties of its ethylacetate extracts (Parejo et al., 2005). *T. maxima* was found to exhibit strong radical scavenging and antioxidant activities (Parejo et al., 2005). There is a great possibility of similar activity in other *Tagetes* species. Antioxidant activity of *T. minuta* from Uganda has not been determined according to literature, but since it belongs to the same family as other *Tagetes* species with strong antioxidant properties, it was necessary to determine its potential as an antioxidant.

In this research, antioxidative compounds of *T. minuta* were isolated both from the Ethyl acetate extract and the essential oil. It was done by determining the scavenging

activity using 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH). The active compounds were tested quantitatively for their radical scavenging activity.

## EXPERIMENTAL

### Plant material

Fresh aerial parts of *T. minuta* were collected from Mabira Forest in the morning hours in the month of November 2012. The sample was transported to Makerere University, Department of Chemistry Laboratory. Essential oils from *T. minuta* were extracted on arrival in the Laboratory. The remaining plant material was dried under shade for 3 weeks, ground in a mortar to obtain fine powder. A voucher specimen (CK001) was deposited at Makerere University Herbarium.

### Hydrodistillation

Essential oil from fresh *T. minuta* was extracted by hydro-distillation in a Clevenger type apparatus for 3 h with a separated extraction chamber. The resulting essential oils were dried over anhydrous-sodium sulphate to extract the water. The oil was kept in refrigerated conditions at 8°C prior to the antioxidant activity determination and GC-MS analysis (Conti et al., 2010; Polatoglu et al., 2012).

### Chemicals

All chemicals and reagents used in extraction, isolation and analysis of the active compounds were obtained from Sigma-Aldrich (Germany). These chemicals and reagents were of analytical grade. The standards were also purchased from sigma-Aldrich.

### Solvent extraction- Cold extraction

*T. minuta* dry powder (1000 g) was extracted four times with 2000 ml of ethyl acetate at 40 to 45°C. The supernatant (extract) was separated from the residue by paper filtration (Whatman No. 1 filter, whatman paper Ltd., UK). It was dried in vacuum using a rotary evaporator at 40°C to remove all the ethyl acetate to give a residue. The powder was dried and re-extracted three times with 2000 ml methanol. The extract was combined and evaporated at 40°C to dryness. Both methanolic and ethyl acetate extracts were kept in a dry place for further testing (Gua et al., 2009).

### DPPH assay

The capacities to donate hydrogen atoms/electrons by the essential oil and solvent extracts from the test samples were preliminarily detected using thin layer chromatography (TLC) and further measured spectrophotometrically.

### TLC screening for antioxidants

Dilutions of volatile oils (5 µl, 1:5 in hexane), and the crude extracts were spotted on silica gel sheets and developed in ethylacetate:hexane (7:3 v/v). The plates were sprayed with 0.2% solution of the stable radical, diphenylpicrylhydrazil (DPPH) (Brand Williams et al., 1995; Burits et al., 2001; Helle et al., 2004; Xiao et al., 2010). Active spots were detected as yellow spots on a purple

background. Zones where the colour changed within 30 min (after spraying) were taken as positive results (Burits et al., 2001).

#### DPPH spectrophotometric assay

This assay uses DPPH as a reagent (Argolo et al., 2004; Brand-Williams et al., 1995; Burits et al., 2001; Helle et al., 2004). 50 µl of various concentrations of the volatile oils were added to 5 ml of 0.004% methanolic solution of DPPH. After 30 min of incubation period at room temperature, the absorbance was read against the blank at 517 nm using a U-1100 UV-VIS Spectrophotometer (Hitachi Ltd; Tokyo Japan). The tests were carried out in duplicate. DPPH solution (1.0 ml; 0.3 mM) plus methanol (2.5 ml) was used as a negative control. After 30 min the absorbance values were measured at 517 nm and converted into the percentage antioxidant activity (AA) using the following formula:

$$\%AA, \text{ which was, } \%aa = ((A_{C(0)} - A_{A(t)}) / A_{C(0)}) * 100$$

Where  $A_{C(0)}$  is the AA for the control solution at  $t=0$  minutes, and  $A_{A(t)}$  is the AA after the given time intervals, for  $t = 5, 10, 15, 20, 25,$  and  $30$  min (Kulisica et al., 2004).

#### GC-MS analysis

GC-MS analysis was used to identify the compounds in the essential oil and solvent extracts that had antioxidant activity. The GC-MS results of *T. minuta* was already determined in a previous research (Kyarimpa et al., 2014).

#### High performance liquid chromatography

The active fractions were purified with HPLC. A Dionex Ultimate 3000 HPLC (Dionex) equipped with a diode array detector and operated by Chromeleon Version 6.80 SR9 software. 2.0 ml each of the active fractions was injected onto a 150 x 2.1 mm, 100 Å, 2.6 µm Phenomenex Kinetex C18-column at 35°C. N-Hexane was used as a mobile phase. The flow rate was 237 µl/min. To selectively detect antioxidants, the detector recorded the signal at 520 nm (Application Note 281, Dionex Corporation, Sunnyvale, CA, USA). HPLC was carried out only samples with antioxidant activity and the solvent system was chosen based on Rf values of the TLC experiments.

#### Nuclear magnetic resonance (NMR) spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is (arguably) the most powerful tool available for determining the structure of organic compounds. It is used to identify and/or elucidate detailed structural information about chemical compounds. In this case it was used to determine the structure of the pure active compound in the sample. All NMR spectra were recorded on a Bruker Avance II 400 (resonance frequencies 400.13 MHz for  $^1\text{H}$  and 100.63 MHz for  $^{13}\text{C}$ ) equipped with a 5 mm broadband observe probe head (BBFO) with z-gradients at room temperature with standard Bruker pulse programmes. The sample was dissolved in 0.6 ml of CDCl<sub>3</sub> (99.8% D). Chemical shifts are given in ppm, referenced to residual solvent signals (7.26 ppm for  $^1\text{H}$ , 77.0 ppm for  $^{13}\text{C}$ ).  $^1\text{H}$  NMR data were collected with 32k complex data points and apodized with a Gaussian window function ( $l_b = -0.3$  Hz and  $g_b = 0.3$  Hz) prior to Fourier transformation.  $^{13}\text{C}$ -jmod spectra with WALTZ16  $^1\text{H}$  decoupling was acquired using 64k data points. Signal-to-noise enhancement was achieved by multiplication of the FID with an

exponential window function ( $l_b = 1$  Hz). All two-dimensional experiments were performed with  $1\text{k} \times 256$  data points, while the number of transients (2–8 scans) and the sweep widths were optimized individually. The resulting FIDs were zero-filled to a  $2\text{k} \times 1\text{k}$  data matrix and apodized with a sine function for COSY in both the  $\omega_1$  and  $\omega_2$  dimensions prior to Fourier transformation.

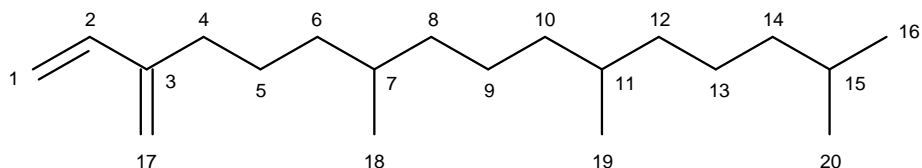
Heteronuclear spectra were zero-filled only in F1 to a  $1\text{k} \times 512$  data matrix, and apodized in both dimensions with a shifted sine function. The heteronuclear single quantum coherence (HSQC) experiment was acquired using adiabatic pulse for inversion of  $^{13}\text{C}$  and GARP-sequence for broadband  $^{13}\text{C}$ -decoupling, optimized for  $1J(\text{CH}) = 145$  Hz.

## RESULTS AND DISCUSSION

The essential oil from *Tagetes minuta* exhibited strong antioxidant activity on TLC. Some components of the crude extract were also found to be active (Figure 2). Track 1 on the first TLC plate from the left (Figure 2) was the essential oil, the other tracks were different extracts from *T. minuta* and *Tephrosia Vogelli*. It was noted that some of the components of the two plants had strong antioxidant activity. These fractions were isolated using Column Chromatography, HPLC. The pure fractions were analysed using NMR spectroscopy. Track 1 had very strong antioxidant activity as shown by the DPPH reaction on the TLC plate (Figure 2). This oil was later used for quantitative measurement again using DPPH and Ultra Violet Spectrophotometer (Table 1). The total antioxidant capacity revealed that the essential oil from *T. minuta* had a high antioxidant activity. Free radical scavenging activity of the extracts was assessed using the stable free radical DPPH. Plant extracts which reduce DPPH by donating hydrogen ions are considered as antioxidants having free radical scavenging activity. The results from Table 1, were used to calculate the amount of DPPH scavenged over a period of time according to the formula  $\{\%AA, \text{ which was, } \%aa = ((A_{C(0)} - A_{A(t)}) / A_{C(0)}) * 100\}$ , and the  $LC_{50}$  was determined (Appendix 1B). DPPH solution alone served as control (A0). It is evident from the study, that the investigated extracts and essential oil have the ability to quench free radicals. This indicates that *T. minuta* is a potential source of natural antioxidants.

#### Nuclear magnetic resonance (NMR) Spectroscopy

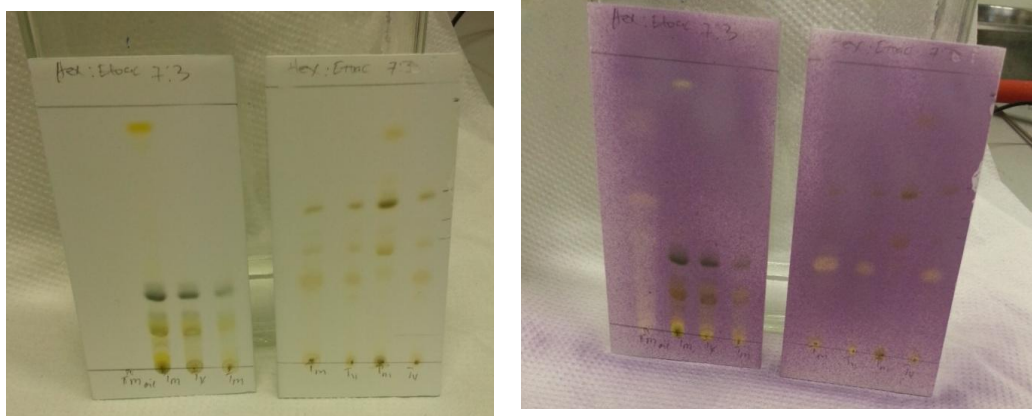
One active pure compound was identified using both the proton NMR and the carbon NMR as shown in Figure 1 and Table 2. The  $^1\text{H}$  NMR spectra of this compound revealed the presence of two isolated olefinic spin systems without any further coupling partners: on the one hand a vinyl group, indicated by its characteristic ABX-system at  $\alpha$  5.05 (d,  $J = 10.8$  Hz),  $\alpha$  5.23 (d,  $J = 17.6$  Hz) and  $\alpha$  6.37 (dd,  $J = 17.6, 10.8$  Hz), and on the other hand an olefinic methylene group with broad singulets at  $\alpha$  4.99 and  $\alpha$  5.00 ppm, respectively. Besides a triplett at



**Figure 1.** The structure of Neophytadiene

**Table 1.** Ultraviolet Spectroscopic results showing the effect of sample concentration on the absorbance of DPPH.

Conc. (g/L)	Absorbance (min)						
	0 (Control)	5	10	15	20	25	30
DPPH( Blank)	1.063	1.037	1.016	0.995	0.975	0.954	0.935
5	0.225	0.151	0.11	0.08	0.057	0.05	0.049
5	0.223	0.14	0.09	0.055	0.046	0.046	0.045
10	0.0132	0.046	0.034	0.034	0.034	0.035	0.035
10	0.191	0.086	0.045	0.045	0.045	0.045	0.045
20	0.179	0.066	0.056	0.055	0.055	0.055	0.055
20	0.206	0.077	0.059	0.058	0.059	0.059	0.06
30	0.134	0.069	0.076	0.081	0.085	0.086	0.085
30	0.156	0.082	0.074	0.064	0.053	0.042	0.032
40	0.16	0.06	0.063	0.066	0.069	0.07	0.072
40	0.166	0.057	0.063	0.063	0.065	0.064	0.062
60	0.118	0.051	0.053	0.053	0.049	0.041	0.032



**Figure 2.** TLC plate before (left) and after applying DPPH (right). The yellow spots on a purple background indicate antioxidant activity of the compounds at that particular spot.

$\alpha$  2.18 ppm, a bulk of aliphatic methylene and methine protons in the region between 1.60 to 1.00 ppm and In combination with the hsqc experiment the j-modulated  $^{13}\text{C}$  nmr spectra showed signals of 4 olefinic carbons – one quaternary, one methine, and two methylenes – 4 methyl, 3 methine and 9 methylene carbons. In addition to the molecular mass peak at  $m/z = 278$  these results indicated that compound is a noncyclic, nonoxidized diterpene with a molecular formula of  $\text{C}_{20}\text{H}_{38}$ . Extensive

signals of different methyl groups at around 0.90 ppm no more signals were found in the  $^1\text{H}$  NMR spectra. analysis of heteronuclear 2D NMR led to the elucidation of that structure which turned out to be neophytadiene, a widespread component of essential oils from different plant sources. Whereas Burkhardt et al. published only NMR data of the olefinic part of the molecule, we present here to our best knowledge for the first time the fully assigned nmr resonances (Table 2).

**Table 2.** NMR resonance of the fully assigned Neophytadiene.

	1H	13C
1 <sup>a</sup>	5.23, d, J = 17.6	113.02
1 <sup>b</sup>	5.05, br.d, J = 10.8	
2	6.37, dd, J = 17.6 + 10.8	139.07
3	-	146.69
4	2.18, m	31.70
5	1.49, m	25.63
6	1.00 - 1.30	36.99
7, 11	1.38, m	32.80 + 32.69
8, 10, 12	1.00 - 1.30	37.44 + 37.39 + 37.30
9, 13	1.00 - 1.30	24.80 + 24.46
14	1.15, m	39.38
15	1.53, m	27.99
16, 20	0.87	22.72 + 22.62
17	5.00, br.s	115.42
	4.99, br.s	
18, 19	0.85	19.75 + 19.74

Neophytadiene, is a fatty acid-related compound which plays an important part in competitive inhibition of cyclooxygenase or lipoxygenase in an inflammation reduction, resulting in decreased production of prostaglandins and leukotriene (Pillai and Nair, 2013).

## Conclusion

DPPH is a free radical, stable at room temperature, which produces a violet solution in methanol. It is reduced in the presence of an antioxidant molecule, giving rise to uncoloured methanol solutions. The use of DPPH provides an easy and rapid way to evaluate antioxidants. According to the results obtained from data in Tables 1 and 2 as well as those of statistical analysis, we can say that extracts from *T. minuta* as well as its essential oil possess strong antioxidant properties with an LC<sub>50</sub> of 1.49 g/l<sup>-1</sup> after 30 min as compared to other antioxidants reported in Parejo et al. (2005) and Xiao et al. (2010). A well-known antioxidant, ascorbic acid, was used as positive control. DPPH scavenging patterns for *T. minuta* versus time, along with IC<sub>50</sub> values, are presented in Appendix 1 A and B.

## Conflict of Interest

We declare no competing interests in this research.

## ACKNOWLEDGEMENT

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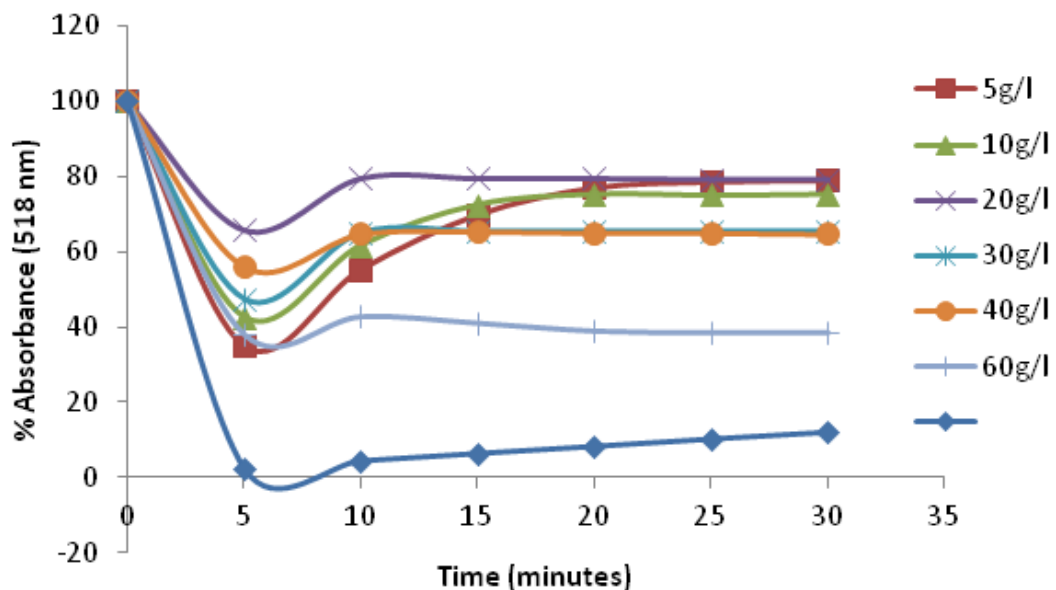
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**APPENDIX A:** Percentage absorbance of DPPH at different concentrations of the Essential oil of *Tagetes Minuta* vs time in minutes



We notice that radical scavenging or absorbance is inversely proportional to time.

**APPENDIX B:** LC<sub>50</sub> Values at Different time Intervals

SN	Duration (Minutes)	LC <sub>50</sub> g/l <sup>-1</sup>
	5	3.08
	10	1.87
	15	1.65
	20	1.55
	25	1.54
	30	1.49