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Antiproliferative, growth inhibitory and antibacterial activities of thymol isolated from the leaf of *Ocimum gratissimum* L.

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Abstract: *Ocimum gratissimum* is a medicinal plant used traditionally in Nigeria for the management of dysentery and cough. Data available on the antiproliferative and antibacterial activities of *Ocimum gratissimum* L are quite sketchy, though used for medicinal remedy. Therefore, the study was aimed at evaluating the cytotoxic and anti-proliferative effects of thymol isolated from the leaves of *Ocimum gratissimum* against tadpoles of *Raniceps ranninus* using a bench-top assay, and the growth inhibitory effect using *Sorghum bicolor* seeds; and antimicrobial effects. Fresh leaves of *O. gratissimum* were hydrodistilled to obtain a golden yellow essential oil which on column chromatography gave a crystalline compound identified as thymol. The cytotoxic and anti-proliferative effects of the isolated thymol against tadpoles of *Raniceps ranninus* were investigated using a bench-top assay, and a growth inhibitory assay was evaluated using *Sorghum bicolor* seeds. The antimicrobial activity was evaluated using broth micro-dilution assay. Thymol exerted cytotoxic and anti-proliferative effects against tadpoles of *Raniceps ranninus* and significantly inhibited *Sorghum bicolor* seed radicle length elongation indicative of its anticancer potential. The antimicrobial assay indicated thymol had pronounced activity with MIC and MBC values of 125µg/ml and 250µg/ml respectively.
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Keywords: *Ocimum gratissimum*, thymol, antimicrobial, essential oil, Antiproliferative, cytotoxic, antibacterial

1. Introduction

Natural products, like plants extract, open a novel frontier for the invention of potent curative agents (Cosa *et al*., 2006). It has been greatly observed that the appl ication of herbal formulations and traditional herbs is important in many developing countries as a curative basis for the sustenance of healthy life-style. Plants consist of a long range of components both chemical and biological that can be used to remedy severe as well as even infectious ailments (Saad *et al*., 2006). Herbal remedy is garnering attractiveness in emerging countries. Herbal medications are time and again are thought to be not hurtful since they are accepted and free negative consequence (Lopes *et al*., 2000). Study attentions have concentrated on countless herbs so as to retain antitumor, antiplatelet, hypolipidemic, or immune-stimulating characteristics that might be beneficial adjunct in facilitating to decrease the threat of cancer and cardio-vascular illness (Craig, 1999). Presently, at hand is an everlasting global green uprising which is chiefly premised on the conviction that traditional medicines are benign and less harmful to the individual body than artificial remedies (Parekh and Chanda, 2006).

Herbal drug shows essential role in sustaining the well-being and affluence of human, bulk of world populace utilise herbal treatments. The World Health Organization (WHO) rumors that about 21,000 vegetation has been utilised in favor of therapeutic purposes (Cathrine and Nagarajan, 2011). Herb has stood the investigation of time meant for their welfare, efficiency, social suitability and marginal side special effects (Chandan *et al*., 2011).

Natural products are crucial to health and well-being of human providing us with, amongst others, viable antibiotics including the penicillins and erythromycins (Baris *et al*., 2006). Isolation as well as structure elucidation of novel natural products involves applying a blend of spectroscopic method (NMR and MS) and a total synthesis as well as comprehending the biosynthetic pathways to these constituents continues to be an important target. There are a lot of plant foods which have very beneficial nutrition properties but have been given little or no attention from researchers. However, one

can best evaluate their importance in the nutritional well-being of our society by further learning about the protein, carbohydrate, mineral and fat content of a plant (Tanaka *et al*., 2006). This is needed to be able to inform public health service providers and nutritionists, the plant that can give the population these nutrients and plants that have been well thought-out to be a reliable supply of ascorbic acid, minerals as well as oil.

Natural products have occupied essential position as drugs source for agelong years and many potent drugs have been synthesized from phytochemicals (Hostettmann *et al*., 1996).

Plants have been regarded for many years, among the most dependable sources of medicaments. Biochemical compounds from herbal plants demonstrate their therapeutic activity (Holetz *et al*., 2002). It have been reported that the larger part of traditional medicines and therapy has to do with the application of plants extract as well as their active components. Bioactive compounds from plant sources have been of high premium to scientists researching on drugs. There has been increasing interest in understudying plants which possess activity against several disease pathogens (Dhir *et al*., 2002). The healthcare services of the larger spectrum of the rural communities in Kenya and most parts of Africa today, relies to a great extent on medicinal plants based on traditional health care delivery programs (Dhir *et al*., 2002). For their primary health care services, in accordance with WHO estimates, herbal medicines are depended on by 80% of the ecosphere's residents (WHO, 2005). The high cost of procuring modern medicines and their insufficient supplies in most health care facilities plus the unpleasant effects coupled with their utilization, and recently, the mindset that plants hold cure for many ailments have resulted to a rekindling of passion in the usage of plants and plant based products.

Plants are medicinal, valuable and treasured local resource for the populace of East Africa (Minja, 1999). The application of the plants in the indigenous cultures are many and diverse (Minja, 1999). The healing power of nearly all herbs is principally as a result of the occurrences of several secondary metabolites, which together are termed phytochemicals (Janifer *et al*., 2010). These phytochemicals possess the prospect to be advanced as herbal remedies or might aid as antecedents for contemporary medication. It is currently extensively agreed that unrestricted radicals are drawn in the pathogenesis of various illnesses. Normal protection mechanisms of the human body averts growth of these ailments but in the events of amplified offensive by reactive oxygen species plus unrestricted radicals, physique's resistance mechanisms requires to be improved by exterior antioxidants (Saha, 2008)..

Ocimum graticimum (Linn) is a valuable multipurpose medicinal plant belonging to the family Lamiaceae (Trombetta *et al*., 2005). It grows semi-wild in village groves around Nigeria. It is commonly used in the treatment of upper respiratory tract infections, diarrhea, headache, fever, eye infections, skin diseases and pneumonia in various African countries (Trombetta *et al*., 2005). When exposed to favorable conditions, meristematic tissues of seeds have the tendency to proliferate, and the extent of proliferation is reflected in the increase in the length of the radicles produced over 96 h in the control seeds. Thus the use of guinea corn seeds (*Sorghum bicolor*) was necessitated. Although, other highly proliferative seeds such as cowpea (*Vigna unguiculata*), maize (*Zea mays*) can be used, *Sorhgum bicolor* was found to be most convenient because of its relatively small size and low dormancy level (Weyermann *et al*., 2005). Hence, the present study evaluates the cytotoxic and anti-proliferative effects of thymol isolated from the leaves of *Ocimum gratissimum* against tadpoles of *Raniceps ranninus* using a bench-top assay, and the growth inhibitory effect using *Sorghum bicolor* seeds; and antimicrobial effects.

2. Materials and Methods

Hexane and ethyl acetate (Analar grade) were obtained from BDH Chemicals Ltd (Poole, England), Silica gel and alumina was from Merck (Darmstadt, Germany).Normal saline, and methylated spirit was obtained from H-Medix, Abuja.

Plant material

Fresh leaves of *Ocimum gratissimum* were collected from Idu, Abuja, Nigeria and were identified and authenticated at the herbarium of the National Institute for Pharmaceutical Research and Development, Idu Industrial Area, Abuja, Nigeria where voucher specimen was deposited (Herbarium/voucher number 5942).

Extraction and isolation

Hydrodistillation of the fresh leaves of the plant (3 kg) for 4 hours yielded 15ml (0.5% v/w) of a golden yellow essential oil using the method of Okhale *et al*. (2015). The essential oil (7 ml, 6.2559 g) was subjected to column chromatography on silica gel (63-200 µm, 70-230 mesh, column dimensions 25 mm id x 300 mm length) using a gradient of petroleum ether-diethyl ether 100:0 (200 ml), 95:5 (200 ml) and 90:10 (1000 ml). Fractions of 100ml each were collected and monitored by TLC and similar fractions were combined. The combined fractions 5-10was further separated on activated alumina (60-325 mesh, column dimensions20 mm id x 250 mm id) using a gradient of hexane-ethyl acetate 100:0 (400 ml), 95:5 (400 ml) and 90:10 (600 ml). Fractions of 200ml each were collected and

monitored by TLC and similar fractions were combined. Fractions I-VII were combined and concentrated to dryness under vacuum at 40°C, and on recrystallization gave white crystals (1.2 g) with melting point of 49.5°C. The melting point remained undepressed when mixed with commercially available thymol (Sigma).

Gas Chromatography-Mass Spectrometry Analysis

According to the method of Okhale *et al*. (2015), GC-MS analysis was carried out on a Shimadzu QP-2010 GC with QP-2010 Mass Selective Detector [MSD, operated in the EI mode (electron energy=70 eV), scan range of 45-700 amu, and scan rate of 3.99 scans/sec], and Shimadzu LCsolution data system using the method of Okhale *et al*. (2015). The Gas chromatography column was HP-5MS fused silica capillary with 5% phenyl-methylpolysiloxane stationary phase, with length of 30 m, internal diameter of 0.25 mm and film thickness of 0.25 µm. The carrier gas was helium with flow rate of 1.61 mL/min. The program used for Gas chromatography oven temperature was 60-160°C at a rate of 10°C/min, then held at 160°C for 2 min, followed by 160-280°C at a rate of 15°C/min, then again held at 280°C for 4 min. The injection port temperature was 250°C while ion source temperature was 200°C; interface temperature was 250°C. 1.0 µL of diluted sample (1% v/v in hexane) was injected using autosampler and in the split mode with ratio of 20:80. The compound was identified by NIST Mass Spectral Library.

Experimental plant (*Sorghum bicolor*)

S*orghum bicolor* seeds of uniform weight (35 ± 1 mg), obtained from a local market in Abuja and their viability were determined by their ability to remain submerged in water. Seeds that remain submerged in water were selected and dried for use. They were sterilized by soaking in 70% alcohol for two minutes and repeatedly washed with distilled water. Twenty seeds were selected for each group (*n* = 20).

Experimental Animals (*Raniceps ranninus*)

Tadpoles were harvested from toad colonies in a small water settlement around the University of Abuja and were identified as tadpoles of *Raniceps ranninus*. Young tadpoles of 5-6 days old from hatching time were selected and used for the study.

Tadpole cytotoxicity assay (*Raniceps ranninus*)

To make 10 different Final Bath Concentration (FBC) of thymol, about 1.0 – 0.1 ml of a stock solution of thymol were added to 9.0 – 9.9 ml of normal saline in 20 ml capacity bottles. A starting Final Bath Concentration of 10.0 – 1.0 µg/ml of thymol was used and based on the results, a second FBC of 0.9 – 0.1 µg/ml was used. Using standard method Ayinde and Agbakwuru (2010), ten tadpoles were selected and placed in the prepared solution of thymol. The procedure was repeated using normal saline as control. The mortality of the tadpoles was observed for a maximum of 96 hours.

Effects of thymol on *Sorghum bicolor* seed growth

Twenty viable seeds each were soaked in 15ml of distilled water and thymol solutions (625, 500, 250, 125, 62.5 and 31.25 µg/ml) in small sample bottles for 24 hours using the method of Shogbaike *et al*. (2002). After 24 hours, each solution was poured into 9 cm wide Petri dishes laid with cotton wool of about 3mm thick sandwiched between two layers of 9 cm wide filter paper (Whatman No 1). The soaked seeds were transferred and spread on each petri dish with their respective solution and incubated in a dark environment favourable for seed germination. The lengths (mm) of the emerging radicles of the root and shoot of the seeds were measured at 24, 48, 72 and 96 hours. Results were expressed as the percentage inhibition of linear growth of the radicle, calculated by the formula:

Percentage Growth Inhibition = **(A – B/A) × 100**

Where: **A** = Mean *Sorghum bicolor* seed radicle length (mm) for normal saline (control)**, B** = Mean *Sorghum bicolor* seed radicle length (mm) for thymol.

Determination of antibacterial activity of thymol

Test organisms

The following bacteria were used as test organisms in the screening: One Gram-positive bacterium, *Staphylococcus aureus* (ATCC 22952) and two Gram negative bacteria (clinical strains) *Escherichia coli* and *Pseudomonas aeruginosa* obtained from the Microbiology and Biotechnology Department of the National Institute for Pharmaceutical Research and Development, Abuja, Nigeria.

Standardization of organisms

Inocula were prepared by direct colony suspension as recommended by CLSI (2006) according to the method described by Pamplona-Zomenhan *et al*. (2011). Strains of bacteria were inoculated in Mueller Hinton agar and incubated at 35°C ± 2°C for 18 to 24 hours. Bacterial suspensions in sterile saline solution were prepared from direct colonies. These suspensions were adjusted to a turbidity level of 0.5 McFarland approximately $1.5x10^8$ cfu/mL.

Agar cup diffusion method

The agar cup diffusion method was adopted for the susceptibility study Oladejo *et al*. (2013). Muller Hinton agar plates were inoculated with the

0.5 McFarland standard innoculum (10^8 cfu/ml). Wells (6mm) were bored into the inoculated plates using a sterile cork borer and 100 µl of the extract were then added into the wells. Ciprofloxacin was used as a positive control while sterile distilled water and DMSO served as negative controls. Diameters of zones of inhibition were determined after incubating the plates at 37°C for 18 - 24 hr. The experiments were carried out in triplicates and the zones of inhibition (mm) expressed as the mean and standard deviations recorded.

Broth micro-dilution assay for minimum inhibitory concentrations (MIC)

The broth micro-dilution method was used according to the method described by Chuah *et al*. (2014), with slight modification, of extracts ranged between 2000 to 3.91 µg/ml. Plates were then incubated at 37°C for 18 h overnight. After incubation, the MIC of each extract was determined as the lowest concentration at which no growth was observed in the duplicate wells. Twenty microliters of a *p*-iodonitro tetrazolium violet solution (0.04%, w/v) (Sigma, USA) was then added to each well. The plates were incubated for a further 30 minutes, and estimated visually for any change in color from yellow to pink indicating reduction of the dye due to bacterial growth. The highest dilution (lowest concentration) that remained yellow corresponded to the MIC. The experiments were performed in triplicate.

Minimum bactericidal concentrations (MBC)

Fifty microliter of the wells that inhibited growth of the bacteria from MIC experiments were inoculated into fresh Muller Hinton broth and incubated for 18-24 h at 37°C. The lowest concentration that inhibited growth was taken as the minimum bactericidal concentration.

Statistical Analysis

Data were expressed as Mean ± SEM. Graph pad Prism version 5.02 was used to analyze data. The differences between means were compared using Two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. $P \leq 0.05$ were considered significant.

3. Results and Discussion

Isolation and characterization of thymol

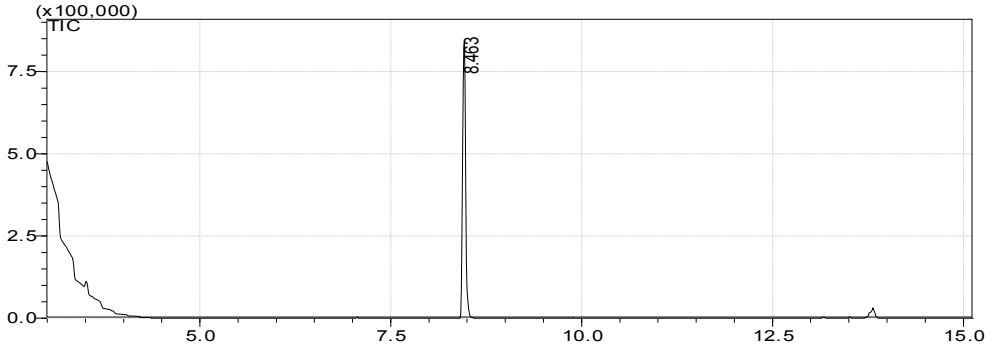

Figure 1: GC chromatograph of reference thymol with retention time of 8.463 minutes

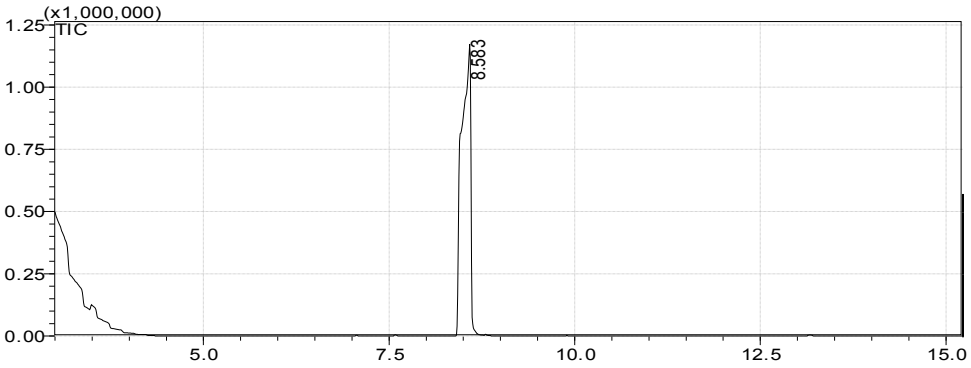

Figure 2: GC chromatograph of isolated thymol with retention time of 8.583 minutes

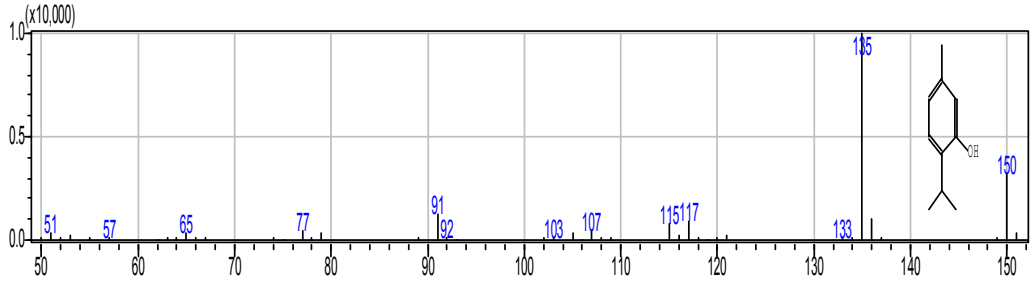

Figure 3: Mass spectrum of reference thymol

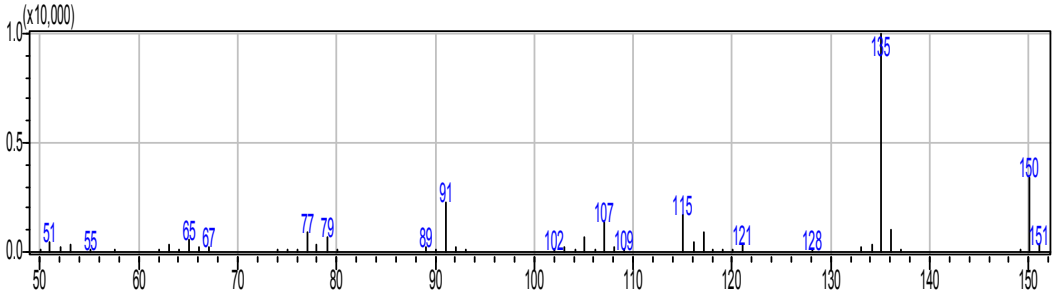

Figure 4: Mass spectrum of isolated thymol

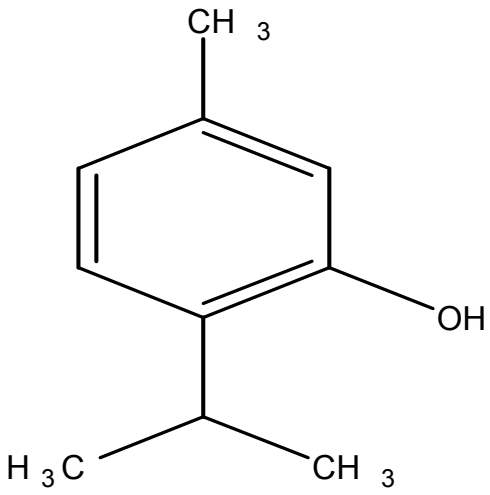

Figure 5: Structure of Thymol

The ^{1}H NMR spectra of thymol had chemical shift (δ) absorptions at 1.17 (6H, d, J=6.5 Hz), 3.2 (1 H, m), 2.26 (3 H, s), 6.75(1 H, s), 6.85 (1 H, d, J=7.8) and 7.15(1 H, d, J=7.8) [12].

The gas chromatograph of the compound gave a single peak at 8.583 minutes, which is comparable with reference thymol at retention time of 8.463 minutes (Fig. 1 and 2). The mass spectra of the compound gave m/z 135 (100%), 115 (18%), and 150 (35%), wherem/z 150 (35%) corresponded to the molecular ion which is characteristic of the reference thymol (Fig. 3 and 4).

Effect of Thymol on *Sorghum bicolor* seed germination

Thymol produced significant ($p<0.01$) dose dependent reduction in the radicle length of *Sorghum bicolor* seed in a time dependent manner (Table 1). Thymol at 500 µg/ml exerted higher inhibitory effect than

the lower concentrations (31.25, 62.5, 125 and 250 µg/ml) at 24, 48, 72 and 96 hours; and produced 100 % growth inhibition at 625 µg/ml.

Table 1: Effects of thymol on *Sorghum bicolor* mean radical length and percentage growth inhibition

Treatment	Mean Radicle Length (mm)				% Growth Inhibition [a]			
	24 hr.	48 hr.	72 hr.	96 hr.	24 hr.	48 hr.	72 hr.	96 hr.
Normal saline	0.87±0.10	1.27±0.16	2.96±0.31	3.29±0.58	0	0	0	0
31.25 µg/ml	0.51±0.08	0.82±0.13	1.45±0.19***	1.49±0.23***	41.38	35.43	51.01	54.71
62.5 µg/ml	0.66±0.07	0.89±0.12	1.48±0.17***	1.57±0.19***	11.14	29.92	50.00	52.28
125 µg/ml	0.65±0.06	1.05±0.12	1.50±0.17***	1.68±0.21***	25.29	17.32	49.32	48.94
250 µg/ml	0.26±0.05*	0.80±0.11	1.29±0.15***	1.58±0.18***	70.11	37.01	56.42	51.98
500 µg/ml	0.04±0.02**	0.16±0.05***	0.31±0.08***	0.62±0.10***	95.40	87.40	89.53	81.16
625 µg/ml	0.00±0.00**	0.00±0.00***	0.00±0.00***	0.00±0.00***	100	100	100	100

[a]Percentage Growth Inhibition = [(mean radicle length of control - mean radicle length of treated) / mean radicle length of control] x 100. Values are presented as Mean ± SEM; n = 20;
* significantly different from Control; * $P< 0.05$, ** $P< 0.01$, *** $P < 0.001$

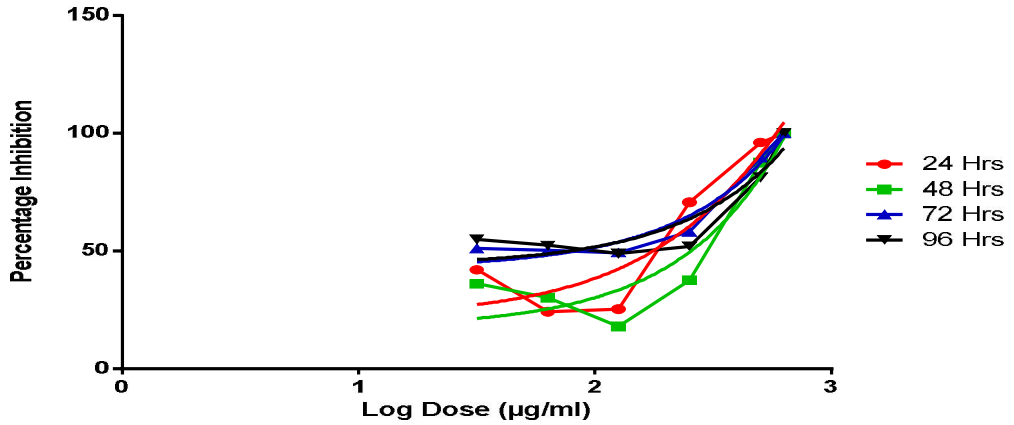

Figure 6: Percentage response versus log dose of thymol in *Sorghum bicolor* seed growth inhibitory assay.

Tadpole cytotoxicity assay

Thymol exerted cytotoxic effects against tadpoles of *Raniceps ranninus* in a concentration dependent manner with IC_{50} of 0.0614 µg/ml and 0.5517 µg/ml at 24 and 48 hours respectively.

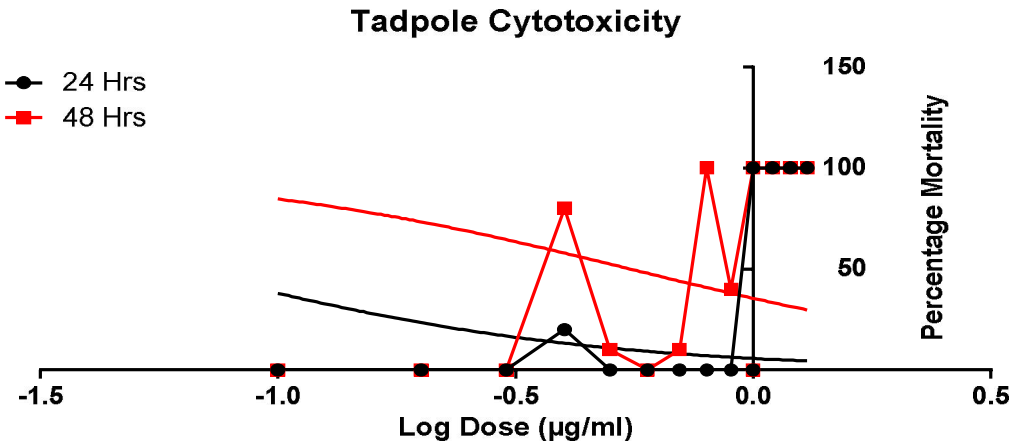

Figure 7: Percentage response versus log dose of thymol in *Raniceps ranninus* tadpole cytotoxicity assay (with IC_{50} of 0.0614 µg/ml and 0.5517 µg/ml at 24 and 48 hours respectively).

Thymol (32-65%) is an active compound present in volatile oils obtained from the leaves of *Ocimum gratissimum*. Essential oils and their components are becoming increasingly popular as naturally occurring bioactive substances. Some essential oil constituents have demonstrated anti-mutagenic effects; in particular carvacrol (isomeric with thymol) and thymol have demonstrated strong antimutagenic effects (Mezzoug *et al*., 2007). In addition, there is evidence that thymol has antitumor properties. Studies have shown that phenolic chemotype (thymol and carvacrol) showed stronger antioxidant properties than the non-phenolics (Mezzoug *et al*., 2007).

Though the exact mechanism of action is unknown, some evidence suggested that thymol exerted some of its biocidal properties by membrane disruption (Trombetta *et al*., 2005). Methotrexate elicit its anticancer effect via disruption of polyamine synthesis by inhibiting dihydrofolate reductase which gives rise to reduced production of tetrahydrofolate and methyltetrahydrofolate, which are methyl donors in chemical reactions resulting in the production of methionine and S-adenosylmethionine and ultimately polyamines. Polyamines are low molecular-weight organic polycations, displaying a broad biological activity in human and plants. The main PAs present in higher plants are putrescine, spermidine and spermine (Kuznetsov *et al*., 2006). In plants PAs exert a broad spectrum of biological activities such as the regulation of gene expression, signal modulation, cell proliferation and membrane stabilization (Igarashi *et al*., 2000). These roles have been associated with the control of cell division, embryogenesis and root formation (Kumar et al., 1997).

Polyamine biosynthesis is up-regulated in actively growing cells, including cancer cells (Kubota *et al*., 1985) therefore polyamine concentration as well as gene expression and activity of enzymes involved in polyamine biosynthesis, are higher in cancer tissues than in normal surrounding tissues (Uehara *et al*., 1980). Interestingly, these levels decrease after tumor eradication and increase after relapse indicating that polyamines levels increased in case of cancerous growth (Weiss *et al*., 2002). Thymol may have also produced its cytotoxic and sorghum seed growth inhibitory effect via inhibition of polyamine synthesis similar to methotrexate and in addition to other mechanisms yet to be understood.

The beneficial health properties of thymol (32-65%) as the main component of *Ocimum gratissimum* leaf have encouraged us to investigate its anticancer activity (Weiss *et al*., 2002). The two bioassay experimental models adopted to examine the probable cytotoxic and growth inhibitory effects of thymol are widely used as valid methods for cytotoxicity studies of chemicals (McLaughlin *et al*., 1991). The principle is that agents that are cytotoxic and inhibits either the growth or reduce the elongation of radicle and shoot of growing seeds of plants such as cowpea, *Sorghum bicolor* and maize have herbicidal or anticancer activity (Ayinde and Agbakwuru, 2010). *In-vitro* cytotoxicity assays can be used to predict human toxicity and for the general screening of chemicals (Okhale *et al*., 2015). It has been previously reported that different cytotoxicity assays can give different results depending on the test agent used and the cytotoxicity assay employed (Weyermann *et al*., 2005).

Antimicrobial activity of thymol

The results of the antibacterial activity of thymol using agar cup diffusion method are given in Table 2. A diameter zone of inhibition greater than 15 mm was considered as a high antibacterial activity. The highest zone of inhibition was produced

against *S. aureus* (20.67 mm) and lowest against *E. coli* (16.0 mm). It was observed that thymol was effective against both Gram positive and Gram negative bacteria. The MIC and MBC values are given in Table 3. Thymol was found to have its highest activity (lowest MIC) against *P. aeruginosa* with a MIC and MBC value of 125 µg/ml and 250 µg/ml respectively. The MIC and MBC values against *E. coli* and *S. aureus* were similar with values of 250 µg/ml and 500 µg/ml respectively.

Table 2: Antibacterial activity of thymol

Sample	Diameter zone of Inhibition (mm)		
	P. aeruginosa	*E. coli*	*S. aureus*
Thymol	17.0±0.0	16.0±0.0	20.67±0.577
Ciprofloxacin	39.67±0.577	29.0±0.0	25.67±0.577

Significantly different at P<0.05 level (mean ± S.D).

Table 3: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration of thymol

Sample	MIC (µg/ml)			MBC (µg/ml)		
	P. aeruginosa	*E. coli*	*S. aureus*	*P. aeruginosa*	*E. coli*	*S. aureus*
Thymol	125.0±0.0	250.0±0.0	250.0±0	250.0±0.0	500.0±0.0	500.0±0.0
Ciprofloxacin	2.44±0.0	2.44±0.0	2.44±0.0	2.44±0.0	2.44±0.0	2.44±0.0

Significantly different at P<0.05 level (mean ± S.D).

The results obtained from the studies carried out on the isolated thymol from *Ocimum gratissimum* showed that the compound has some antiproliferative, cytotoxic, growth inhibitory and antimicrobial effects.

Thymol produced a dose-dependent cytotoxic effect against tadpole and inhibition of radicle growth of *sorghum bicolor* used in the growth inhibition assay. Compared to the control (normal saline) thymol produced cytotoxic and growth inhibitory effects in a concentration dependent manner such that 625 µg/ml completely inhibited *sorghum bicolor* seed growth. The index of potency is the inhibitory concentration. Thymol produced an IC_{50} of 631 mg/ml, 90 mg/ml and 38 mg/ml at 24, 48, 72 and 96 hours respectively. The discrepancy of the IC_{50} could be as a result of the time span for absorption and germination of the seeds.

Thymol is an essential oil used in mouth wash because of its antimicrobial activity (Vimal *et al*. 2013). Several studies have documented its antimicrobial activity. The antibacterial activity observed for thymol in this study agrees with other reports (Vimal *et al*. 2013).

Cancer and tumor related ailments known to be among the leading causes of death are characterized by uncontrolled cell proliferation in the body. Cellular proliferation depends on the rates of cell division and death and, thus, many anticancer drugs have been used to prevent cancer cell division in order to inhibit cancer cell proliferation. In order to curb the high cost of treating the disease and the life threatening side effects which usually accompany orthodox drugs, there is need for research into medicinal plant with probable antiproliferative effects.

Conclusion

The antiproliferative effect of thymol was evident compared to the control. Thymol significantly inhibited *Sorghum bicolor* seed growth, radicle length in a concentration dependent manner. Thymol and the leaves of *Ocimum gratissimum* could be explored as potential anticancer agents.

Conflict of Interest

The authors declare no conflict of interest.

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