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Pleurotus ostreatus photo by Aldy

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Book:

Rai MK, Carpinella C. 2006. Naturally Occurring Bioactive Compounds. Elsevier, Amsterdam.

Chapter in book:

Webb CO, Cannon CH, Davies SJ. 2008. Ecological organization, biogeography, and the phylogenetic structure of rainforest tree communities. In: Carson W, Schnitzer S (eds) Tropical Forest Community Ecology. Wiley-Blackwell, New York.

Abstract:

Assaeed AM. 2007. Seed production and dispersal of *Rhazya stricta***.** 50th Annual Symposium of the International Association for Vegetation Science, Swansea, UK, 23-27 July 2007.

Proceeding:

Alikodra HS. 2000. Biodiversity for development of local autonomous government. In: Setyawan AD, Sutarno (eds.) Toward Mount Lawu National Park; Proceeding of National Seminary and Workshop on Biodiversity Conservation to Protect and Save Germplasm in Java Island. Universitas Sebelas Maret, Surakarta, 17-20 July 2000. [Indonesian]

Thesis, Dissertation:

Sugiyarto. 2004. Soil Macro-invertebrates Diversity and Inter-Cropping Plants Productivity in Agroforestry System based on Sengon. [Dissertation]. Universitas Brawijaya, Malang. [Indonesian]

Information from internet:

Balagadde FK, Song H, Ozaki J, Collins CH, Barnet M, Arnold FH, Quake SR, You L. 2008. A synthetic *Escherichia coli* predator-prey ecosystem. Mol Syst Biol 4: 187. www.molecularsystemsbiology.com. DOI:10.1038/msb.2008.24

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Effects of selected medicinal plant extracts on mitochondrial function

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Abstract. *Achampong AS, Ocloo A, Appiah-Opong R. 2019. Effects of selected medicinal plant extracts on mitochondrial function. Biofarmasi J Nat Prod Biochem 17: 1-13.* Mitochondria are organelles that produce approximately 90% of the energy required by the eukaryotic cell through oxidative phosphorylation. Mitochondria also play vital roles in other metabolic processes in the cell. Mitochondria have unique features that make them sensitive to some xenobiotics. Thus, they serve as primary or secondary targets for these xenobiotics. Medicinal plants are the source of phytochemicals that also have the potential to regulate mitochondrial function. Therefore, it is necessary to study the effects of these medicinal plants, especially those in Ghana, on mitochondrial function. The objectives of this study were to determine the effects of *Taraxacum officinale*, *Morinda citrifolia,* and *Millettia thonningii* on mitochondrial function. The extracts' antioxidant properties, phenolic content, and phytochemicals were determined. We determined the effect of the extract on cell viability and mitochondrial respiratory chain activity. All extracts significantly stimulated basal respiration and also caused an increase in complex IV respiration. The extracts exhibit considerable antioxidant activity, and all contain phytochemicals that have been shown to have antioxidant properties. *M. thonningii* partially blocked the effects of rotenone, while Noni juice partially blocked the effects of antimycin A. The extract, however, had a weak cytotoxic effect on Jurkat leukemia cells and MCF7 breast cancer cells.

Keywords: Mitochondria, *Millettia thonningii*, *Morinda citrifolia*, *Taraxacum officinale*

INTRODUCTION

Mitochondria are essential organelles present in the eukaryotic cell, which produce about 90% of the energy needed by the cell in the form of adenosine triphosphate (ATP) through a process known as oxidative phosphorylation (Nadanaciva et al. 2007). Mitochondria also play essential roles in fatty acid β-oxidation, apoptosis, heme synthesis, and calcium metabolism (Scatena et al., 2007).

Mitochondria also have unique properties that enable them to carry out their functions and make them sensitive to chemical compounds. For example, they have a double membrane consisting of the outer and the inner membrane. The cholesterol-rich outer membrane describes all the organelles, while the cardiolipin-rich inner membrane encloses the matrix. Moreover, mitochondria are the only organelles in animal cells containing their DNA and have a basic interior (Wallace and Starkov 2000).

These unique properties serve as the primary or secondary targets for drugs and other xenobiotics (Wallace and Starkov 2000). Consequently, several drugs produce pharmacological effects through their interaction with the mitochondria. For instance, the immunosuppressant, Cyclosporin A, is known to prevent the induction of the mitochondria permeability transition pore (MPTP) and hence provide protection against ischemic-reperfusion injuries (Smith et al. 2012). Idebenone is another drug that acts as an antioxidant. The drug increases the complex I activity of the electron transport chain (ETC) and protects against neurodegeneration and cardiomyopathy (Smith et al., 2012). Methylene blue is reported to increase complex IV activity in Alzheimer's models (Smith et al., 2012).

However, some compounds exhibit their toxic effects by interfering with mitochondrial function. Widely used as a drug in treating epilepsy and other seizures, Valproic acid, is a substrate for the fatty acid β-oxidation pathway, primarily in the mitochondrion. The toxicity of valproate is considered primarily due to its interference with mitochondrial β-oxidation (Silva et al. 2008). Other antiretroviral drugs, such as zidovudine, have also induced mitochondrial toxicity (Lewis et al., 2003). Zidovudine inhibits the function of DNA polymerase polymerase-γ (DNA pol-polymer)—the enzyme that replicates mtDNA. This pol-γ inhibition leads to depletion of mtDNA and thus to mitochondrial dysfunction (Lewis and Dalakas 1995). Well-characterized drugs are associated with side effects where oxidative stress may contribute to nonsteroidal anti-inflammatory drugs (NSAIDs), antipsychotics, and analgesics (Deavall et al., 2012).

The toxic effect produced by some of these drugs on the mitochondria subsequently leads to failure of energy generation and mitochondrial dysfunction (Wallace and Starkov 2000). Impaired mitochondrial function induces uncoupling oxidative phosphorylation, ATP depletion, and excessive reactive oxygen species (ROS) production. Mitochondrial dysfunction has been linked with a range of pathological conditions, including diabetes, obesity, Parkinson's disease, Alzheimer's disease, lactic acidosis, cancer, and retinopathy (Scatena et al., 2007; Wallace, 2013). Therefore, although mitochondria serve as critical primary targets for drugs, some medications lead to toxicity. Thus, in drug development, including the development of medicinal plant products, it is essential to

study the compound's effect on mitochondrial function to predict and prevent the severe side effects of the mixtures and the herbal preparations. Gohil et al. (2010) have screened an extensive array of drugs already in clinical use and classified several interfering with mitochondrial function.

Medicinal plant extracts contain many phytochemicals, including alkaloids, flavonoids, polyphenols, and tannins. These phytochemicals can interfere with mitochondrial function, just like synthetic compounds and drugs (Wallace and Starkov 2000; Forbes-Hernandez et al. 2014). *Phyllanthus urinaria* has been found to inhibit complex I and complex II of the ETC and significantly induces a decline in mitochondrial respiration (Huang et al., 2014). Dichloromethane extract from the seeds of *Millettia thonningii* has been shown to inhibit complex I activity, and this inhibition is believed to be responsible for its molluscicidal and schistosomicidal actions (Lyddiard and Whitfield 2001). Similarly, the ethanolic extract of *Paulinia pinnata* has been reported to inhibit the activity of complex II, which may explain the reported toxicity in fish (Ocloo et al., 2015). Although there has been some research on several medicinal plants on their effects on mitochondria, there is currently little information on therapeutic plant yields in sub-Saharan Africa on mitochondrial function.

The objectives of this research were (i) to determine the effects of aqueous leaf extracts of *T. officinale*, *M. citrifolia* and *M. thonningii*, and *M. citrifolia* fruit juice (Noni juice) on the mitochondria complexes I-IV activity *in situ* in permeabilized cardiac fibers; (ii) to assess the effects of the aqueous leaf extracts of *T. officinale*, *M. citrifolia* and *M. thonningii*, and Noni juice on mitochondrial function in leukemia (Jurkat) and breast cancer (MCF7) cell lines using the MTT assay; (iii) to determine the antioxidant activities, phenolic content, and phytochemistry of the leaf extracts of *T. officinale*, *M. citrifolia*, *M. thonningii*, and Noni juice.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals were purchased from Sigma-Aldrich (Bournemouth, U.K.) except $MgCl_2.6H_2O$ (Barcelona, Spain), sucrose (Karlsruhe, Germany), imidazole (Belgium), and KH_2PO_4 (Frankfurt, Germany).

Plant materials

The leaves were harvested at different locations. *T. officinale* Weber leaves were harvested at Adabraka, Accra, Ghana, while *M. citrifolia* leaves were harvested at Ayikuma, Greater Accra Region of Ghana. *M. thonningii* (Schum. & Thonn) Baker leaves were harvested near Botany, University of Ghana, Ghana. Noni juice was purchased from the Royal Noni Factory at Dzorwulu, Accra, Ghana. Prosper Avekor confirmed the identity of each plant specimen at the Department of Botany, University of Ghana, Legon. The voucher specimen were *T. officinale* (GC45929), *M. citrifolia* (ASMC1014), and *M. thonningii* (CSRPM/406).

Preparation of plant materials

The leaves of three medicinal plants, *T. officinale*, *M. citrifolia*, and *M. thonningii*, were air-dried for three weeks and subsequently pulverized with a blender. To obtain the aqueous extract, the pulverized samples were dissolved in distilled water (500 mL) to 50 g of the powdered sample (10% of a mixture prepared). The resulting mixture was heated at 80ºC for 1 hour, cooled to room temperature, and centrifuged at 4500 x g for 20 minutes. The supernatant was removed by decantation. The pellet was re-suspended in 500 mL distilled water. The supernatant obtained was decanted, collected, and mixed with the previous one. The heating and centrifugation of the extracts were repeated. The leaf extracts and Noni juice were frozen-dried in a Supermodulyo freeze-dryer (Thermo Electron Corporation, USA).

Analysis of the effect of the extracts on mitochondrial complexes

Preparation of tissues for permeabilization

Male ICR mice (3-5 months) with an average weight of 28.75 g were obtained from the Centre for Plant Medicine Research (CPMR), Akuapem-Mampong, Ghana. The mice were acclimatized in the laboratory for 24 hours and then sacrificed.

The myocardial tissues were isolated using scissors and forceps, then placed in ice-cold isolation buffer (7.23 mM K_2EGTA, 2.77 mM CaK_2EGTA, 5.77 mM Na_2ATP, 20 mM taurine, 6.56 mM $MgCl_2.6H_2O$, 15 mM Na_2Phosphocreatine, 20 mM imidazole, 0.5 mM dithiothreitol, 50 mM MES). The samples were then transferred to a plastic petri dish containing a small amount of the ice-cold isolation buffer. Fat and connective tissues were removed. Next, the myocardial tissue was dissected into muscle strips under a dissecting microscope. Next, these strips were further dissected into thin muscle fibers.

Permeabilization of mouse cardiac tissues

The fiber bundles were permeabilized by transferring them into a vial containing isolation buffer (2 mL) and 50 µg/mL of saponin and mixed gently on a rocker-shaker at 4°C for 20 minutes. Next, the permeabilized fibers were washed by transferring them into another vial containing the respiration medium (0.5 mM EGTA, three mM $MgCl_2.6H_2O$, 20 mM taurine, ten mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, one g/l fatty acid-free BSA) and mixed gently at 4°C for 5 minutes to wash out the saponin (Kuznetsov et al. 2008). This step was repeated three more times.

Measurement of oxygen consumption

Oxygen consumption rates were monitored using a Clark-type oxygen electrode (Strathkelvin Instruments Limited, Scotland) in a sealed chamber at 37°C. The Clark-type oxygen electrode consists of a platinum or gold cathode and silver/silver chloride anode separated by a potassium chloride electrolyte solution. An oxygen-permeable membrane separates the two-half cells from the

experimental assay media. When a voltage is applied across the two half-cells, oxygen passes through the membrane and is reduced at the cathode to the hydroxyl radical ($O^{\cdot H,-}$). The KCl electrolyte is buffered to remove the $O^{\cdot H,-}$produced at the cathode. The reduction of each oxygen molecule is accompanied by 4 electrons of current flow in the circuit.

The signal generated by the electrode is linear concerning the molecular oxygen flux to the cathode. This oxygen flux is also proportional to the partial pressure of oxygen (PO_2) of the respiratory medium, membrane permeability, temperature, and cathode surface area (Strathkelvin Instruments Ltd. 2012). During respiration testing, the output signal from the oxygen meter is digitally collected every second by the Strathkelvin 782 system software. The data files are converted to absolute values based on the oxygen content of 210 mol O_2 and the amount of mitochondrial protein used.

For oxygen consumption measurements, Permeabilized fibers (2.5-4 mg) were added to a continuously stirred respiration medium (500 µL). The fibers were incubated with the extracts (2 µL) at a 10mg/mL concentration for 2 minutes. Functional analysis of the respiratory chain enzyme complexes was performed using specific substrates and inhibitors in a substrate-inhibitor titration (Kuznetsov et al. 2008).

In this titration, 1.25 µL of 0.8 M malate and 2.5 µL of 2 M glutamate (complex I substrates) were first added to the polygraph chambers to measure complex I respiration. This step was continued by adding 5 l 0.5 M ADP to obtain maximum mitochondrial respiration. Inhibition of complex I was achieved by adding 0.25 l 0.01 M rotenone. Next, 5 l of 1 M succinate was added to stimulate respiration of complex II. Next, an aliquot of 0.25 µL of 0.02 M antimycin A was added to inhibit complex III respiration. This was followed by measuring the complex IV respiration by adding 0.2 M TMPD (N, N, N', N'-tetramethyl-p-phenylenediaminedihydrochloride) (1.25 µL) and 0.8 M ascorbate (artificial substrates of complex IV) (1.25 µL). Finally, the efficacy of the preparation was evaluated by confirming mitochondrial integrity through the addition of cytochrome C (1.25 µL; 4 mM).

Determination of the effects of the extracts on cell viability

The cytotoxic effect of the extracts was evaluated using a tetrazolium-based colorimetric [3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] (MTT) assay based on Ayisi (2011). The cell lines used in this study were the Jurkat leukemia and MCF7 breast cancer cell lines. The MCF7 cells were maintained in DMEM-10% FBS, while the Jurkat cells were maintained in RPM I 1640 supplemented with 10% FBS. The cells were incubated at 37°C in the presence of 5% C.O. They were then seeded (1 d x 10^5cells/mL) into 96-well plates pre-treated with varying concentrations (0-1000 µg/mL) of t h e crude extracts. Curcumin was used as the positive control. The experiment was carried out in triplicate.

After incubating cells with the extract for 72 hours, 20l of 2.5 mg/mL MTT solution was added to each well, and incubation lasted for 4 hours. The reaction was then stopped with acidified isopropanol (150 l), after which the plates were incubated overnight in the dark. Optical density was read using a Tecan Infinite M200 (Austria) plate reader at a wavelength of 570 nm. Cytotoxicity was determined by the concentration of the extracts, which inhibit 50% cellular proliferation (IC_{50}). In addition, percent cell survival was evaluated, and IC50 values were determined.

Determination of antioxidant activities of the extracts

The free radical scavenging activities of the samples were evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method with slight modification (Blois 1958). Each extract (10 mg/mL) was prepared by dissolving the dry extract in distilled water. An amount of 10 mM butylated hydroxytoluene (BHT) was applied as the positive control. Extracts were prepared at various concentrations ranging from 0-5 mg/mL. The reaction mixture was 100 µL of plant extract or BHT and 100 µL of 0.05 mM DPPH in 96-well plates. Triplicate experiments were performed. The samples were incubated in the dark for 20 minutes (RT). The absorbance was read at 517 nm using a microplate reader (Tecan Infinite M200, Austria).

The free radical scavenging capacity of each sample was calculated as the percent DPPH radical scavenging effect using the below formula:

$$\% \text{ DPPH Scavenging effect} = [(A_0-A_1)/A_0] \times 100,$$

Where: A_0 is the absorbance of the blank solution, and A_1 is the absorbance of the extract. The EC_{50} (extract concentration to produce 50% reduction of DPPH) values were calculated from a plot of the % DPPH scavenging effect versus log concentration of extract; the lower the EC_{50} value, the higher the antioxidant activity and vice versa.

Determination of total phenolic content of extracts

The total phenolic content of the sample was calculated based on the Folin-Ciocalteau assay (Marinova et al. 2005) with slight modification. TFolin-Ciocalteu reagent is a mixture of tungstate and molybdate (Jadhav et al., 2012). The polyphenols in plant extracts react with the Folin-Ciocalteu reagent to create a blue complex (a phosphotungsticphosphomolybdenum complex) quantified by visible light spectrophotometry (Schofield et al. 2001). The blue color produced has maximum absorption in the region of 750 nm and is proportional to the total amount of phenolic compounds present.

5 mg/mL of each extract was prepared to determine the total phenolic content. The aliquot of extract (10 µL) was added to 790 µL of distilled water in each well. To this solution, Folin-Ciocalteau reagent (50 µL) was added. The resulting solution was thoroughly mixed and incubated in the dark for 8 minutes. Next, the solution was added with 150 µL of 7% Na_2CO_3 and further incubation for 2 hours in the dark at room temperature. The experiment was performed in duplicates. Absorbance was read at a wavelength of 750 nm using a microplate reader (Tecan Infinite M200, Austria). Meanwhile, Gallic acid was used as the standard phenolic compound to make a calibration curve (Figure 1).

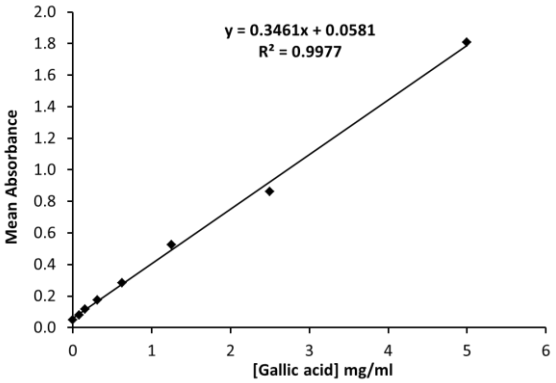

Figure 1. Calibration curve for gallic acid

Phytochemical analysis

The crude extracts and Noni juice were assayed for the presence of six phytochemicals: alkaloids, flavonoids, cardiac glycosides, saponins, tannins, and terpenoids. As described elsewhere, the alkaloids were tested (Sofowora 1993; Evans 2002) with slight modification. Finally, tests for the other phytochemicals were carried out as described (Mir et al., 2013).

Determination of the presence of alkaloids in the extracts

0.1 g of each crude extract was weighed into 5 mL of 2 M hydrochloric acid (HCl) solution. The mixed solution was stirred, warmed, and filtered. The filtrate from each extract was then divided into three portions. Dragendorff's reagent was added to one part of each test solution, while Mayer's reagent was added to another amount. Wagner's reagent was added to the third portion of the test solutions. The presence of a yellowish or reddish-brown precipitate indicated the presence of alkaloids.

Determination of the presence of glycosides

An aliquot of aqueous extract (5 mL) containing 0.1 g of extract was mixed with 2 mL of glacial acetic acid (CH_3COOH) having one drop of ferric chloride ($FeCl_3$). Concentrated sulfuric acid (H_2SO_4) (1 mL) was added carefully to the above mixture so that concentrated H_2SO_4 was at the bottom of the mixture. The appearance of a brown ring indicates the presence of glycoside constituents.

Determination of the presence of flavonoids

An amount of 0.1 g of each crude extract was added to 5 mL of 80% (v/v) ethanol. The resulting solution is filtered and divided into two parts. In one part of the filtrate, a magnesium lathe is added. This step was followed by adding 0.5 mL of concentrated HCl, and the solution was monitored for 10 minutes to observe color changes. Next, concentrated HCl (5 mL) was added to another portion, and the solution was warmed for 5 minutes. Observation of light pink color is an indication of the presence of flavonoids.

Determination of the presence of saponins

Each extract of as much as 0.5 g was boiled together for 30 seconds with 5 mL of distilled water and then filtered. The filtrate (2.5 mL) was then mixed with 1.25 mL of distilled water and shaken vigorously to obtain a stable and persistent froth. Three drops of olive oil are added to the foam. The formation of an emulsion indicates the presence of saponins.

Determination of the presence of tannins

To detect the presence of tannins, 0.25 g of extract was boiled in 10 mL of distilled water for 30 seconds and then filtered. This is followed by adding 3 drops of 0.2% $FeCl_3$ to filtrate. The observation of a brownish-green or a blue-black coloration indicated the presence of tannins.

Determination of the presence of terpenoids

Aqueous extract (5 mL) containing 0.1 g of the extract was mixed with 2 mL of chloroform ($CHCl_3$). 3 mL of concentrated H_2SO_4 was carefully added to the mixture to form a layer of the aqueous extracts. An interface indicated the presence of terpenoids with reddish-brown coloration.

Data analysis

The data for the antioxidant, cell viability, and mitochondrial respiration measurements were analyzed as described below:

The EC_{50} values obtained in the DPPH assay were calculated from plots of%DPPH scavenging effects versus log extract concentrations using GraphPad Prism version 5.0 (GraphPad Prism Software Inc., San Diego, CA). The DPPH radical scavenging effect was calculated using the formula:

$$\% \text{ DPPH Scavenging effect} = [(A_0-A_1)/A_0] \times 100,$$

Where: A_0 represents the absorbance of the blank and A_1 is the absorbance of the extract

Nilai IC50 yang diperoleh dalam uji MTT ditentukan dari plot persen viabilitas sel versus konsentrasi ekstrak menggunakan Microsoft Excel. The percent cell viability was calculated using the formula:

$$\% \text{ Cell viability} = (A_1/A_0) \times 100$$

Where: A_0 is the absorbance of the blank and A_1 is the absorbance of the extract

The respiration test was analyzed using the Strathkelvin 782 system, which converts the respiratory rate to an absolute value in Microsoft Excel. Each plant extract was evaluated five times, and the values were recorded as mean ± SEM. Comparisons between means were made, and significance was assessed by one-factor analysis of variance using Microsoft Excel. The probability value of $P < 0.05$ was used as a criterion for a significant difference.

The respiratory control ratio (RCR) was calculated as state 3/state 2 to measure mitochondrial coupling. State 3 respiration was stimulated by adding 0.5 M ADP, while State 2 respiration was produced by adding 0.08 M malate and 2 M glutamate. Leakage respiration was also determined from G+M-stimulated respiration (second

respiratory status) in the absence of ADP. Leaky respiration measures the leakage of protons across the inner mitochondrial membrane.

RESULTS AND DISCUSSION

Effects of the selected plant extracts on mitochondrial function in saponin-permeabilized cardiac fibers

The effects of the selected extracts on mitochondrial respiratory chain activity are displayed in Figure 2. The first pair of rods showed incubation of permeabilized fibers only in the presence of solvents or extracts. This breathing proved minimal. Complex I respiration is stimulated by adding glutamate and malate (G+M) and is represented by the second pair. The third pair showed the activation of oxidative phosphorylation with the addition of ADP. The fourth pair shows inhibition of complex I by rotenone, a known complex I inhibitor. Complex II respiration is stimulated by the accumulation of succinate and is indicated by the fifth pair. The sixth pair represents complex III inhibition by antimycin A, a known complex III inhibitor. The seventh pair represents the addition of ascorbate and N,N,N′,N-tetramethyl-p-phenylenediamine (TMPD) to stimulate respiration via complex IV. Ascorbate and TMPD are artificial electron donors to complex IV. The last pair of bars represents cytochrome C, a test for the intactness of the outer mitochondrial membrane.

Effects of *T. officinale* on mitochondrial respiratory chain activity

In the presence of the *T. officinale* extract alone, there was significant minimal respiration, while no respiration was observed in the presence of the control/solvent alone (Figure 2). Subsequent addition of substrates and inhibitors resulted in insignificant changes. G+M stimulated respiration appeared unaffected by the T. officinale extract compared to the solvent. The extract did not significantly decrease ADP-stimulated respiration, rotenone-inhibited respiration, succinate-stimulated respiration, and antimycin A-inhibited respiration compared to the solvent. However, the ascorbate+TMPD-stimulated respiration increased non-significantly in the presence of the *T. officinale* extract compared to the solvent. The addition of cytochrome C appeared to elicit a non-significant increase in respiration rate in the presence of the *T. officinale* extract compared to the solvent.

Effects of *Morinda citrifolia* on mitochondrial respiratory chain activity

In the presence of the solvent alone, no respiration was observed. However, when the *Morinda citrifolia was* independently added, significant minimal respiration was observed (Figure 3). The *M. citrifolia* extract also caused a substantial increase in the complex IV respiration compared to the solvent. On the other hand, in the presence of this extract, a non-significant decrease in respiration was observed with the addition of G+M, ADP, succinate, and antimycin.

Compared with the solvent, rotenone-inhibited respiration did not appear to be affected in the presence of *M. citrifolia* extract. The addition of cytochrome C seemed to cause an insignificant increase in respiration in the presence of *M. citrifolia* to extract compared to the solvent.

Effects of noni juice on mitochondrial respiratory chain activity

There was significant minimal respiration observed in the presence of the Noni juice extract alone (Figure 4). Subsequent addition of substrates and inhibitors resulted in insignificant changes. Noni juice extract did not significantly increase antimycin A-inhibited respiration and ascorbate + TMPD-stimulated respiration. However, Noni juice extract did not significantly decrease G+M stimulated respiration, ADP stimulated respiration, rotenone-inhibited respiration, and succinate stimulated respiration insolvent. The addition of cytochrome C seems to cause an insignificant increase in respiration compared to the solvent.

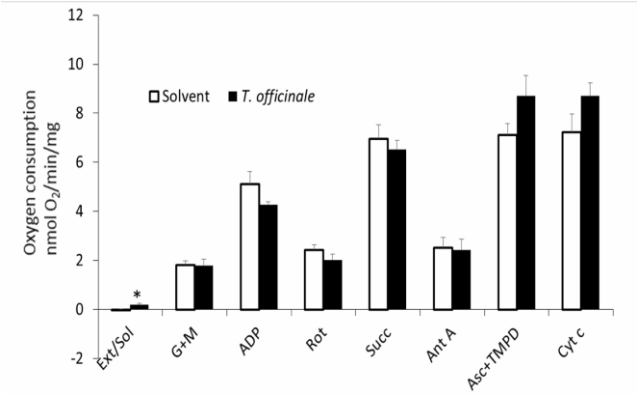

Figure 2. Effects of *T. officinale* and solvent on respiration rates; Values are means ± SEM (n=5): 2 µL of solvent, 2 µL of 10 mg/mL of extract; Black bars represent respiration rates in the presence of the extracts (ext); White bars indicate respiration rates in the presence of the solvent (sol) *-Values significantly different from control ($p<0.05$).

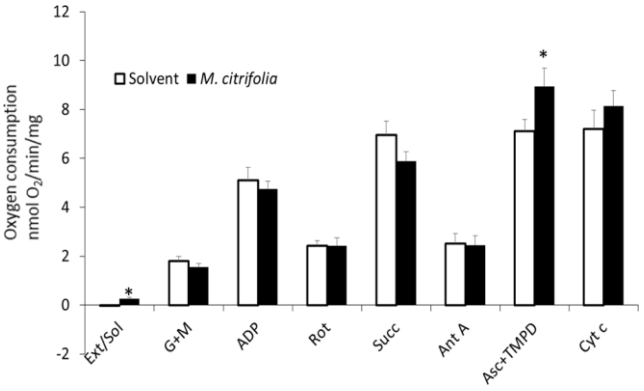

Figure 3. Effects of *M. citrifolia* and solvent on respiration rates; Values are mean ± SEM (n=5): 2 µL of solvent, 2 µL of 10 mg/mL of extract; Black bars indicate respiration rates in the presence of the extracts (ext); White bars indicate respiration rates in the presence of the solvent (sol) *-Values significantly different from control ($p<0.05$).

Effects of *M. thonningii* on mitochondrial respiratory chain activity

Similar to the trend observed with the other extracts, the *M. thonningii* extract alone appeared to have stimulated significantly minimal respiration compared to the solvent (Figure 5). Non-significant changes were observed with the addition of the substrates and inhibitors. G+M stimulated the M. thonningii extract but did not alter respiration compared to the solvent. *M. thonningii* extract did not significantly increase ADP-stimulated respiration, rotenone-inhibited respiration, and ascorbate+TMPD stimulated respiration. However, succinate-stimulated respiration and antimycin A-inhibited respiration did not appear to be significantly decreased in the presence of *M. thonningii* extract compared to the solvent. The addition of cytochrome C caused an insignificant increase in respiration in the presence of *M. thonningii* extract compared to the solvent.

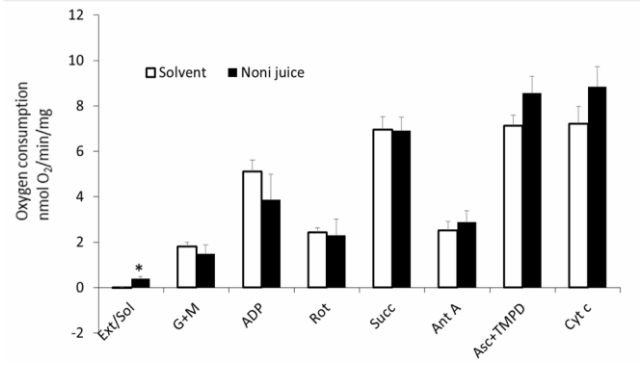

Figure 4. Effects of Noni juice and solvent on respiration rates; Values are mean ± SEM (n=5): 2 µL of solvent, 2 µL of 10 mg/mL of extract; Black bars indicate respiration rates in the presence of the extracts (ext); White bars denote respiration rates in the presence of the solvent (sol) *-Values significantly different from control ($p<0.05$)

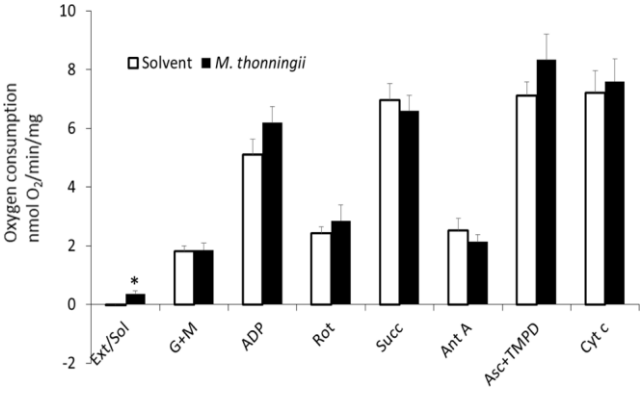

Figure 5. Effects of *M. thonningii* and solvent on respiration rates; Values are mean ± SEM, (n=5): 2 µL of solvent, 2 µL of 10 mg/mL of extract; Black bars showed respiration rates in the presence of the extracts (ext); White bars indicate respiration rates in the presence of the solvent (sol); *-Values significantly different from control ($p<0.05$)

Effects of the plant extract on respiratory control ratio (RCR) and leak respiration.

The RCR values obtained for the extracts are indicated in Table 1. *Taraxacum officinale, Millettia thonningii, and Morinda citrifolia* increased RCR values more than the solvent and seemed to have non-significantly increased coupling. However, the RCR in the presence of the Noni juice was lower than the solvent, indicating that the Noni juice appeared to have non-significantly decreased coupling.

Figure 6 shows a graph of the effects of the extracts on the leak respiration. The leak of respiration was not observed in all tested samples. The M. thonningii and T. officinale extracts appeared to have caused slightly lower leak respiration than the solvent. The M. citrifolia and Noni juice extracts appeared to have caused much lower leak respiration than the solvent.

Effects of plant extracts on cell viability

Curcumin, a positive control, had IC_{50} values of 11.4 M and 74.24 M (Fig. 7A) in Jurkat leukemia cells and MCF7 breast cancer cells, respectively. However, the IC_{50} values of the extracts obtained in the Jurkat leukemia cells were more significant than 100 µg/mL, although dose-dependent responses were observed (Figure 7). No IC_{50} values were obtained for Noni juice in Jurkat leukemia cells. At the highest concentration tested, noni juice could inhibit the growth of Jurkat cells by about 40%. In addition, no IC_{50} value was obtained for any of the extracts in MCF7 breast cancer cells, and a flat curve was observed (Fig. 8). The IC_{50} values have been summarized in table 2.

Table 1. Effects of plant extracts on mitochondrial respiratory control ratio (RCR)

Extracts	RCR
Solvent	2.82 ± 0.16
T. officinale	3.23 ± 0.80
M. citrifolia	3.15 ± 0.37
Noni juice	2.49 ± 0.70
M. thonningii	3.52 ± 0.83

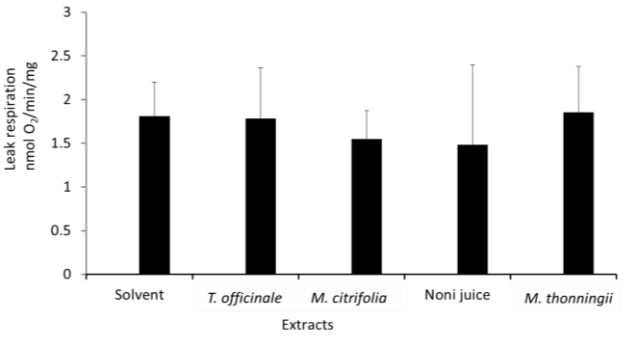

Figure 6. Effects of the extracts on leak respiration (proton leak across the inner membrane)

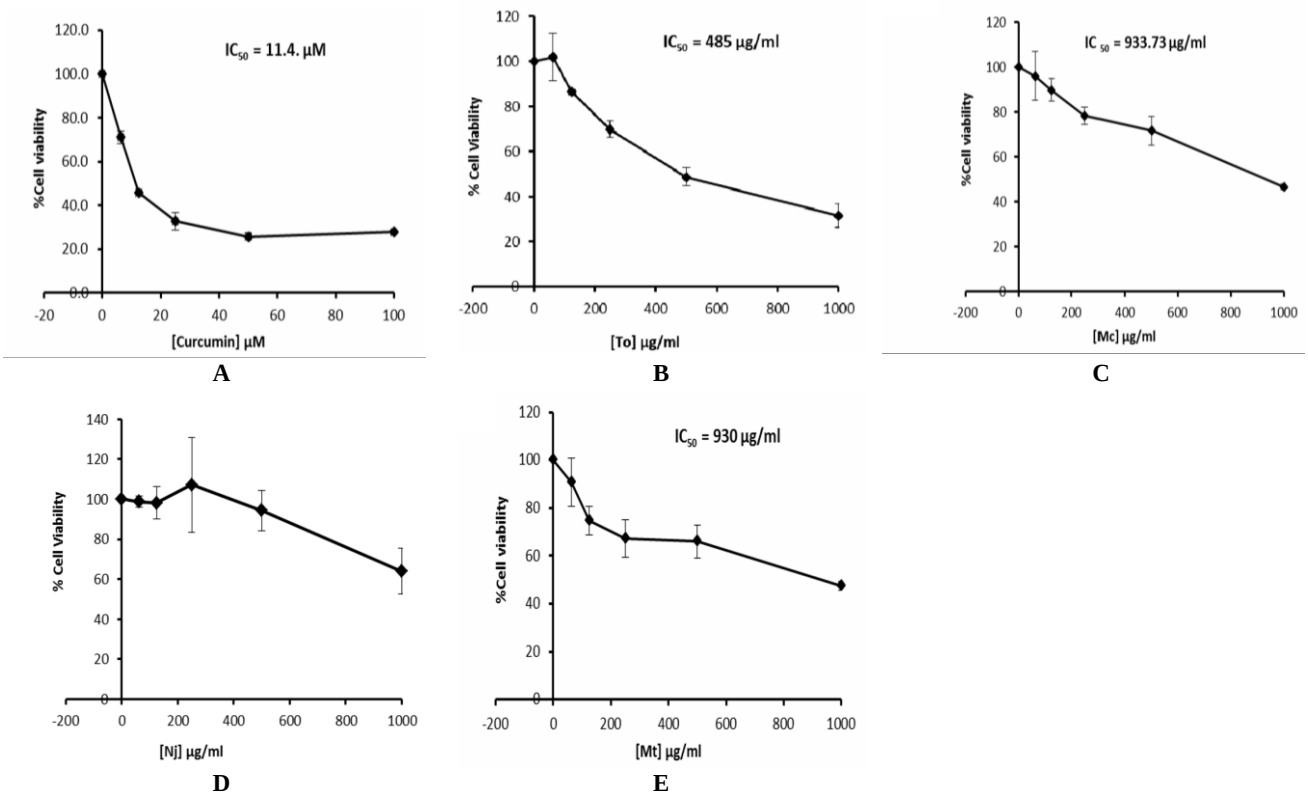

Figure 7. Dose-response curves showing the effects of extracts on cell viability in Jurkat leukemia cells: A. Curcumin (positive control), B. *T. officinale* (To), C. *Morinda citrifolia* (Mc), D. Noni juice (Nj), E. *Millettia thonningii* (Mt); Data represent mean ± S.D. of n = 3.

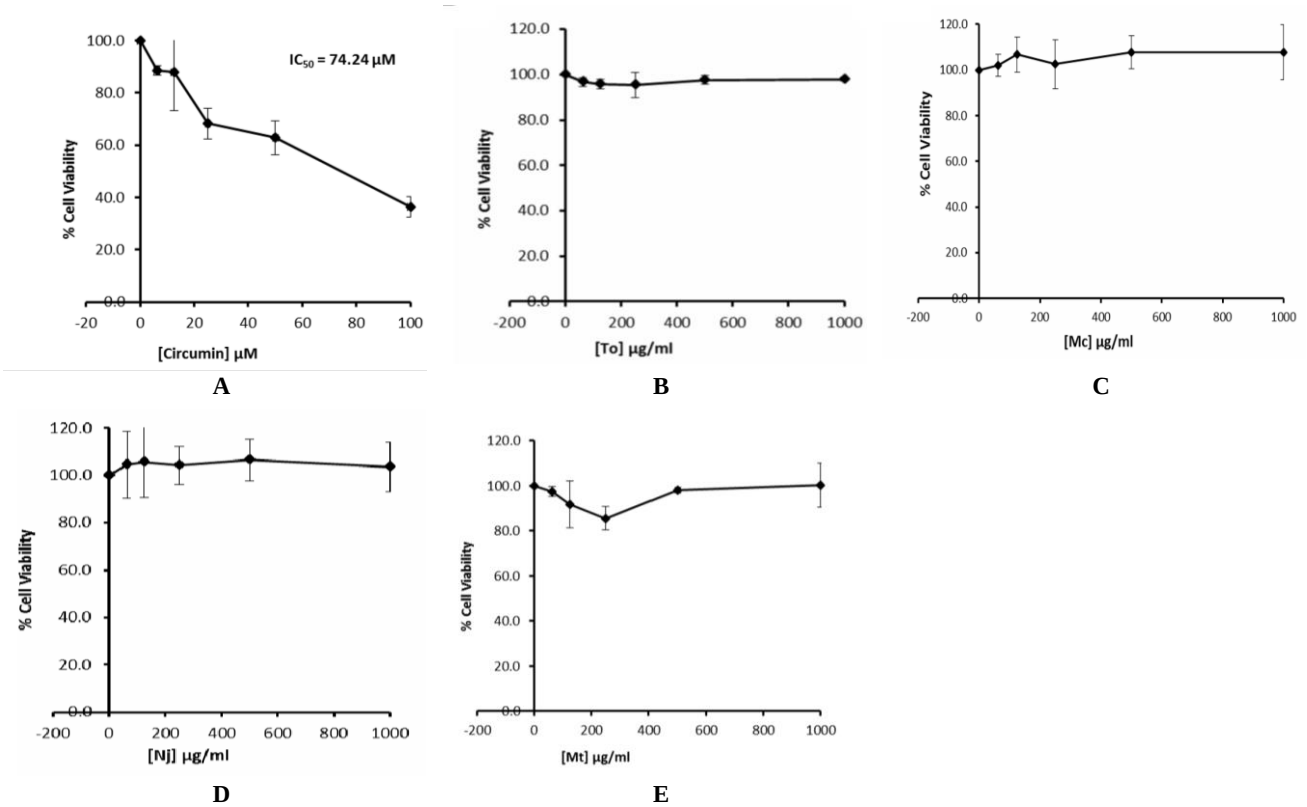

Figure 8. Effects of extracts on MCF7 breast cancer cell viability: A Curcumin (positive control) B. *T. officinale* (To) C. *M. citrifolia* (Mc) D. Noni juice (Nj) E. *M. thonningii* (Mt); Data represent the mean ± S.D. of n = 3.

Table 2. IC_{50} of plant extracts and curcumin (positive control)

Extract	I.C. 50	
	Jurkat leukemia cells	MCF7 breast cancer cells
Curcumin	11.4 µM	74.74 µM
T. officinale	485 µg/mL	-
M. citrifolia	933.33 µg/mL	-
Noni juice	-	-
M. thonningii	930 µg/mL	-

Note: IC_{50}; Fifty percent inhibitory concentration values of the plant extracts and the positive control (curcumin)

Antioxidant activities of the selected plant extracts

Figure 9 and Table 3 show the extracts and Noni juice's DPPH scavenging activities. Figure 9 shows the DPPH scavenging activities of the extracts and the standard over the concentration range of 0-5 mg/mL. Table 3 summarizes the EC50 values. All extracts scavenged DPPH radicals in a dose-dependent manner (Fig. 9). The EC_{50} value for *M. thonningii* (0.21 mg/mL) was smaller compared to that of BHT (0.43 mg/mL). *T. officinale* also had a lower EC_{50} value of 0.31 mg/mL than the standard BHT. *Noni juice and M. citrifolia* had higher EC_{50} values of 1.98 mg/mL and 0.60 mg/mL, respectively, compared to BHT. Among the extracts, *M. thonningii* had the smallest EC_{50} value, while Noni juice had the highest EC_{50} value.

Phenolic content in the plant extracts

The phenolic content of the four extracts was determined from the gallic acid calibration curve and is indicated in Table 4. *M. thonningii* had the highest phenolic content, followed by *M. citrifolia* and *T. officinale,* and Noni juice which had the lowest phenolic content.

Table 3. EC_{50} of plant extracts and standard (BHT)

Extract	E.C. 50
BHT	0.43
T. officinale	0.31
M. citrifolia	0.60
Noni juice	1.98
M. thonningii	0.21

Note: EC_{50}; Fifty percent effective concentration values of the plant extracts and the standard (BHT)

Table 4. Phenolic content of the extracts

Extract (5 mg/mL)	[Phenolics] mg GAE/100g
T. officinale	7127.98 ± 0.16
M. citrifolia	7338.92 ± 0.17
Noni juice	3036.69 ± 0.17
M. thonningii	23880.38 ± 0.14

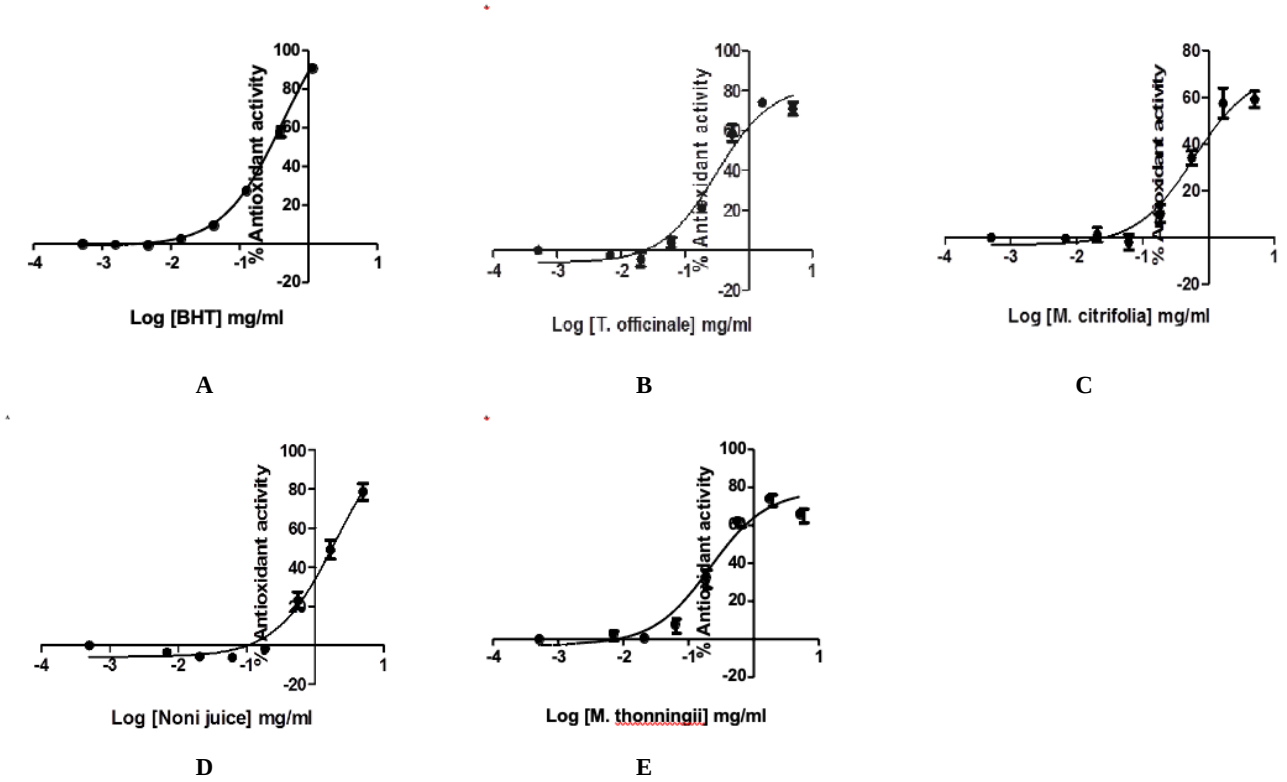

Figure 9. Antioxidant activities of BHT and extracts: A. Butylated hydroxyl toluene (BHT) B. *T. officinale* C. *M. citrifolia* D. Noni juice E. *M. thonningii*; Radical scavenging capacity of each extract calculated as the percent DPPH radical scavenging effect; Each data point is mean ± S.D. of n = 3

Phytochemical constituents of the selected plant extracts

Qualitative tests were performed to determine the extracts' alkaloids, flavonoids, cardiac glycosides, saponins, tannins, and terpenoids. Table 5 shows the results from the phytochemical analysis. All the plant extracts harbored saponins, as evidenced by the presence of emulsion. All except Noni juice tested positive for the presence of tannins. Only *M. citrifolia* and *Taraxacum officinale* tested positive for flavonoids, while only *M. citrifolia* tested positive for the presence of alkaloids. Terpenoids and glycosides were not found in all four extracts.

Discussion

Mitochondria are crucial and unique organelles sensitive to several xenobiotics, including drugs and phytochemicals. Some xenobiotics exhibit pharmacological effects and toxicity by interfering with mitochondrial function. Since ancient times, medicinal plants have contributed to health care and contain several phytochemicals. The present study extracts three medicinal plants to determine their phenolic content, antioxidant activities, phytochemistry as well as their effects on mitochondrial respiration and cell viability. The data indicated that all the extracts showed powerful antioxidant activities and contained phytochemical compounds known to have antioxidant properties. Nevertheless, all the extracts affected mitochondrial respiratory chain activity.

Effects of the selected plant extracts on mitochondrial respiratory chain activity

All extracts demonstrated some effect on mitochondrial respiratory chain activity. This observation agrees with previous findings that plant xenobiotics interfere with mitochondrial function (Forbes-Hernandez et al., 2014). All the tested extracts significantly stimulated basal respiration (Figures 2-5), indicating that they may contain active compounds that act as substrates and donate electrons to the enzyme complexes stabilizing their activities. Furthermore, an increase of 5-20% in respiration was observed in the extracts with the addition of cytochrome C. This phenomenon indicates that the outer mitochondrial membrane remained intact throughout the respiration assays (Figures 2-5).

Table 5. Qualitative screening of the phytochemicals in the extracts

Phyto-chemicals	Extract			
	T. officinale	***M. citrifolia***	**Noni juice**	***M. thonningii***
Alkaloids	-	+	-	-
Flavonoids	+	+	-	-
Glycosides	-	-	-	-
Saponins	+	+	+	+
Tannins	+	+	-	+
Terpenoids	-	-	-	-

Note: + = present; -= absent

Effects of the extracts on respiratory chain enzyme complexes

Taraxacum officinale seemed to have caused a decrease in complex I respiration. This phenomenon suggests that it may contain phytochemical compounds that inhibit complex I activity (Figure 2). *T. officinale* also seemed to have decreased the rotenone-inhibited respiration, which contradicted the previous finding (Ocloo et al. 2015), where the aqueous extract of *T. officinale* caused an increase in the rotenone-inhibited respiration and blocked the effect of rotenone. Complex II respiration was decreased in the presence of *T. officinale* extract, indicating that this extract may contain phytochemical compounds that inhibit complex II activity. Similar findings were made by Ocloo et al. (2015), where the aqueous extract of *T. officinale* caused a decrease in complex II respiration. *However, consistent with previous findings* (Ocloo et al. 2015), the extract of *T. officinale* causes an increase in complex IV activity and thus may contain electron-donating phytochemicals to complex IV stabilizing its activity. The discrepancy between the results obtained in this study and obtained by Ocloo et al. (2015) may be due to varied chemical constituents in the *T. officinale* extracts due to differences in plant location, time of harvesting, and preparation method.

Morinda citrifolia appears to have decreased complex I respiration, suggesting that it may contain phytochemical compounds that inhibit complex I activity (Figure 3). *M. citrifolia* also appears to have decreased complex II respiration, suggesting that it may contain active compounds that inhibit complex II activity. However, *M. citrifolia* was the only extract that significantly increased complex IV respiration. This suggests that *M. citrifolia* might contain phytochemical compounds that donate electrons to complex IV and stimulate its activity.

Noni juice appears to have decreased complex I respiration, suggesting that it may contain phytochemical compounds that inhibit complex I activity (Figure 4). However, it does not appear to alter complex II respiration. Noni juice was the only extract that caused an increase in antimycin A-inhibited respiration. This suggests that the juice may contain compounds that donate electrons downstream of complex III to complex IV and block the effect of antimycin A. Respiration of complex IV is also increased in the presence of Noni juice, suggesting that Noni juice extracts may contain phytochemical compounds that stimulate complex IV activity by donating electrons to complex IV.

Figure 5 shows that *M. thonningii* did not affect complex I respiration. This result contradicts the previous study, which showed *M. thonningii* seeds inhibited complex I respiration (Lyddiard and Whitfield 2001). Among other extracts, *M. thonningii* was the only extract that caused an increase in the rotenone-inhibited respiration, suggesting that it may contain phytochemicals that donate electrons downstream of complex I, II, III, and IV and block the effect of rotenone.

Millettia thonningii appears to have decreased complex II respiration, suggesting that it may contain phytochemical compounds that inhibit complex II activity. *M. thonningii leaves* are known to have molluscicidal activity (Harrison

et al., 2011). Inhibition of complex II activity by *M. thonningii* extract may partly explain the mechanism involved in leaf mollusk activity. In addition, *M. thonningii* stimulated complex IV activity, suggesting that *M. thonningii* may contain phytochemical compounds that deliver electrons to complex IV and stabilize its activity.

Effects of the extracts on ATP synthesis

ADP-stimulated respiration was increased only in the presence of *M. thonningii* extract, suggesting that this extract may contain phytochemicals that stimulate the synthesis of ATP (Figure 5). However, the other extracts appeared to have decreased ADP-stimulated respiration, indicating that they may contain compounds that inhibit ATP synthesis (Figures 2-4). Previously, the aqueous extract of *T. officinale* did not affect ADP-stimulated respiration (Ocloo et al., 2015).

Effects of the extracts on respiratory control ratio (RCR)

The respiratory control ratio can be defined as the ratio of state three respiration to state two respiration. It is a way of measuring coupling efficiency. All the extracts appeared to have increased coupling (Table 1). On the other hand, Noni juice appeared to have decreased coupling compared to the solvent, casting doubt on its effectiveness as an energy booster. Similar to these results, a previous study showed that the aqueous extract of *T. officinale* also increased coupling (Ocloo et al., 2015).

Effects of the extracts on leak respiration

Oxidative phosphorylation is not perfectly coupled because protons can leak across the inner membrane. Leaked respiration is an indirect measure of the leakage of protons across the inner membrane. It is also an indirect estimate of the coupling efficiency. It is measured as mitochondrial respiration in the absence of ADP but in the presence of reducing substrates (Gnaiger 2012). High leakage respiration indicates high uncoupling. None of the extracts significantly altered respiratory leakage compared to the solvent (Figure 6), indicating low proton leakage across the inner mitochondrial membrane.

Cytotoxicity of the selected plant extracts

The cytotoxicity of the selected plant extracts was measured by the effects of the extracts on mitochondrial function using the MTT assay. The principle of MTT assay is based on the enzymatic reaction in the mitochondria that converts MTT to formazan in viable cells (Slater et al. 1963). The extracts appeared to show dose-dependent cytotoxic effects on the Jurkat leukemia cells (Figure 8). However, according to the U.S. National Cancer Institute, a plant extract is considered to have an active cytotoxic effect if the IC_{50} value is 30 µg/mL or less (Geran et al. 1972; Lee and Houghton 2005). All the extracts demonstrated IC50 values greater than 100 µg/mL in the present study. Based on the MTT result, the extracts had weak cytotoxic effects on the Jurkat leukemia cells. Thus, they lack the potential to alter mitochondrial function significantly. The extracts also appeared to have had no cytotoxic effect on the MCF7 breast cancer cells (Figure 8), suggesting that mitochondrial function might be unaffected by the extracts.

Anecdotal information on the anticancer activity of *T. officinale* has been reported previously (Sigstedt et al. 2008). The aqueous leaf extract of *T. officinale* has been shown to reduce the growth of MCF-7/AZ breast cancer cells in a dose-dependent manner (Sigstedt et al. 2008). This inhibitory effect is ascribed to the presence of phenolic compounds in the Dandelion leaves (Schütz et al., 2006). However, *T. officinale* showed weak cytotoxic effects on the cell lines used in this study. It is also possible that *T. officinale* acts as a pro-drug; thus, *in vitro* assays alone may be inappropriate for investigating its anticancer property.

There are also reports of the anticancer properties of Noni juice. Fruit juices have been reported to have significant antitumor activity against peritoneal carcinomatosis LLC (Hirazumi et al. 1996). In addition, the methanol extract of the fruit showed cytotoxic activity against breast cancer (MCF7) and neuroblastoma cell lines (LAN5) (Arpornsuwan and Punjanon 2006). Another study showed that polysaccharide-rich substances precipitated ethanol from Noni juice had immunomodulatory and antitumor activity against ascitic tumors Sarcoma 180 in mice (Furusawa et al. 2003). However, Noni juice had a weak cytotoxic effect against the cancer cell lines based on this study. This result does not agree with the observations in the earlier studies. The difference in cytotoxic effect is probably due to the juice preparation methods since the practice is quite elaborate. Noni juice is fermented for two months, during which light intensity, pH, and temperature are carefully monitored. This may or may not be followed by pasteurization. Furthermore, this study did not investigate methanolic extracts of the fruit reported to possess cytotoxic activity.

Although there have been reports of anticancer activity of extracts of *T. officinale* and Noni juice, the findings obtained in this study do not support these claims. The weak cytotoxic effects of the extracts on the cell lines are unclear due to the lack of information on the anticancer properties of the *M. citrifolia* and *M. thonningii* leaves.

Antioxidant activities and phenolic contents of the selected plant extracts

Free radicals such as reactive oxygen species (ROS) are typical by-products of respiration. However, an overload of free radicals may lead to oxidative damage. Imbalances of cellular redox homeostasis contribute to the pathogenesis of diseases such as diabetes, atherosclerosis, immunosuppression, and neurodegeneration (Hensley et al., 2000; Gupta et al., 2012). Antioxidants are chemical compounds that include nutrients, vitamins, and minerals, scavenging free radicals and protecting cellular DNA, proteins, and lipid membranes (Osawa et al. 1990). In addition, antioxidants have been implicated in maintaining human health and preventing and treating several diseases (Halliwell and Gutteridge 1981).

Of the plant extracts analyzed, *M. thonningii* showed the strongest antioxidant activity (Table 3). On the other hand, noni juice showed the lowest antioxidant activity

among the four extracts. However, Noni juice is considered a good source of antioxidants and polyphenols (Bramorski et al., 2010; De-Lu et al., 2013). *M. citrifolia* leaves are well known for their strong antioxidant activity and are safe in acute, subacute, and subchronic oral toxicity tests in rats (West et al., 2007; Serafini et al., 2011). The results obtained in this study confirm the potent antioxidant activity of the *M. citrifolia* leaves. *T. officinale* is an easily accessible source of natural antioxidants (Amin et al., 2013). In a previous study, *T. officinale* effectively scavenged free radicals released in the liver in rats with CCl4-induced hepatotoxicity (Gulfraz et al. 2014). Dandelion also reduced atherosclerosis by reducing oxidative stress in cholesterol-fed rats (Choi et al. 2010). The results obtained in this study agree with previous findings and confirm the antioxidant properties of *T. officinale*.

Phenols have been shown to have antioxidant activity against free radicals. The antioxidant action of phenolic compounds is due to their high tendency to chelate metals. Phenolics have hydroxyl and carboxyl groups, able to bind mainly iron and copper (Jung et al. 2003). Therefore, measuring the concentration of phenolic hydroxyl groups in plant extracts is another way to confirm antioxidant capacity. In addition, a high linear correlation has been observed between the radical scavenging activity of DPPH and the total phenolic content (Ghafar et al., 2010). Li et al. (2009) also identified a significant positive correlation between antioxidant effects and the presence of phenolic compounds in *Radix angelica sinensis.* The results obtained in this study are in accordance with previous findings. Intriguingly, *M. thonningii*, which showed the most potent antioxidant activity, had the highest phenolic content. On the contrary, Noni juice which showed the weakest antioxidant activity had the lowest phenolic content in the present study (Table 3).

Phytochemical constituents of the selected plant extracts

Studies on medicinal plants have indicated phytochemicals such as phenolics, flavonoids, and tannins (Hill 1952). These phytochemicals often determine the physiological action of medicinal plants on the human body and have beneficial effects on health (Pamplona-Roger, 1998; Ayoola et al., 2008). Some phytochemicals are antioxidants and thus attenuate the deleterious effects of reactive oxygen species. The plant extracts were screened for six classes of phytochemicals commonly found in most plants in the current study. These phytochemicals include alkaloids, flavonoids, saponins, glycosides, tannins, and terpenoids. The result showed that each extract contained at least one of these phytochemicals.

The aqueous extract of *Millettia thoninngii* leaves contained saponins and tannins (Table 5). Saponins and tannins are phytochemicals known to have antioxidant properties (Chen et al., 2014; Forbes-Hernández et al., 2014). Both phytochemicals, as well as glycosides, alkaloids, flavonoids, and terpenoids, are in the leaves of *M. Thonningii* (Borokini and Omotayo (2012). In another study, isoflavonoids were placed in the deuterochloroform extract of hexane-defatted seeds of *M. thonningii* (Lyddiard and Whitfield 2001).

Saponins, flavonoids, and tannins were identified in the aqueous extract of *T. officinale* leaves (Table 5). These phytochemicals are well-known plant components (Yarnell and Abascal 2009). Flavonoids are polyphenols with antioxidant properties (Pietta 2000; Forbes-Hernández et al. 2014). In the earlier studies, *T. officinale* leaves were rich in phenolics and other antioxidants such as vitamin A, vitamin C, zinc, and copper (Schmidt 1979; Jackson 1982; Yarnell and Abascal 2009).

Morinda citrifolia leaves contain alkaloids, flavonoids, saponins, and tannins (Table 5). These secondary metabolites have been identified in previous studies on the *M. citrifolia* plant (Singh et al., 2012; Pandy et al., 2014). Other constituents identified in the *M. citrifolia* products include vitamin A, vitamin C, rutin, and flavone glycosides (Higa and Fuyama 1993; Farine et al. 1996). Several alkaloids have been shown to have antioxidant properties (Maiza-Benabdesselam et al., 2007). Noni juice is a rich source of antioxidants such as flavonoids (Nijveldt et al. 2001). Studies on the chemical composition of noni juice also show that the dominant phenolic compounds in noni juice function as free radical scavengers and prevent several diseases (Dixon et al. 1999; Chan-Blanco et al. 2006). Contrary to these findings, only saponins were identified in the juice.

The extract contains various classes of phytochemicals and has been shown to affect mitochondrial function. However, the phytochemical analysis carried out in this study was only limited to qualitative analysis because the quantitative analysis was not carried out. Therefore, the relationship between the phytochemicals of the extract and the observed effects on mitochondrial function is unclear. *M. thonningii*, for example, had the highest antioxidant activity and the highest phenolic content, including saponins and tannins. Saponins and tannins have antioxidant properties. Therefore, it is not surprising that *M. thonningii* extract appears to have stimulated complex IV activity and removed inhibition of complex I by rotenone. However, because the concentrations of the saponins and tannins in the extract were not determined, it is difficult to tell if these effects result from these phytochemicals alone or a combination of other factors.

Comparing the four extracts showed a similar effect on complex IV by stimulating complex IV activity. They also increase basal respiration in the absence of a substrate. However, the extract results in complexes I, II, and III differed. Each extract contains saponins. *T. officinale*, *M. citrifolia*, and *M. thonningii* also contain tannins. *T. officinale* and *M. citrifolia* contained flavonoids and saponins, and tannins and alkaloids were also detected in *M. citrifolia*. The amount of each phytochemical that contributes to the observed antioxidant effects of the mitochondrial complex was not determined in this study. The varied impact on complexes I, II, and III may be attributed to different concentrations of the phytochemicals in each extract. For example, although four phytochemicals were identified in *M. citrifolia* and only two were identified in *M. thonningii*, the latter had a higher antioxidant activity,

probably due to higher concentrations of the phytochemicals identified in this extract. No clear association was observed between the phytochemicals identified in each extract and their observed effects on mitochondrial function.

A positive linear relationship between antioxidant activity and the anticancer effect of the aqueous extracts of five herbal plants *(L. chinensis, R. officinale, S. officinalis, A. Pilosa, and P. polyphylla)* was identified by comparing the percentage free radical scavenging capacity and percentage growth inhibition on MCF-7 and A549 cells (Li et al. 2007). This result suggested that the antioxidants present in the herbal water extracts might have contributed to their anticancer effects on both cell lines. However, no relationship between antioxidant and anticancer activity was observed in this study. Although the extract exhibited prominent antioxidant activity, its growth inhibitory effect on Jurkat leukemia cells and MCF-7 cells was generally weak.

In conclusion, all the extracts significantly stimulated basal respiration, and they all appeared to increase complex IV respiration, albeit only *Morinda citrifolia* produced a significant increase. All the extracts showed vigorous antioxidant activities, and all of them contained phytochemicals exhibiting antioxidant properties. *M. thonningii* partially blocks the effects of rotenone. Noni juice partially blocks the effects of antimycin A. Finally, all the extracts demonstrated weak cytotoxic effects on Jurkat human leukemia and the MCF7 human breast cancer cell lines.

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The potential of white-oyster mushroom (*Pleurotus ostreatus*) as antimicrobial and natural antioxidant

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Abstract. *Egra S, Kusuma IW, Arung ET, Kuspradini H. 2019. The potential of white-oyster mushroom* (Pleurotus ostreatus) *as antimicrobial and natural antioxidant. Biofarmasi J Nat Prod Biochem 17: 14-20.* White-oyster mushroom (*Pleurotus ostreatus*) is a favorite meal in Indonesia. Previously, this fungus was known as a useless plant, but everything changes after the nutrition is known. People try to cultivate it because the nutrients contents are very good for body health. Therefore, this research needs to be done to support this added value in health, especially antimicrobials and antioxidants. This research used successive extraction with hexane solvent, acetate ethyl, ethanol, water, and crude ethanol by antimicrobial assay, antioxidants assay (DPPH), total antioxidant content, and total phenolic content. The highest results on the antimicrobial barrier test against Candida albicans bacteria was 47.60 % with 100 ppm concentration. While on antimicrobial assay using Propionibacterium acnes bacteria, there was no significant inhibition. Regarding the antioxidant test against DPPH, the result showed the occurrence of free radicals by 25 % on water extraction at a concentration of 100 ppm. Continuously, the total antioxidant content assay showed that ethyl acetate had the highest value of 368.708 mg gae/gr. The total content phenolic assay results showed that the solvent hexane had a 78.495 mg gae/gr value. These findings indicated that the mushroom has an active phenolic compound with no contribution to impede its working on *Candida albicans* assay.

Keywords: Antimicrobial, total antioxidant, total phenol, white-oyster mushroom

INTRODUCTION

Mushrooms became more popular in Indonesia when people realized their benefits. Over the years, people have consumed several varieties of mushrooms, such as white-pink oysters, shitake, black jelly, and straw mushrooms, because of their benefits. Today, mushrooms have been processed and consumed in various forms as vegetables, crackers, and herbs for health purposes. Most Indonesian people start cultivating oyster mushrooms because of their efficacy and high economic value, and they provide high income for cultivators. Nasrul (2004) stated that historically, it had been used as food since 3,000 years ago, exclusively presented to the King of Egypt, while the Chinese have used it as herbs since 2,000 years ago.

Mushrooms are highly nutritious and cholesterol-free foods (Nasrul 2004). Sumarmi (2006) states that 100 grams of oyster mushrooms contain protein (19-35 %) which consists of 9 amino acids; fat (1,7-2,2 %), in which 72 % are unsaturated fatty acids, carbohydrates, vitamins B (thiamin, riboflavin, and niacin), vitamin D and C, minerals (K, P, Na, Ca, Mg, Zinc, Fe, Mn, Co, and Pb), and the levels of metal-microelements are very low, so it is safe to be consumed every day.

According to Chang and Buswell (1996), mushrooms are delicious food. Some mushrooms have been known to have biological activities such as anti-cancer, anti-diabetes, overcoming hyperlipidemia, and increasing the immune system. According to Bobek et al. (1998), oyster mushrooms are good for cardiovascular patients and can control cholesterol levels. The community owns limited knowledge about the benefits of mushrooms, so they consume them only as additional dishes. This research was conducted to determine the potential of white-oyster mushrooms (*Pleurotus ostreatus*) as antimicrobial and natural antioxidants.

MATERIALS AND METHODS

Plant materials and chemicals

This research was carried out at the Chemistry Laboratory, Department of Forest Products Technology, Faculty of Forestry, Mulawarman University, Samarinda, Indonesia. The research material (fresh oyster mushrooms) was obtained from the Faculty of Forestry, Mulawarman University, Samarinda, Indonesia. The chemical used are *n*-hexane, ethyl acetate, ethanol, acetone, isolates of *Propionibacterium acnes* and *Candida albicans* provided by Forest Products Chemistry Laboratory in Mulawarman University, nutrients agar (Difco, USA), chloramphenicol, and glucose (Merck, Germany). The equipment used in this research was Shimadzu UV-VIS 1240 spectrophotometer (Shimadzu, Japan), an evaporator (Eyela, Japan), and an autoclave provided by all American Model 25X-2.

Extraction

White-oyster mushroom (*Pleurotus ostreatus*) (500 grams) was cut into small pieces and dried at 39oC for 48

hours in the oven. The dried mushroom was ground into a coarse powder in a blender. The powder was extracted successively with n-hexane, ethyl acetate, and ethanol. The powder was extracted separately with ethanol to obtain crude ethanol. The extraction was conducted at room temperature with continuous shaking on a shaker (7400 Tübingen; EdmunBuchler, Germany) for 48h., followed by filtration of suspension with Whatman filter paper No. 2 (Maidstone, UK). The crude alcohol extracts were evaporated at 40ºC and put in a vacuum oven to near-dried extracts to yield the mushroom extracts.

Antimicrobial assay

Antimicrobial assays were conducted using the agar well disk diffusion method of Kuspradini (2012). *Propioni bacterium acnes* and *Candida albicans* were used in all experiment. Nutrient agar (Difco) was the main media to culture bacteria. 20 mL aliquots of sterile media were transferred to Petri dishes and allowed to solidify. The media were inoculated with 20 µL of microbial suspension spread uniformly on the surface of the plates. After that, the media agar plates were cut using a sterile cork-borer with a size seven-mm well, and a 20 µL acetone solution containing 25-100 µg extracts was added to the well. Chloramphenicol was used as a positive control at the concentration of 10 µg/20 µL in each well. The plates were incubated in the dark at 32ºC for 24 h. Microbial activity was measured (mm) by the existence inhibiting zone around agar diffusion well-containing extract. Microbial activity was calculated as the mean inhibition zone for the test sample divided by the mean inhibition zone for the standard drug. (Kuspradini 2012).

Total antioxidant capacity

Total antioxidant capacity was evaluated according to the method described by Prieto et al. (1999). 5 mg sample was dissolved in 1000 µL ethanol. 100 µL sample solution was added by 1 mL Reagent (0.6 M sulfuric acid + 28 mM sodium phosphate + 4 mM ammonium molybdates). Then, the sample was incubated in a water bath shaker at 95ºC for 90 minutes and cooled to room temperature. Measurement was made using a spectrophotometer with an absorbance wavelength of 695 nm. The gallic acid was used as a standard on 0.02 in 0.1 mg/mL and ethanol as a blank.

Total phenolic content

Total phenolic content was determined using the method by Slinkard and Singleton (1977). The sample (5 mg) was dissolved in 1000 µL ethanol. 20 µL sample solution was added to the tube with 100 µL (2 N Reagan Folin-Ciocalteu) and 300 µL from 0.2 mg/mL solution of sodium carbonate and 1180 µL aquadest, and the volume growth of 1600 µL in the reactive tube. Incubation was done at a temperature of 37ºC for 2 hours. The measurement was conducted with a spectrophotometer with a wavelength absorbance of 760 nm. Gallic acid was used as standard.

Total flavonoid content

Total flavonoid content was determined using the method of Choi et al. (2006). 5 mg sample was dissolved in 1000 µL ethanol. 250 µL of the sample solution was put into a test tube and added with 1.25 mL of distilled water and 75 µL of 5% $NaNO_2$ and incubated at room temperature for 5 minutes. 150 µL was added from 10% $AlCl_3$ and incubated at room temperature for 6 minutes. Then it was added with 500 µL of 1 M NaOH and 275 µL of distilled water, and was incubated for 20 minutes for a reaction. Measurements were carried out with a spectrophotometer with an absorbance of 510 nm using distilled water as a blank.

RESULTS AND DISCUSSION

Extraction

The extraction method used was successive maceration at room temperature which three different solvents (*n*-hexane, ethyl acetate, and ethanol). The ideal solvent for the extraction process must have some conditions, namely: (i) it must be able to dissolve the extractive substances, (ii) it must have a similar boiling level to the substance, and (iii) it must be inert (it does not react with substances that will be extracted, (iv) it must have a low boiling point for easy evaporation, but not too low that it can cause the loss of some solvents as a result of the evaporation (Guenther 1987). The extraction is started by macerating the sample for 24 hours with *n*-hexane. The filtrate was filtered with filter paper and evaporated to obtain concentrated hexane extract. The powder of mushroom, which had been extracted with hexane, was re-extracted with ethyl acetate followed by ethanol.

The extract yield can be used as a reference to find out the amount of simplicial needed to make a certain number of thick extracts. According to Lo et al. (1983), the extraction method is one of the factors that will affect the yield of an extract. Extraction using solvents consists of several methods, including maceration, percolation and heat methods, reflux, soxhletation, infusion, decadence, and digestion. Besides, the amount of extract is also influenced by the polarity index in the solvent; the lowest to highest polarity index in this study is, respectively, hexane, ethyl acetate, and ethanol.

Table 1 showed that the highest yield of extract was with the ethanol extract (7.88%), while with the hexane extract, it was less than 1% (0.51%). The yield on extracting mushrooms with acetate ethyl was 6.02%. The *n*-hexane solvent has 0.51% yield.

Table 1. Yield of oyster mushroom extract in several solvents

Extract	Sample weight (g)	Extract yield (g)	Extract (%)
n-Hexane	40	0.18	0.51
Ethyl acetate	40	2.14	6.02
Ethanol	40	2.80	7.88
Crude ethanol	23	0.08	0.39

Note: The percentage of dried-mushroom extract based on the weight

Antimicrobial activity

Antimicrobial assay on white-oyster mushroom (*P. ostreatus*) was done against *P. acnes* and *C. albicans* with agar diffused method. Chloramphenicol was used as a positive control because it has broad spectrum as antibacterial, while acetone was used as a negative control. Microbial resistance to antibiotics is a major problem today. Many biological active components released from plant species are commonly used as drugs, because they can offer a new source of antimicrobial activity. The search for antimicrobial bioactivity from natural materials gives the result of antimicrobial activity from white oyster mushroom (*Pleurotus ostreatus*) which is shown in Table 2.

Chloramphenicol with the zone barriers 24.5mm has presented the results of the test activity of bacteria *P. acnes.* This study showed the extract has no resistance to *P. acnes.* This indicates that there is no active anti-bacterial compound towards *P. acnes.* The absence of active compounds in the extract is thought to be due to very little concentration, perhaps if the concentration is raised it will increase inhibition. Antimicrobial activity of oyster mushroom extract has also conducted against one of the fungus, *C. albicans*. The results of the assay displayed that oyster mushroom extract indicated the formation of barriers on some level zone concentration of extract. Mushroom extract on the concentration of 25 µg showed the lowest activities of anti-candida with inhibition of 8.53 mm width. Increasing inhibition activity was shown on the concentration of 50 µg with inhibition of 10.5 mm width. The best activities were indicated by mushroom extract on the concentration of 100 µg with inhibition of 10.8 mm width. Davis and Stout (1971) reported the inhibiting diameter regions on 5 mm or less then inhibiting activities is categorized as weak; 5-10 mm is categorized as medium; 10-19 mm is categorized as strong, whilst 20 mm or more is categorized as very strong.

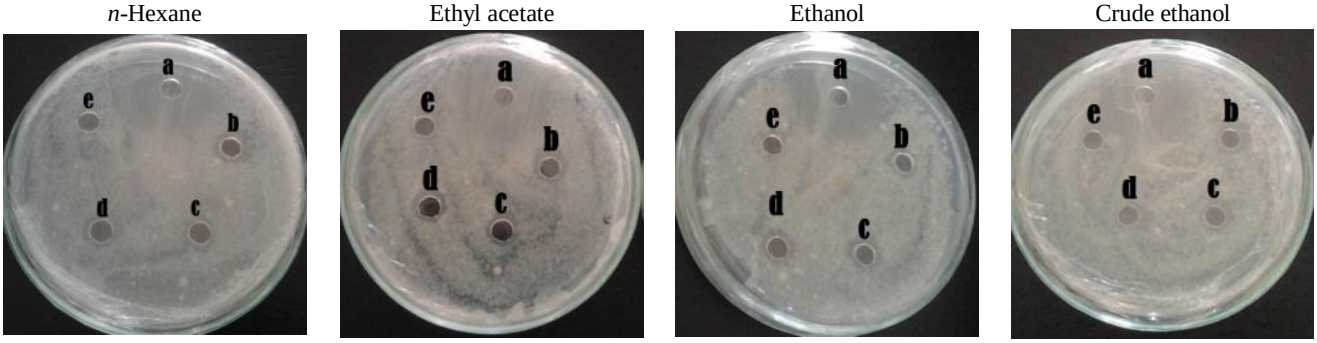
Table 2 showed the inhibition as a whole in medium category as it is around 8-10 mm. Of all solvent, the highest barriers was produced on the rough extract ethanol with 10.8 mm. It was identified on the concentration 100 µg. Oyetayo (2009) reported that fungus extracts are able to inhibit the growth of all kinds of organisms on the concentration between 12.5 mg/mL until 100 mg/mL. This research used the less concentration, 25 µg, 50 µg, 100 µg, and these concentrations have been able to inhibit the growth of the mushroom *C. albicans*.

Nwachukwu and Uzoeto (2010) found that the hot water extract of the *R. vesca* mushroom is able inhibiting the growth of *E. coli*, *S. typhi*, *P. mirabilis*, and *C. albicans*. The ethanol extraction of *A. auricular* showed a broad spectrum of microbial effect against microorganisms experiment with the exception of *S. tophi* and *P. aeruginos. P. squarrosulus* displaying the microbial activity against *K. pneumonia* (6,14 mm), *S. pneuoniae* (5.12 mm), and *C. albicans* (4.10 mm). *P. aeruginosa* has swapped off almost all extract from four species of fungus except hot water extraction from *P. squarrosulus* which shows the inhibition zone (3.41 mm). *V. vulvae* showed the microbial activity against *S. typhi* (4.60 mm). However, Vamanu (2012) reported, the *P. ostreatus* is able to inhibit *Candida albicans* with MIC 12.5, 12.5, 25, 25 mg/mL, continuously on a different nitrogen source, corn, malt, and yeast extraction, and peptone used on the culture media.

Table 2. antimicrobial activity of oyster mushroom against *Candida albicans*

Sample extracts	Inhibition (mm)				Inhibition (%)			
	25 µg	50 µg	100 µg	(+)	25 µg	50 µg	100 µg	(+)
n-Hexane	9.86	9.46	8.63	23.76	41.5	39.8	36.3	100
Ethyl acetate	9.76	9.96	9.7	21.86	44.65	45.56	44.4	100
Ethanol	8.53	9.16	9.06	23.96	35.6	38.2	47.6	100
Crude ethanol	9.3	10.5	10.8	22.7	40.96	46.3	37.8	100

Note: (+) is a positive control (Chloramphenicol)



Picture 1. Inhibition of oyster mushroom against *Candida albicans*. (a) control (-); (b) control (+); (c) concentration 100 µg; (d) concentration 50 µg and (e) concentration 25 µg

Hapsari et al. (2012) reported that the chemical composition at oyster mushroom is alkaloid, saponin, phenolic, and tannin. Tannin plays important role in degradation of microbial cell wall. Ajizah (2004) reported, tannin also has antibacterial capability by precipitating proteins. The effect of antibacterial tannin is through the reaction with the membrane cell, inactivation enzymes, and function of genetic material. This condition makes some fungi used extensively as food for its nutrition in traditional medicine (Stamets 2000; Lindequist et al. 2005). The strong activity of plant extracts against *C. albicans* shows the potential for use as a drug for candidemia, nosocomial infections, and diseases caused by other *Candida infections*

DPPH antioxidant activity

Antioxidant assay was done by scavenging free radicals which are measured at spectrophotometer with DPPH free radicals agent. The positive control used vitamin C because it is known as the best natural antioxidant. The number of free radical scavenging activity is presented as the percentage of the inhibition with an indication of color changes of DPPH. The result of the antioxidant activity mushroom extract oysters is presented in Table 3.

Table 3 displayed that the antioxidants activity by scavenging free radical DPPH is influenced by the type of extraction and concentration levels. The crude ethanol provided the highest inhibition by 21.43% on the concentration of 100 ppm. The results indicated that the use of higher concentrations is potential to improve antioxidant activity. These active compounds were soluble on the ethanol extraction.

Antioxidant activity assay aimed to find out how big a plant will be able to scavenge free radicals. Mechanism of scavenging free radicals is by inhibiting lipid oxidation that can be used to calculate the activity of antioxidant (Pornariya 2009). The crude ethanol extract showed relatively better inhibition with 21.43 % on 0.1 mg/mL even though it was still lowest than vitamin C which was used as a positive control. Mau et al. (2001) and Lo (2005) found that free radical activity in oysters mushroom towards DPPH was 81.8 % on 6.4 mg/mL and 68.4% on 5 mg/mL. It specified that extracting concentration on antioxidant activity assay plays an important role to produce the high antioxidant activity.

White Oyster mushroom (*Pleurotus ostreatus*) is able to be an antioxidant because it provides phenolic, L-ergotien, selenium compound and vitamin C (Jayakumara et al. 2006). Phenolic compounds are capable to inhibit the oxidation reaction and reduce hydroxyl radical, superoxide, and peroxide. Phenolic also has an effect on the process of synthesis transcription of endogenous antioxidant, namely glutation (Khotimah 2008). 250 mg/kg BW white oyster mushroom extraction has a great antioxidant effect because it contains antioxidant substances, phenol, ergotien, vitamin C, selenium, and beta-carotene. Phenol compound is the component with the highest antioxidant activity on white oysters mushroom. The research to find antioxidant compounds must continue to be done, because it is very important in finding solutions to diseases caused by free radicals, in testing oxidative reactions in food, and in protecting against DNA damage and carcinogenesis. In the future this substance will function in many ways such as pharmacological activity; anti-inflammatory, anti-bacterial, and anti-fungal.

Total antioxidant capacity

Total antioxidant content assay has been done to find out how many active compounds are able to scavenge free radicals on the mushroom. Surekha et al. (2011) concluded that mushroom is healthy food, moreover its rich protein and antioxidant compounds which are essential compound are able to fight disease. Total antioxidant content assay results from several extraction methods on mushroom are presented in Table 4.

Table 4 showed that the oyster mushroom extractions have good antioxidant activity. The ethyl acetate has the highest antioxidant content values which are followed by hexane extract, ethanol, and crude ethanol extract. Table 4 displayed the different colors on the assay solvent indicating the existing active compounds. Polyphenol is one of contributed antioxidant activity on the fruit, vegetables, and fungus (Ferreira et al. 2007).

Table 3. Antioxidant activity of oyster mushroom extract against DPPH

Sample	Inhibition (%)		
	25 ppm	50 ppm	100 ppm
Vitamin C (positive control)	96.84	96.84	96.85
n-hexane	3.92	2.41	0.30
Ethyl acetate	2.72	2.72	6.34
Ethanol	2.25	8.20	13.88
Crude ethanol	17.85	17.85	21.43

Table 4. The total antioxidant capacity of *Oysters mushroom*

Samples	Absorbance			Antioksidan capacity (mg GAE/g)
	Rep. 1	Rep. 2	Rep. 3	
n-hexane	1.283	1.238	1.352	313.625
Ethyl acetate	1.485	1.523	1.526	368.702
Etanol	0.548	0.563	0.544	128.792
Crude Etanol	0.479	0.502	0.505	114.708

Note: Rep.= Repetition

Table 5. The total phenolic content of oyster mushroom

Samples	Absorbance			Antioksidan capacity (mg GAE/g)
	Rep. 1	Rep. 2	Rep. 3	
n-hexane	0.28	0.3	0.28	78.495
Ethyl acetate	0.23	0.2	0.21	57.733
Etanol	0.07	0.09	0.1	22.495
Crude Etanol	0.09	0.08	0.09	21.352

Note: Rep.= Repetition

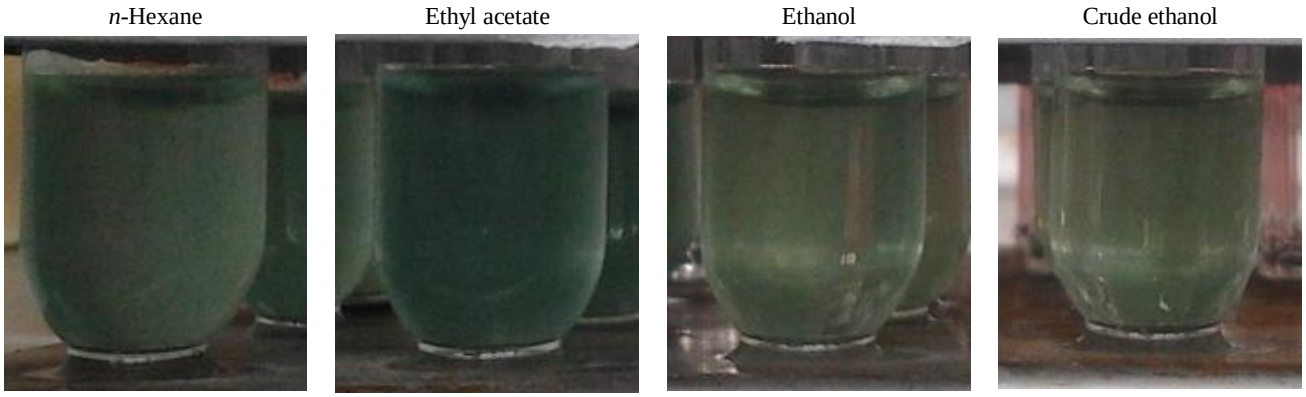


Figure 2. The result of total antioxidant capacity assay

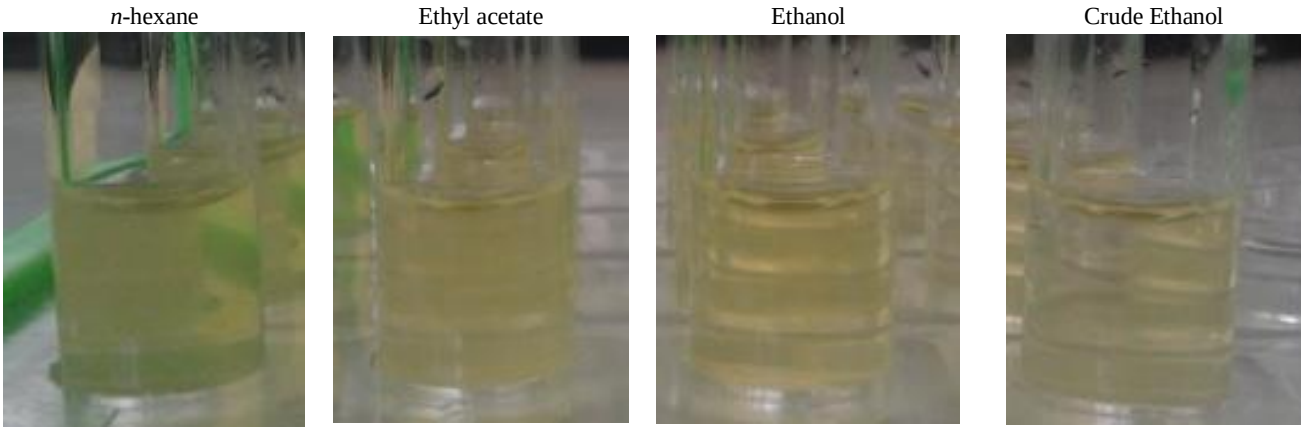


Figure 3. The result of total antioxidant content

The high antioxidant content on oyster mushroom extract allegedly because the polyphenol is found on the oyster mushroom. Hapsari et al. (2012) reported that oyster mushroom extraction has antioxidant and tyrosine activities with the result that the ethanol extraction has higher activity than water extraction. It indicated that solvent ethanol is more dissolving antioxidants active compounds than water which is polar. The Culinary-medicinal mushroom research which found a good antioxidant content and potentially antihypertensive can be seen from the occurrence of inhibition of oyster mushroom to ACE (angiotensin I-converting enzyme).

Total phenolic content

Total phenolic content assay have been done to find out how much the content of active phenol compounds on the oyster mushroom. Phenol components, flavonoid, anthocyanin, and carotenoids develop the main components of the natural antioxidant which scavenging free radicals due to its ability to divide the hydrogen atoms or electron and the balance of radical compound (Shahidi and Wanasundra 1992). Table 5 shows the results in total phenolic content from oyster mushroom extract on some of the solvents.

Table 5 presented that the highest rate of phenol content is from the extract by hexane followed by ethyl acetate, ethanol and crude ethanol which indicates that oyster mushroom has active phenolic compounds. Alvarez (2007) reported that mushroom can be used as the source of low calories and fat on food with the high polyphenol and antioxidants activity level. Phenol compound is the component with the highest antioxidant activity on the white-oyster mushroom. The activities are not only mainly caused by the capability in reducing hydrogen and singlet oxygen quencher but the component also has the potential metal chelation effect (Polite 2010).

Phenolic acid plays the main role to phenolic components in the mushroom (Ferreira 2009). According to Puttaraju (2006), galic, tannin, protocatechuic, and gantesik acids are some main phenolic content detected in the water extraction from some Indian mushroom traditional food. Abdullah et al. (2012) stated that the total phenolic from some varieties of mushroom extraction assay started at 6.19 to 63.51 mg GAE/g with G lucidum having the highest phenolic content (63.51 ± 1.11 mg GAE/g). Iwalokun (2007) reported the phenolic content and the antioxidant content on acetone extract of *P. ostreatus* is equal to petroleum ether extract of *P. ostreatus*.

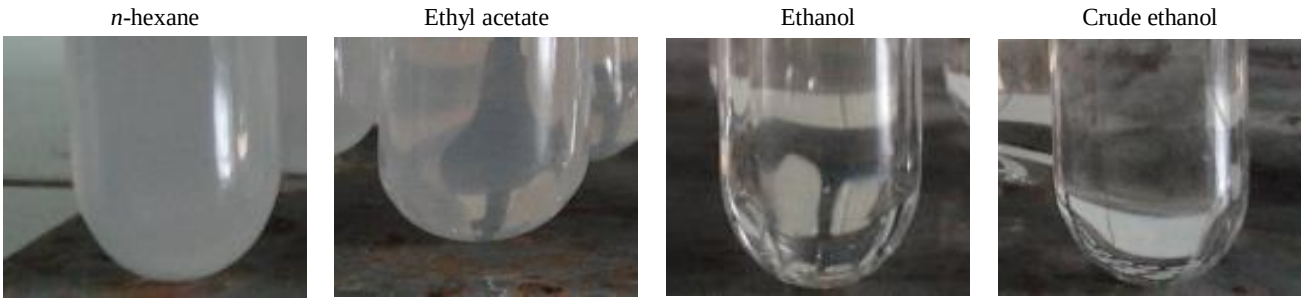


Figure 4. The result of total flavanoid content

Table 6. The total flavonoid content of white oyster mushroom

Samples	Absorbance			Antioksidan capacity (mg GAE/g)
	Rep. 1	Rep. 2	Rep. 3	
n-hexane	1.758	1.905	1.461	653.5
Ethyl acetate	0.357	0.394	0.231	122.474
Etanol	0	0	0	0
Crude Etanol	0	0	0	0

Total flavonoid content

Total flavonoid content assay has been done to find out how much the active compound of flavonoids in oyster mushrooms. Flavonoids are usually glycosylated and can be classified as anthocyanidins, flavanols (catechins), Flavone, flavanone, and flavonol, which are commonly found in fruits and vegetables in orange, red, and blue. Generally, they are found in light-colored fruit, vegetables or foods that are known to be healthy for the human body (Lin and Tang 2007). Table 6 shows that oyster mushroom extract has flavonoid levels. The highest level of flavonoid is in hexane solvent and is followed by ethyl acetate.

The highest flavonoid content in hexane is probably due to the density that occurs because of the reaction of the reagent solvent as shown in Figure 5. It is caused by hexane which has non-polar properties, so it has no ability to attract or dissolve active flavonoid compounds in oyster mushrooms. Hapsari et al. (2012) reported that from the chemical composition of the oyster mushroom simplicia, the presence of alkaloids, saponins, phenols, and tannins has been detected, but the presence of flavonoids was undetected. This was confirmed by Kim's research (2009) reporting that carotenoids such as lutein, lycopene, β-carotene, zeaxanthin staining were not detected in oyster mushroom. The absence of flavonoids in oyster mushrooms is probably a biological factor and a factor in the ecology of oyster mushrooms because the bioactive components inhibit enzyme activity (tyrosinase) for the development process and growth of oyster mushroom pigmentation (Xie et al. 2003).

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Screening and partial characterization of δ-endotoxins from some local *Bacillus thuringiensis* isolates for insecticidal activity against the spotted stem borer

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Abstract. *Kimani PG, Nyambaka H, Kasina M. 2019. Screening and partial characterization of δ-endotoxins from some local* Bacillus thuringiensis *isolates for insecticidal activity against the spotted stem borer. Biofarmasi J Nat Prod Biochem 17: 21-38.* Prolonged use of synthetic chemical pesticides is environmentally undesirable, causing the rapid development of resistance among insect pests. Kenya has unexplored potential in controlling lepidopteran pests by using proteinous *delta*-endotoxins sourced from local isolates of a naturally occurring bacterium, *Bacillus thuringiensis* Berliner (*Bt*). This study attempted to identify the insecticidal proteins in some Kenyan *Bt* isolates, characteristic of Cry1 and/or Cry2 proteins. It also aimed to test their efficacy as affected by different temperatures and their specificity on an invasive and prevalent lepidopteran stem borer, *Chilo partellus* (Swinhoe). Using froth floatation and centrifugation, delta-endotoxin crystals were isolated and purified from cultures of twenty unidentified local Bt isolates. Total protein in the resulting suspensions was quantified using the Bradford assay method, and the approximate protein yield was 3.14 ±0.084 mg/mL of nutrient broth culture with a purity level of 54.8 % ±15.3 %. Leaf-dip bioassays were used for testing the efficacy of the δ-endotoxins against *C. partellus*. Among the isolates evaluated, *Bt* 44 and *Bt* 48 had the most potent δ-endotoxin crystals towards the 1st instar larvae, leading to mortality of 62.6 % and 64.8 %, respectively, after 72 h. The effect of the δ-endotoxins' concentration and temperature on larval mortality was examined for 72 hours at temperatures of 24°C, 27°C, and 31°C and levels of 0.01 mg/mL, 0.1 mg/mL, and 1.0 mg/mL. The resulting LC_{50} was 52.3 µg/mL and 42.0 µg/mL, while LT_{50} values were 76.7h and 60.9h for *Bt* 44 and *Bt* 48, respectively. Higher efficacy was found at 24°C and 31°C than at 27°C, an indication that these δ-endotoxins are tolerable for local conditions where temperatures are higher than in temperate regions. The relationship between concentration and temperature was significant for δ-endotoxins of *Bt* 48 but not those of *Bt* 44. A major protein component of the δ-endotoxins had a molecular weight of M_r ~ 130 kDa, which generates a trypsin-resistant core of M_r ~ 70 kDa. Cry protein analysis detected more Cry1 in *Bt* 44 than *Bt* 48 δ-endotoxins and no Cry2 in either. However, *cry* gene analysis using PCR detected the presence of both *cry1* and *cry2* genes in the DNA of *Bt* 44 but none in *Bt* 51, a negative control from toxicity tests against the pest. The chromatographic analysis revealed some differences in the elution profiles of δ-endotoxins of both *Bt* 44 and *Bt* 48, indicating that there may be different types and amounts of Cry toxins in the crystals or even novel proteins. These findings indicate that the two local *Bt* isolates expressed Cry1 and probably Cry2 proteins can control *C. partellus* and may, therefore, become promising sources for δ-endotoxins for biopesticide development for controlling the pest.

Keywords: δ-endotoxins, *Bacillus thuringiensis*, insecticidal activity, stem borer

INTRODUCTION

Lepidopteran stem borers are the most important group of insect pests that attack sorghum, maize, and sugarcane in many parts of the world (Mugo et al., 2001; Tende et al., 2010). Stem borer species that attack cereal crops in Kenya include *Chilo orichalcociliellus*, *Chilo partellus*, *Busseola fusca*, *Sesamia calamities*, and *Eldana saccharina* (Songa et al. 2002). *C. partellus* (Lepidoptera: Pyralidae) is Africa's most prevalent stem borer pests, which has colonized much of Eastern and South Africa (Hutchison et al. 2008) due to its high invasiveness.

Chilo partellus may be displacing native stem borer species in several areas of Kenya, including the low-altitude and coastal maize regions (Mbapila et al. 2002; Ofomata 2003). In severe infestations, *C. partellus* attacks entire parts of the maize plant but the roots and causes losses of up to 75% of maize seedlings (Khan and Amjad 2000). In Kenya, pest populations are abundant not only in the dry mid-altitude and dry coastal areas but also in the moist-transitional and moist mid-altitude (ca.<1500 m) agro-ecological zones. In this area, yield losses fall at US$ 34-75/hectare (De Groote et al. 2003; Muhammad and Underwood 2004). These losses have made up a large portion of the estimated yield loss of 13.5% annually in Kenya (De Groote 2002), translating to 400,000 tonnes of maize, equivalent to the amount imported annually; besides, the areas are most prone to food insecurity.

The maize ruined by pests is highly susceptible to mycotoxin contamination causing aflatoxin and fumonisin poisoning generally experienced in Kenyan maize growing zones (Betran and Isakeit 2003; Campa et al. 2005). Nowadays, Kenya lost a bumper harvest of maize to aflatoxin contamination in the Coast and Eastern provinces, which the government had to mop up to decrease eminent poisoning (Muthomi et al. 2010; Ngetich 2010).

Broad-spectrum synthetic chemical pesticides have been mainly used to manage agricultural pests (Muhammad

and Underwood 2004), but their application has not been maintained due to their high cost for small-scale Kenyan farmers (Bonhof et al. 2001; Tarus et al. 2010). Other issues of concern have been environmental degradation, disruption of the pests' natural enemies, and the development of secondary pests. Many reports showed cases of resistance to target insects. It is, therefore, essential to develop alternative pest management strategies that are safe, environmentally friendly, and can be used in the Integrated Pest Management systems (Camilla 2000). Biological control using entomopathogenic bacterium such as *Bacillus thuringiensis (Bt)* is a promising option.

The microbial pesticide, based on *Bacillus thuringiensis* (*Bt*), can, in an integrated control program, be used rapidly and efficiently to control insect pests (Dulmage 1993). Products based on *Bt* are the most successful microbiological pesticides used in forestry, agriculture, and public health (Burges 1982). Despite their success, local people use it in a limited amount, accounting for only about 2% of the world's insecticide market (Cetinkaya 2002). The main limiting factors include the fact that many of the well-characterized *Bt* strains have been isolated in temperate zones and may not be well suited for application in pest management programs in the tropics. Therefore, local isolates may be better because they possess useful attributes such as greater field persistence at high temperatures (Brownbridge 1991). Further, the impact of the environment on *Bt* efficacy is not unexpected because secondary products are induced or increase in concentration during periods of physiological stress or physical damage (Dixon and Paiva 1995).

Specific objectives: (i) To determine the insecticidal activity of local *Bt* isolates against the spotted stem borer, *C. partellus*, (ii) To assess the effect of temperature and concentration of δ-endotoxin from local *Bt* isolates on insecticidal activity, (iii) To characterize the local *Bt* isolates for the presence of Cry1 and Cry2 proteins.

MATERIALS AND METHODS

Research design

The study was carried out at three levels. First, determine the most active isolates by culturing each *Bt* isolate sampled on agar plates and multiplying it by fermentation in liquid broth. The two most active strains were identified by leaf-dip insect bioassay. Second, to determine the effects of *Bt* isolate, δ-endotoxin concentration, temperature, and their interaction on larval mortality using leaf-dip insect bioassay on neonate *Chilo partellus* (Lepidoptera: Pyralidae). Third, protein profiles such as molecular weights, relative quantity, and identity in each active isolate were examined using denaturing gel electrophoresis (SDS-PAGE), liquid chromatography (RP-HPLC), and Enzyme-linked Immunosorbent Assay (ELISA), and Polymerase Chain Reaction (PCR) experiments as illustrated in Figure 1.

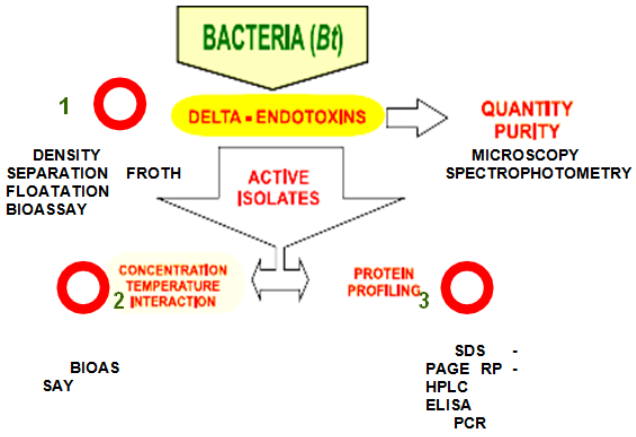


Figure 1. Illustration of the research design

Bt isolates and the sampling procedure

Local *Bt* isolates were isolated from KARI Biotechnology Centre (Mwathi 2007). Meanwhile, the standard control reference isolates, *B. thuringiensis* subsp. *aizawai* HD-133 (*Bta*) and *B. thuringiensis* subsp. *kurstaki* HD-1 (*Btk*) was kindly provided by Prof. Zeigler, D. R. of *Bacillus* Genetic Stock Centre, USA. Simple random sampling was used to select twenty local *Bt* isolates from a pool of 68.

Activation and multiplication of *Bt* isolates

Twenty local *Bt* samples and a reference standard *Bt. aizawai* (*Bta*) isolate was renewed by streaking aseptically on agar plates following the manufacturer's instructions and earlier report (Poinar and Thomas 1978). The *Bt* inoculants were incubated at 30ºC for seven days. The formation of bi-pyramidal-shaped protein crystals by a phase-contrast microscope confirmed the growth of inoculants. A loopful of the sporulated culture of *Bt* was inoculated into 50 mL liquid nutrient broth (Biotec Laboratories Ltd Ipswich, United Kingdom). Separate inoculation processes were repeated for all the isolates and the standard. The inoculated flasks were incubated on a rotary shaker for 96 h at 30ºC at 200 rpm.

Isolation of the delta-endotoxin crystals

The δ-endotoxin crystals were collected by centrifugation at 1,300 rpm for 10 min at 4ºC (Eppendorf 5810C®, Eppendorf-Nether-Hinz, Hamburg, Germany). The pelleted crystals were rinsed by low-speed centrifugation (4,000 rpm, 4ºC, 5 min) in sterile 0.85 % saline, air-dried, weighed, and suspended in 5-mL 0.85 % saline to minimize exoprotease activity, and stored at-20ºC. The purity of the crystals was confirmed by phase-contrast microscopy.

The total protein concentration of the crystals was measured using the Bradford-protein determination method (Bradford 1976) using a UV-Vis spectrophotometer (SmartSpec Plus®, Bio-Rad laboratories Inc. USA). The Bradford reagent consists of 10 mg coomassie brilliant blue (G-250) in 5 mL of 95 % ethanol, adding 10 mL of 85 % (w/v) phosphoric acid, diluting it to 100 mL, and filtering

the solution through Whatman's no.1 before use. Bovine Serum Albumin (BSA, fraction V) served as a standard at a stock solution concentration of 2 mg/mL. Into appropriately labeled test tubes, 0.1 mL of each standard and selected *Bt* samples were mixed, 5.0 mL of the Bradford reagent added, mixed well, and incubated at room temperature for five minutes. Absorbance was calculated at 595 nm for dilutions ranging from 0.1 to 2 mg/mL of standard. A calibration curve of the standard protein was made to determine the total protein concentration of *Bt* isolates. These measurements resulted in protein yield and percentage of total protein in the pellets of the *Bt* isolates.

Tests for insecticidal activity

Experimental insects

Yellow *C. partellus* eggs were collected from the International Centre for Insect Physiology and Ecology (ICIPE) and Kenya Agricultural Research Institute (KARI), Katumani insectaries. The eggs were surface sterilized with a 10% sodium hypochlorite solution and placed in plastic hatching jars at 28°C. It required 5-6 days for eggs to hatch to neonate larvae. The physiological age of larvae in all bioassays was determined to be 1-day-old old neonates.

To ensure a continuous supply of neonate larvae, a colony was maintained at 28°C and 65 ± 5 % at a photo-period of 12L:12D hours without exposure to any xenobiotics in the Insect Bioassay Laboratory, Biotechnology section of KARI, NARL, Nairobi from the initial eggs to minimize physiological variability of the larvae.

Experimental diet

The natural diet for the larvae was maize leaves collected from two to four-leaved CML 216 maize plants grown in the KARI Biosafety Greenhouse Level 2. All bioassay experiments refer to the leaf-dip method by Yee et al. (2008) and Insecticide Resistance Action Committee (IRAC) method No. IV, with some modifications. The neonates were collected using a camel hairbrush to avoid injury and were classified as dead when they did not respond to prodding utilizing the brush. The corpses were surface sterilized and crushed in a Petri dish to confirm that the larvae had died from Bt infection. The homogenate was inoculated on a nutrient agar plate and incubated for 24 h at 30°C. At the end of each bioassay, all dead larvae were sterilized in 70 % ethanol, autoclaved at 121°C, and disposed of. The Petri dishes and other reusable pieces of equipment were cleaned by soaking in sodium hypochlorite for one hour and washed with a detergent. All plant tissues, dead insects, and other disposable items were placed in biohazard bags, autoclaved for one hour, and burnt in an open pit.

Preliminary bioassay

A preliminary study was carried out to select the optimum concentration treatments for screening the toxicity of local *Bt* isolates. At the 2-4th leaf stage (2 cm), Maize leaf disks were dipped in the *Bta* δ-endotoxin suspension for 3 minutes at various concentrations (13.5, 1.35, 0.135, 0.0135, and 0.00135 mg/mL) and allowed to air dry on for 30 minutes. 0.85 % saline in place of δ-endotoxin suspension served as the negative control. One larva per leaf disk grew in a Petri dish (diameter = 8.5 cm) lined with a wet filter paper aseptically. All Petri dishes were sealed with an absorbent paper towel for proper aeration. The room temperature was maintained at 27 ± 1°C and relative humidity (R. H.) at 65 ± 5 % at a photoperiod of L12: D12.

Three replicate assays were carried out on alternate days for a total of 30 larvae at each dose level. The monitoring of the experiment was carried out daily for seven days, with larval mortalities recorded every 24 h. The mean lethal concentration causing 50 % larval mortality (LC_{50}) was determined by averaging the replicates, converting mortalities to percentage mortality, transforming percentage larval mortalities to probits, and plotting these against log-transformed concentration values, then reading off the concentration corresponding to the probit = 5 using probit analysis. The LC_{50} obtained was used as the toxin concentration in the screening assay.

Screening bioassay

Nineteen *Bt* isolates were screened to determine the two most effective strains against *C. partellus*. The larvae were subject to a treatment of one dose of toxin. Maize leaf discs were treated with 11.0 µg/mL of *Bt* δ-endotoxin suspension (LC_{50} at 72 h obtained for *Bta* standard), and the bioassay setup is referred to as above mentioned. The average lethal time taken for 50 % larval mortality (LT_{50}) was determined by transforming percentage larval mortalities to probits and plotting these against log-transformed time values. The LT_{50} result was used to rank the *Bt* isolates to increase adequate median lethal time, which suggests decreasing toxicity of δ-endotoxin to *C. partellus* neonate larvae. The two isolates showing the highest larval mortality and the shortest LT_{50} were selected for further bioassay and protein analysis.

Concentration and temperature bioassays

The principal technique used to assess concentration-mortality relationships was similar to Yee et al. (2008), with slight modifications to accommodate the temperature variable. Neonate *C. partellus* larvae were exposed to maize leaf disks dipped in *Bt* suspensions with concentrations ranging from 1.0, 0.1, and 0.01 mg/mL. The duration of exposure was maintained for 72 h, after which untreated leaf disks replaced the inoculated ones for the remainder of the assay. The larvae were incubated at 24°C. Thirty larvae were examined per concentration per isolate per temperature. The entire experiment was carried out on two other alternate days resulting in three replicate assays for each temperature variation. The same was repeated for two different temperatures (27 and 31°C), giving about 270 larvae being exposed to each *Bt* isolates.

For each bioassay, 90 larvae (in groups of 30 per temperature treatment) were treated similarly as a negative control by exposing them to distilled water. The larvae were assigned to treatments in a complete randomized design. Dead larvae were documented daily up to six days after treatment (DAT). Probit analysis was applied to

estimate median lethal concentration (LC_{50} values) and median lethal time (LT_{50} values) for each isolate. To determine the effects of level, temperature, and their interaction for each strain on percentage mortality of *C. partellus* neonate larvae, one and two-way analyses of variance were carried out using Statistical Package for Social Scientists (SPSS Inc. 2004) program.

Protein profiling

The characteristics of *Bt* δ-endotoxins from the efficacious isolates were investigated using electrophoresis, ELISA, liquid chromatography, and polymerase chain reaction (PCR).

Electrophoresis

Electrophoresis using discontinuous 12 % SDS-PAGE was conducted under reducing conditions on a vertical electrophoresis unit (Vertical slab gel unit, Hoefer scientific instruments, SE 600 series) following Laemmli (1970) with modifications. The running buffer comprises 15.0 g/l Tris base, 72.0 g/l glycines, and 5.0 g/l SDS at pH 8.3. The polyacrylamide gel slab was discontinuous with an upper stacking gel to concentrate the sample, increase band resolution, and resolve the lower gel for protein separation. The resolving gel was cast first using 30 mL of a solution containing acrylamide mix (30 % total monomer concentration and 2.67 % cross-linking monomer concentration, 7.5 mL of 1.5 M Tris-HCl (pH 8.8), 0.3 mL of 10 % SDS, 9.9 mL distilled water, 0.3 mL of 10 % APS, 12 µL TEMED) and allowed to polymerize at room temperature (25ºC) for 90 minutes. The stacking gel was cast on top of the resolving gel using a 10 mL solution containing acrylamide mix (1.7 mL), 1.0 M Tris-HCl (pH 6.8) of 1.25 mL, 10 % SDS (100 µL), 6.8 mL distilled water, 10 % APS (100 µL), 10 µL TEMED and allowed to polymerize at room temperature for 90 minutes.

Samples which contain the 15 µL *Bt* δ-endotoxin suspensions of *Bt* 44, *Bt* 48, *Bt* 51 (negative control) and *Bta* (standard control) were diluted at a ratio of 1:3 with sample/loading buffer containing 10 % SDS (1.6 mL), 2-β-mercaptoethanol (0.4 mL), glycerol (0.8 mL), 0.2 mL of 0.05 % (w/v) bromophenol blue dye (tracker dye), 0.5 M Tris-HCl pH 6.8 (1.0 mL), 1.6 mL of 1 M 1,4-dithiothreitol (DTT) in 4.0 mL distilled water and repeated using 20 µL and 30 µL of samples for optimization. Samples were boiled for five minutes and centrifuged at 10000g for 5 min before loading 60 µL per well alongside a standard molecular weight marker (See Blue® Plus 2 prestained standard, Range: 4-250 kDa, Invitrogen).

Electrophoresis was performed at 80mA for about two hours. The gel was submerged in a staining solution for 30 minutes at room temperature with very gentle shaking on a rocker. The staining solution was made of 0.1 % (w/v) Coomassie Brilliant Blue (R250) in 40 % methanol, and 10 % (v/v) acetic acid and then filtered after the dye had dissolved. Destaining the gel was carried out in an excess solution containing 40 % (v/v) methanol, and 10 % (v/v) acetic acid at shallow speed shaking while making three changes to the destaining solution until a transparent background was obtained. The procedure was repeated the same as above, using samples containing solubilized *Bt* δ-endotoxins.

The samples were solubilized by suspending the *Bt* δ-endotoxins crystals in 50 mM Na_2CO_3.$NaHCO_3$ buffer (pH 9.5) containing ten mM DTT and incubated for 30 h at 37ºC. The solution was centrifuged for 15 minutes at 10000g. The supernatant was mixed with porcine trypsin solution (cell-culture grade, Sigma Chemical, St. Louis, USA) at an enzyme/toxin ratio of 1:20 (w/w) for 24 h at 37ºC to obtain trypsin-resistant fragments. The suspension was centrifuged for 10 minutes at 10000 g at 4ºC, and the supernatant was diluted with the loading buffer as previously described.

Crystal protein solubilization and tryptic digestion

The *Bt* δ-endotoxins crystals of the local isolates (*Bt* 44 and *Bt* 48) and the standard strain *Bta*) were solubilized and digested as described previously in section 3.6.1 to obtain trypsin-resistant fragments. The resulting solution was centrifuged at 15000 g for 15 minutes before injection into the HPLC system.

Reverse phase-High-performance liquid chromatography

A protein mapping method was modified from the original method, according to Fullmer and Wasserman (1979), that included a Beckman 126 HPLC system equipped with a reversed-phase C_{18} (Beckman ODS, 250 mm x 4.6 mm i.d.) analytical column. A 100 µL aliquot of trypsin-digested δ-endotoxins was combined with 25 µL acetic acid, then 20 µL of the sample, which had been centrifuged (15000 g, 15 min.), manually injected into the HPLC apparatus. After optimization, chromatographic separations were done by isocratic elution using a mobile phase containing acetonitrile and water at a ratio of 70 %: 30 %, respectively (the water was acidified with 0.2 % acetic acid) at ambient room temperature (25ºC). The flow rate was set at 1 mL/min at 10 MPa, and the separations were monitored by UV absorption at 254 nm using a photodiode array detector for 10 minutes. The data obtained was analyzed automatically using the integrated computer program.

Sandwich enzyme-linked immunosorbent assay (ELISA)

Sandwich ELISA analysis was carried out according to the EnviroLogix QualiPlate® kit (EnviroLogix Inc, Portland, USA) for non-quantitative laboratory detection of Cry1 and Cry2 proteins. The kit set was supplied with micro-titer plates, enzyme conjugates, substrates, and positive controls. The samples of δ-endotoxin suspensions of *Bt* isolates (*Bt* 44, *Bt* 48, *Bt* 51, and *Bta*) were made by adding a buffer solution containing phosphate-buffered saline (PBS) / 0.55 % Tween-20 at a ratio of 1:10. The solution was left to stand overnight, centrifuged at 15,000 rpm for 10 mins, and the supernatant was decanted and retained. The experiment was carried out following the manufacturer's kit protocol.

In brief, separate microtitre plates with wells precoated with antibodies raised against Cry1 and Cry2 proteins. Cry enzyme conjugate (horseradish peroxidase) (50 µL) was mixed immediately, followed by 50 µL or buffer blank, 50 µL of Cry positive control, and 50 µL of *Bt* δ-endotoxin

supernatant solution. The solutions of the wells were thoroughly mixed by shaking the plates in a rapid circular motion on the benchtop for 30 seconds. The high precaution was observed to avoid cross-contamination between wells. The wells were sealed with Parafilm to prevent evaporation and incubated at ambient temperature (25ºC) for 2 hours. The Parafilm was carefully removed, and the contents of the wells were vigorously shaken off into a sink. The wells were soaked with PBS / 0.05 % and Tween-20 wash buffer, then shaken off to empty contents. This wash step was repeated three times. The plates were dried using a blotting paper towel to remove as much water as possible.

100 µL of the substrate was added to each well, and the contents were thoroughly mixed. The plates were sealed with new parafilm and incubated further for 30 minutes at ambient temperature. Finally, 100 µL of 1 M hydrochloric acid stop solution was mixed thoroughly in each well. The experiment was carried out in three replicates (three wells per sample, blank, and positive control). The plates were read using UV absorption at 450 nm using a microtitre plate reader (Biohit® BP 800, Biotek Instruments Inc. USA) within 30 minutes of the addition of the stop solution, and the absorbance was recorded.

Polymerase Chain Reaction

The more toxic local *Bt* isolate *Bt* 44 was analyzed for genes encoding Cry1, Cry2, and Cry3 proteins. *Bt aizawai (Bta)* and *Bt kurstaki* (*Btk)* isolates served as positive control. A local strain, *Bt* 51, which had shown no toxicity against *C. partellus* at the screening stage and had previously been used in screening assays on coleopterans (Mwathi 2007), served as a negative control in the experiments.

DNA extraction

High purity plasmid DNA was isolated from *Bt* isolates using Pure Link® plasmid miniprep Kit (Invitrogen). *Bt* culture was incubated at 30ºC overnight in LB media while shaking at 200 rpm. Cells were pelleted by centrifugation for 5 minutes at 14000 g and resuspended in 250µL of suspension buffer (R3) with RNase A until no cell clumps remained. An equal amount of lysis buffer was mixed with the cells, and the solutions were mixed gently, five times without vortexing. The suspension was incubated for 5 minutes at room temperature (25ºC), and after that, 350µL of precipitation buffer was added until the solution was homogenous. Centrifugation of the mixture was carried out at 12000 g for 10 minutes to separate lysate from lysis debris. The supernatant was loaded on a spin column, and DNA was purified using centrifugation as per the manufacturer's instructions.

In brief, the supernatant in the spin column was placed into a wash tube. About 700 µL of wash buffer (W9) with Ethanol was added to the column. The mixture was centrifuged for 1 minute at 12,000g. The flow-through was discarded, and the column was placed back into the wash tube. The same step was repeated for a minute. Both the wash tube and the flow-through were discarded. Plasmid DNA was eluted using 75µL of preheated TE buffer in the middle of the spin column. The DNA concentration was estimated using Nanodrop. This plasmid DNA was used for all Polymerase Chain Reactions (PCR).

Oligonucleotide PCR primers

Amplification of the *cry1 and cry2* homology groups utilized one pair of universal primers designed to amplify a specific fragment by simultaneous alignment with all previously described genes in that group by using the Amplify 1.0 program (Bill Engels, University of Wisconsin, Madison) as a defined by Ben-Dov et al. (1997). The primers and the expected sizes of their PCR products are displayed in Table 1. A single universal primer and several specific primers for each *cry* class (selected from their highly variable regions) were applied together in two reactions. The oligonucleotide primers were obtained from Ransom Hill Bioscience, Inc. (Ramona, Calif., USA); each pair was highly specific and yielded a PCR product of the predicted size that was easily identified by electrophoresis in agarose gels (0.8 to 2.5 %).

DNA templates and PCR analysis

Template DNA was denatured for 5 min at 94oC, annealed to primers for 40 s at 55oC, and extended at 72oC for 90 s for 2.15 h in a DNA Thermocycler (MJ Research, Inc., Watertown, Mass.) by 30 reaction cycles. Each reaction was carried out in 25 µL; (1 µL of template DNA was mixed with 2 X GC of 12.5 µL reaction buffer, 150 mM (each) deoxynucleoside triphosphate (3.75 µL), ten µM (each) primer (Forward and reverse), and 0.25 U of *Taq* DNA polymerase (Appligene).

Table 1. The characteristics of the universal primers for Cry1 and Cry2 and specific primers for Cry2Aa1, Cry2Ab2, and Cry2Ac protein-encoding genes

Gene	Primer[a]	Primer sequence[b]	Product size (Bp)
cry1	Un1 (d)	5'-CATGATTCATGCGGCAGATAAAC-3'	277
	Un1(r)	5'-TTGTGACACTTCTGCTTCCCATT-3'	
cry2	Un2 (d)	5'-GTTATTCTTAATGCAGATGAATGGG-3'	698
	Un2(r)	5'-CGGATAAAATAATCTGGGAAATAGT-3'	
cry2Aa1	Un2 (d)	5'-GTTATTCTTAATGCAGATGAATGGG-3'	498
	EE-2Aa(r)	5'-GAGATTAGTCGCCCCTATGAG-3'	
cry2Ab2	Un2 (d)	5'-GTTATTCTtAATGCAGATGAATGGG-3'	546
	EE-2Ab(r)	5'-TGGCGTTAACAATGGGGGGAGAAAT-3'	
cry2Ac	Un2 (d)	5'-GTtATtCTTAATGCAGATGAATGGG-3'	725
	EE-2Ac(r)	5'-GCGTTGCTAATAGTCCCAACAACA-3'	

Note: **a** (d) and (r) direct and reverse primers, respectively; **b** Bases that do not match the appropriate sequences are shown by lowercase letters

The reliability of the primers was verified with the following *B. thuringiensis* reference strains: *B. thuringiensis* subsp. *kurstaki* HD-1 (*Btk*) and *B. thuringiensis* subsp. *aizawai* HD-133 (*Bta*) for the *cry1* and *cry2* classes against samples (*Bt* 44 and *Bt* 51). Electrophoresis of the PCR products was carried out in agarose gels (1.5%) for 30 minutes, followed by staining in Coomassie Blue for 15 minutes. Gels were photographed under UV.

Statistical analysis

The bioassay data were compiled, tabulated, and analyzed using Microsoft Office Excel 2003 (Microsoft Corporation, 1983-2003) and Statistical Package for Social Sciences (SPSS) version 13 (SPSS Inc. 2004, USA). The calibration curve using BSA as the standard protein was used to estimate the total protein concentration of the *Bt* δ-endotoxins. Dixon's Q-test was used to reject outliers, while the accuracy of results was expressed using standard deviation, standard error, coefficient of variance, and confidence intervals (Harvey 2000; Rorabacher 1991; Skoog et al. 1992). The percentage of larval mortalities was calculated every 24 h for 144 h in replicates. Where the mortalities of the control were between 5 % and 10 %, the larval mortalities were corrected using Abbott's formula (Equation below) before analysis, while those >10% were excluded (Abbott 1925).

$$P_t = \frac{P_o - P_c}{100 - P_c} \times 100\ \%$$

Where;
P_t = corrected mortality (%);
P_o = observed mortality (%);
P_c = control mortality (%):

Mean lethal time causing 50 % larval mortality (LT_{50}) and mean lethal concentration causing 50 % larval mortality (LC_{50}) values were measured using the probit analysis method by transforming percentage cumulative larval mortalities to probits and plotting these against log-transformed time and concentration values, respectively. The data were subjected to one and two-way ANOVA to evaluate the effect of each treatment and their interactions and thus test the null hypothesis. The average value was separated across different isolates, temperature levels, and concentrations levels with Tukey's HSD (Honestly Significant Difference) test and used to determine the main effects and interactions of the treatments (DeCoster and Claypool 2004). Where no statistical significance was observed, data were subjected to Bonferroni *post hoc* tests. The main factors and interaction effect size were calculated using partial *Eta* squared (η^2) and categorized as 0.01 for a small effect, 0.06 for a medium effect, and 0.14 for a large effect, according to Cohen (1988). The correlations were investigated using a two-tailed Pearson product-moment correlation coefficient, and the strength of the relationship was categorized as 0.5-1.0 for strong, 0.3-0.49 for medium, and 0.1-0.29 for weak relationships (Cohen 1988). The significance level of all tests was p = 0.05. For chromatograms, peak properties were measured by the integrated HPLC computer program and aligned for comparison between samples. For Electrophoresis, a calibration curve was used to determine the molecular weights of the separated proteins in the samples.

RESULTS AND DISCUSSION

Isolation of *Bt* delta-endotoxin

During the period of activation and multiplication, the growth of the local *Bt* isolates and *Bta* was fast, producing smooth creamish-white colonies that were rough-edged and slightly raised from the nutrient agar (Figure 2.A), except for *Bt* 20, which showed yellowish fluffy growth. The Bt grew with slight foaming in the nutrient broth to form a pale yellow opaque suspension that thickened over time and settled to reveal white sediment. However, isolate *Bt* 20 developed a fast-settling gelatinous yellow suspension whose pellet mass weighed 0.873 g. The Dixon's Q-test revealed that pellet mass from *Bt* 20 was an outlier as Q_{exp} = 0.592, which was higher than Q_{crit} = 0.342 at a 95 % confidence level and hence excluded from further analysis due to its different characteristics (Figure 2.B).

The autolysis of the *Bt* cells was complete; thereby, most of the released protein crystals precipitated at the bottom of the flask. Nearly complete separation of the δ-endotoxin protein crystals from the spores and cell debris was carried out by decantating the frothy spent culture and low-speed centrifugation at 1,300 rpm. The crystals precipitated as a white pellet at the bottom of the tube, separating the spores and cell debris in the supernatant fraction. Serial washing, decanting, and centrifugation removed the crystals of spent culture components, spores, and cell debris.

Microscopy showed a high concentration of the bi-pyramidal-shaped crystalline inclusions in the pellets obtained. Table 2 displayed the pellet masses, total protein concentrations, and yields. The mass of the resulting pellets r from 0.489 g for *Bt* 16 to 0.225 g for *Bt* 47. The protein mass of the pellets was different significantly among the isolates. The percentage of total protein content in the pellets ranged from 26.5 to 92.1 %. Isolates *Bt* 31 and *Bt* 47 recorded higher contents, while *Bt* 5, *Bt* 16, and *Bt* 8 had low protein content. The protein yield from the nutrient broth ranged from 2.223 mg/mL to 4.603 mg/mL of nutrient broth and was significantly different across the different *Bt* isolates.

A **B**

Figure 2.A. Agar plate of *Bt* culture. B. Suspended pellets of *Bt* after centrifugation (yellow one is *Bt* 20)

The growth of the Kenyan *Bt* isolates on nutrient agar, and the nutrient broth was similar to that reported by others (Brownbridge 1991; Brownbridge and Onyango 1992; Wang'ondu 2003; Mwathi 2007), which illustrates the viability of these isolates at KARI germplasm. Nevertheless, the growth of *the Bt* 20 strain showed a deviation from the rest to its exclusion from further analysis. The protein purity and yield values reported in this study varied, although the culturing conditions were similar, indicating that the local isolates tested were diverse. Mohan and Gujar (2001) reported that although *Bt* strains in their study were cultured under the same conditions, their total yield spore counts differed even for the same strain obtained from two different sources.

The total protein yield reported in the literature also varies significantly from as low as 0.634 mg/mL (Ghribi et al. 2004) of culture broth to 15.33 mg/mL (Liu et al. 2000) using different culture conditions and crystal protein extraction protocols. Furthermore, it is documented that δ-endotoxin yields are affected by culture conditions and may vary within different *Bt* strains cultured in similar circumstances due to the enormous diversity of isolates that may have differences in optimum growing conditions and the variety of insecticidal crystal protein produced (Aronson et al. 1995).

Insecticidal activity of *Bt* δ-endotoxins

Toxicity of Bta

The neonate larvae range was fed from the underside of the leaf disk soon after placing them into the Petri dish. However, feeding frequency slowed with time, and some larvae moved away from the meal after 24 h. Most larvae, especially with higher δ-endotoxin concentrations, stopped feeding after 48 h, appeared weak, and stunted in growth compared to the control upon where death was also observed. For example, with the 13.5-mg/mL δ-endotoxin treatments, 60 % of the larvae were found to be away from the diet after 24 h. After 48 h, only 10 % of larvae were still feeding, 40% were located away from the leaves, 20 % looked weak, and 40 % of the larvae were dead. By contrast, all the larvae were actively feeding in the control group g.

Leaf damage was observed to be less on treated leaf disks than on the control. The larvae appeared dark and shrunk upon death compared to the live larva in control (Figure 3.A). A dead larva was washed, ground, and aseptically inoculated onto a nutrient agar plate. Creamish growth was observed around the larva, confirming that larval mortality was due to the ingestion of *Bt* endotoxins (Figure 3.B). In the set of starved larvae, mortality was 100 % in 48 h.

On the control treatment, a 10 % larval mortality was observed after 72 h, while 30 % was recorded after 144 h (Table 3). The LC_{50} value estimate for reference isolates *Bta* was 11.0 µg/mL after 72 h when larval mortality was 10 to 90 %.

Screening of Bt isolates

Among the different *Bt* treatments, only *Bta* and *Bt* 48 recorded 10% mortality at 24 h of observation. Although *Bt* 44 marked the first mortality of 40 % at 48 h, it is the only isolate that recorded 100 % mortality by 120 h. The *Bta* recorded 100 % mortality at 96 h. No larval mortality was found with *Bt* 51 and the control throughout the observation period. Calculations of LT_{50} showed that *Bt* 44 had the most toxic δ-endotoxins, causing 50 % mortality after only 56 h, followed by *Bta* at 64 h and *Bt* 48 at 73 h. The activity of δ-endotoxins from sample isolates compared to standard *Bta* (LT_{50} standard/LT_{50} sample) was 1.13 and 0.87 for *Bt* 44 and *Bt* 48, respectively, compared to the standard isolate *Bta* (Table 4), which was 1.00. One-way ANOVA (for repeated measures) revealed that the difference in percentage larval mortalities of the standard isolate *Bta* and the *Bt* isolates was statistically significant except for the two strains, *Bt* 48 and *Bt* 44, which were retained for further investigation.

$$* \text{Activity} = \frac{LT_{50}(\text{standard})}{LT_{50}(\text{sample})}$$

The toxicity of the local isolates tested towards the first instar of *C. partellus* varied from 70 % for *Bt* 44 to 0 % for *Bt* 51 after 72 h. Thus, reject the null hypothesis that there are no differences in the relative toxicity of δ-endotoxins from local *Bt* isolates against *C. partellus*. Besides, there was no correlation between the endotoxin yield and toxicity in this study, which illustrates that toxicity is probably due to the type of Cry proteins present in the crystals and not so much the protein amount. Indeed, this agrees with the finding that different *Bt* isolates produce different δ-endotoxins, which may differ in toxicity against different target pests (Uribe et al., 2003).

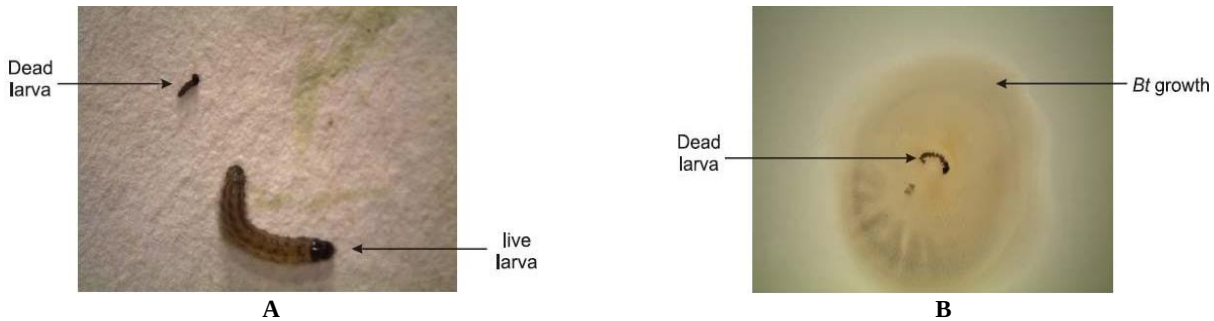


Figure 3.A. *C. partellus* larvae (live and dead). B. Culture of dead *C. partellus* larva

Therefore, culturing conditions need to be optimized for each *Bt* strain for large-scale production. More information is required on standardized culturing techniques, endotoxin extraction, and purification if comparisons are made. For example, Ghribi et al. (2004) reported that for *Bt var kurstaki*, the toxin yield increased by 30 % after 20 min of heat shock at 42 °C together with the addition of 5 g/l NaCl in a gruel-based culturing medium.

The δ-endotoxins from two isolates (*Bt* 44 and *Bt* 48) were selected for further investigation since they exhibited the highest larval mortalities. Although these endotoxins differed significantly from the other samples, they did not vary significantly with the standard reference isolate used (*Bta*). This selection was later confirmed by ranking the LT_{50} values obtained and retaining those that did not differ considerably from the standard reference isolate. This study also revealed that the δ-endotoxins from *Bt* 44 were 1.13 times more toxic than the standard control (*Bta*). Interestingly, an earlier study by Mwathi (2007) revealed that *Bt* 44 showed significant insecticidal activity against *P. truncatus* (the larger grain borer), suggesting that this isolate expressed toxins that may have efficacy towards both lepidopterans and coleopterans. The *Bt* strains in commercial formulations for control of lepidopterans are primarily *Bt kurstaki* and *Bt aizawai*, with the latter strain showing better larval control in situations where *Bt kurstaki* has become less effective due to the resistance development of the pests like the diamond black moth (Schnepf et al. 1998; Polanczyk et al. 2000).

Table 2. Masses of pellets of δ-endotoxins from *Bt* isolates and their protein quantities

***Bt isolates* no.**	**Mass of pellet (g)**	**Protein Conc. (mg/mL)**	**Protein in pellet (%)**	**Protein yield per mL broth (mg)**
31	0.250	1.151	92.1	4.603
19	0.318	1.070	67.3	4.282
70	0.460	1.007	43.8	4.042
44	0.338	0.930	55.0	3.718
74	0.351	0.910	51.9	3.642
47	0.225	0.867	77.1	3.468
48	0.267	0.864	64.7	3.456
5	0.452	0.856	37.9	3.426
41	0.245	0.831	67.8	3.324
12	0.346	0.829	47.9	3.316
16	0.489	0.824	33.7	3.295
Bt aizawai	0.270	0.807	59.8	3.227
66	0.258	0.780	60.5	3.121
3	0.277	0.767	55.3	3.066
24	0.265	0.732	55.2	2.926
58	0.230	0.722	62.8	2.888
45	0.279	0.692	49.6	2.770
8	0.443	0.588	26.5	2.350
51	0.234	0.556	47.5	2.223
60	0.280	0.556	39.7	2.223
Mean	0.314		54.8	3.268
SD	0.084		15.3	0.627
CV	26.8 %		27.9 %	19. 2%

Table 3. Percent mortality of neonate *C. partellus* larvae on treatment with different levels of *Bt aizawai* δ-endotoxins and the LT_{50} values from each concentration treatment

Toxin conc. (mg/mL)	**Mean larvae mortality (%) after time (h)**							
	0 h	**24 h**	**48 h**	**72 h**	**96 h**	**120 h**	**144 h**	**LT_{50} (h)**
0	0	0	0	10	10	20	30	nd
13.5	0	0	40	90	100	100	100	51.4
1.35	0	0	50	70	90	100	100	53.6
0.135	0	0	10	50	70	100	100	73.1
0.0135	0	0	20	60	60	90	100	70.7
0.00135	0	0	20	40	40	60	60	105.0

Table 4. Percent cumulative mortality of neonate *C. partellus* larvae exposed to 0.011 mg/mL δ-endotoxins from *Bt* isolates, LT_{50} values, and relative activity of each isolate

Treatment	**Percentage mortality after time (h)**							**LT_{50} at 72 h**	***Activity**
	0	**24**	**48**	**72**	**96**	**120**	**144**		
44	0	0	40	70	80	100	100	56.3	1.13
Bta	0	10	30	60	100	100	100	63.5	1.00
48	0	10	30	50	50	50	50	73.1	0.87
24	0	0	10	30	40	70	90	90.8	0.70
31	0	0	10	30	30	50	60	90.8	0.70
60	0	0	10	20	30	50	70	121.7	0.52
66	0	0	10	10	30	30	90	314.4	0.20
70	0	0	10	10	30	30	30	314.4	0.20
41	0	0	10	10	10	20	20	314.4	0.20
16	0	0	0	10	10	10	10	567.0	0.11
45	0	0	0	10	10	10	10	567.0	0.11
12	0	0	0	0	20	40	40	-	-
8	0	0	0	0	20	20	20	-	-
19	0	0	0	0	20	20	20	-	-
47	0	0	0	0	10	40	70	-	-
74	0	0	0	0	10	30	60	-	-
58	0	0	0	0	10	10	10	-	-
5	0	0	0	0	0	30	40	-	-
3	0	0	0	0	0	20	40	-	-
51	0	0	0	0	0	0	0	-	-
Control	0	0	0	0	0	0	0	-	-

Note: -: not determined

Information on its efficacy on local *C. partellus* or its comparison with local isolates has not been widely reported, despite numerous reports on the high toxicity of Bta on various lepidopterans. Consequently, this study emphasized the need to include it as a standard reference isolate when screening for toxicity.

Effect of temperature and concentration of δ-endotoxins on insecticidal activity

Bt isolates-mortality response

After exposing the neonate larvae for 72h to the *Bt* δ-endotoxins, the cumulative mean mortality recorded for *Bt* 48 was 64.8 %, and Bt 44 was 62.6 % compared to the standard control *Bta* of 87.0 %. The two isolates did not differ significantly concerning larval mortalities (Tukey HSD); however, the standard isolate (*Bta*) differed considerably with both isolates *Bt* 44 and *Bt* 48 (Bonferroni test). The LT_{50} values were 76.7 h and 60.9 h for *Bt* 44 and *Bt* 48, respectively (Table 5).

Table 5. Larval mortality, LC_{50}, and LT_{50} values for δ-endotoxins from local *Bt* and standard *Bt* isolates

Isolate	No. of larvae	Mortality (%)	(SE)	LC_{50} (µg/mL)	(SE)	Slope (SE)	LT_{50} (h)	(SE)
Bt 44	270	62.6	(7.1)	52.3	(31.2)	1.6 (0.3)	76.7	(13.7)
Bt 48	270	64.8	(6.6)	42.0	(21.8)	1.1 (0.4)	60.9	(9.0)
Bta	270	*87.0	(4.1)	*6.0	(3.5)	0.7 (0.3)	*37.0	(5.0)
Control	90	**0.0	(0.0)	nd	(nd)	nd (nd)	nd	(nd)

Note: Means (SE) in the same column differ significantly (p = < 0.05, Tukey HSD test) nd: not determined

Concentration-mortality response

There was a general increase in larval mortality as the δ-endotoxin level increased from 0.01 mg/mL to 1.00 mg/mL for the tested *Bt* isolates and standard control (Figure 4). The effect of the δ-endotoxin level on the percentage cumulative mean larval mortality was primarily significant for *Bt* 44 and *Bt* 48 compared to *Bta*. This trend also appeared as a strong positive considerable correlation (using Pearson product-moment correlation coefficient) between mean percentage larval mortality and concentration of δ-endotoxins from isolates *Bt* 44, *Bt* 48, and *Bta*, respectively. There was no significant difference between the effect of δ-endotoxin concentration from isolates *Bt* 44 and *Bt* 48 on larval mortality. Still, they differed significantly from the standard *Bta* at 0.01 mg/mL and 0.10 mg/mL.

Temperature-mortality response

The larval mortality was lowest for the δ-endotoxins from the two local isolates at 27ºC (*Bt* 44: 47.8 %; *Bt* 48: 51.1 %) and differed significantly from the mortality at 24ºC (*Bt* 44: 71.1 %; *Bt* 48: 68.9 %) and 31ºC (*Bt* 44: 68.9 %; *Bt* 48: 74.4 %). However, this trend was reversed for *Bta*, having the highest larval mortality at 27ºC (92.2 %) but did not differ significantly with mortality at 24ºC (77.8 %) and 31ºC (91.1 %). Generally, although larval mortalities due to δ-endotoxins from local isolates did not differ significantly between themselves, they differed significantly from those of the standard, *Bta* at 27ºC (Figure 5).

The correlation between temperature and mean cumulative percentage mortality due to *Bt* δ-endotoxin was negative, weak, and not statistically significant for *Bt* 44, positive, weak, and not statistically significant for *Bt* 48, and positive, weak, and not statistically significant for *Bta* (Pearson product-moment correlation coefficient).

Interaction between concentration and temperature effects

There was no statistically significant interactive effect between the δ-endotoxin concentration from *Bt* 44 and temperature on mean cumulative larval mortality (Two-way ANOVA: $F_{4,18}$ = 1.797, p = 0.173) (Figure 6). There was a significant interaction between temperature and concentration of δ-endotoxin from *Bt* 48 on the percentage mean cumulative larval mortality (Figure 7). The interaction between temperature and concentration of δ-endotoxin from *Bta* on the percentage mean cumulative larval mortality was not significant (Figure 8).

The susceptibility observed for neonate *C. partellus* larvae to *Bt* δ-endotoxins was in line with findings of Obonyo and Ogola (2011), that reported a five-day larval mortalities of 81.9 % for neonates and 25.6, 28.0, 14.9 and 9.2 % for 2nd, 3rd, 4th and 5th instar of *C. partellus* larvae fed on *Bt* maize expressing Cry1Ab. Furthermore, he observed that neonate larvae of *C. partellus* and *S. calamistis* showed significantly higher mortalities compared to later instars. At the same time, there was no significant difference between the mortalities of the 2nd to 5th instar larvae. Others have reported higher neonatal stem borer larvae susceptibility to *Bt* δ-endotoxins (Mugo et al. 2004). Yee et al. (2008) found that in most cases, the third instar larvae required a lesser amount of *Bt* and a shorter time to kill compared to the fifth instar larvae satisfactorily. Two *Bt* Cry proteins Cry1Ab and Cry1Ba, controlled *C. partellus* and showed stability in control, with no indication of a change in susceptibility over generations (Tende et al. 2010).

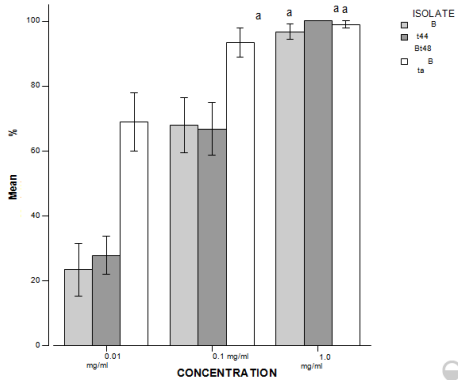


Figure 4. Mean cumulative larval mortality (%) due to concentration effects after 72 h of *Bt* δ-endotoxin exposure. For each isolate, bars labeled with the same letter are not significantly different (p < 0.05, Tukey HSD)

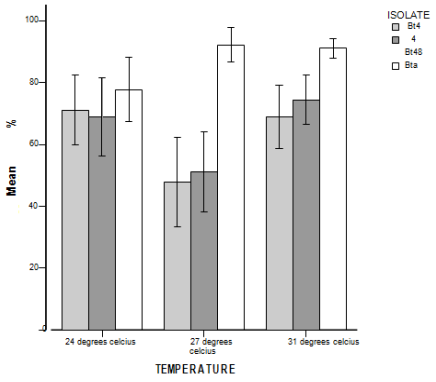


Figure 5. Mean cumulative larval mortality (%) due to temperature effects after 72 h of *Bt* δ-endotoxin exposure. For each isolate, bars labeled with the same letter are not significantly different (p < 0.05, Tukey HSD)

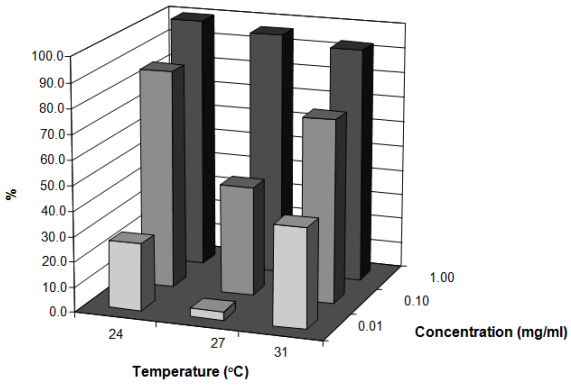


Figure 6. Mean cumulative larval mortality (%) due to interactions between *Bt* 44 δ-endotoxin concentration and temperature effects after 72 h of larval exposure

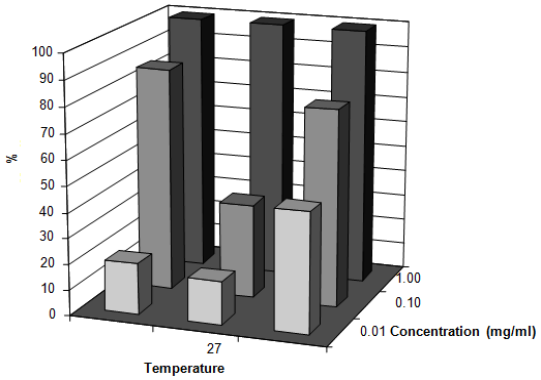


Figure 7. Mean cumulative larval mortality (%) due to interactions between *Bt* 48 δ-endotoxin concentration and temperature effects after 72 h of larval exposure

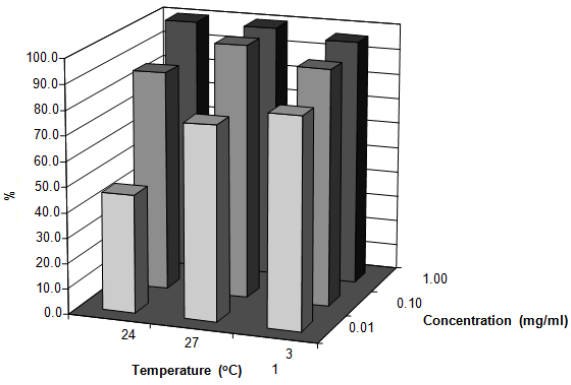


Figure 8. Mean cumulative larval mortality (%) due to interactions between *Bta* δ-endotoxin concentration and temperature effects after 72 h of larval exposure

There was a large and significant main effect of δ-endotoxin concentration on the percentage of cumulative average larval mortality for the *Bt* isolates used in this study. Dose-mortality relationship of *C. partellus* neonates to *Bt* δ-endotoxins also revealed a strong positive correlation between the larval mortality and δ-endotoxin concentration from the *Bt* isolates used. This observation was consistent with most lepidopteran species studied, where an increase in δ-endotoxin concentration resulted in increased mortalities. The phenomena had been observed for *C. partellus* (Osir and Vundla 1999), *C. partellus, S. calamistis, B. fusca* (Wang'ondu 2003), *P. truncatus* (Mwathi 2007), and *M. plana* (Yee et al. 2008). Mean cumulative larval mortalities for the isolates used revealed that, generally, δ-endotoxins from *Bt* 48 were equally toxic to *C. partellus* neonates as those from *Bt* 44. However, both were less toxic than the reference standard Bta, which showed a significantly higher larval mortality of 87.0 % after 72 h, as expected. The high toxicity of *Bta* had been previously demonstrated by Basri et al. (1994; 1996) and Polanczyk et al. (2000), which formed the basis of its choice as a standard reference isolate for this study.

Temperature similarly affected larval mortality for the local isolates tested but differed from the control as expected. These results indicated that the larval mortality was significantly higher at 24 and 31°C, at dose levels of 0.01 and 0.1 mg/mL of local *Bt* δ-endotoxins. At the same time, there were no differences at the highest dose level tested (1.00mg/mL). This observation differed from that of Van Frankenhuyze et al. (2008). Using a different method, they reported that neonate larval mortality of gypsy moth, *Lymantria dispar* L. increased when the temperature was raised from 13 to 25°C at each dose level tested (0.0125-0.049 IU). However, the control in this study had 0% mortality and, therefore, did not exhibit any temperature susceptibility within the study period.

Generally, the neonate larvae showed higher susceptibility to the tested isolates at temperatures higher or lower than 27°C, indicating that the larvae were more tolerant of tested strains at 27°C. The fact that the larvae were reared at 27°C before δ-endotoxin exposure may have conferred tolerance or enhanced immunity towards *Bt* efficacy at this temperature. This phenomenon may be explained in various ways; either the *C. partellus* gut proteases fully activate local isolates' δ-endotoxins faster and/or more entirely at 24 and 31 than at 27°C, or the reduced toxicity at 27°C may result from a reduced rate of protein site activation and reduced toxin binding to the larvae midgut. It may also indicate a situation of altered larvae physiology at temperatures higher or lower than 27°C, which probably suppresses the immunity of the larvae towards *Bt* δ-endotoxins.

These findings, however, do not agree with those of Van Frankenhuyzen et al. (2008), who reported that the final level of mortality attained by each instar decreases with increasing rearing temperature. Rearing temperature is well known to affect mortality progression in larvae infected with *Bt*, which is believed to be mediated by the rate of bacterial cell multiplication (septicemia) in the diseased host, at least in some species (Van Frankenhuyzen et al. 1994); this effect across instars may need to be investigated further for *C. partellus*. The Cry proteins administered to the larvae were also unlikely to be thermally denatured at the highest temperature used (31°C). It has been reported that 90 % of Cry1Ab protein was

denatured after heat treatment at 77°C for 10 mins (Perferoen 1998) and that 75 and 77°C for 2 min were sufficient to denature 50 and 70 % of Cry1Ab, respectively (De Luis et al. 2008). Further, no decrease in the insecticidal activity of Cry9C was found after heat treatment at 90°C for 10 min although, Cry1Ab completely lost its insecticidal activity after heating at 80°C for 10 min (Perferoen 1998).

Therefore, Cry proteins could lose their insecticidal activity at the temperatures experienced in the maize adaptation zones in Kenya, which were tested in this study. Deviation of findings from those of *Bta* δ-endotoxins may be because of a difference in Cry protein composition between that in the tested isolates from *Bta*, necessitating protein profiling or gene characterization of these isolates. Even minor differences in the amino acid sequences of the same toxin produced by different *Bt* strains may influence the specific toxicity of a test insect (Crickmore et al. 1998; Feldmann et al. 1995). Indeed, different Cry proteins may act synergistically or antagonistically on various larvae, or the same cry proteins may behave differently on different larvae (Rouis et al. 2008).

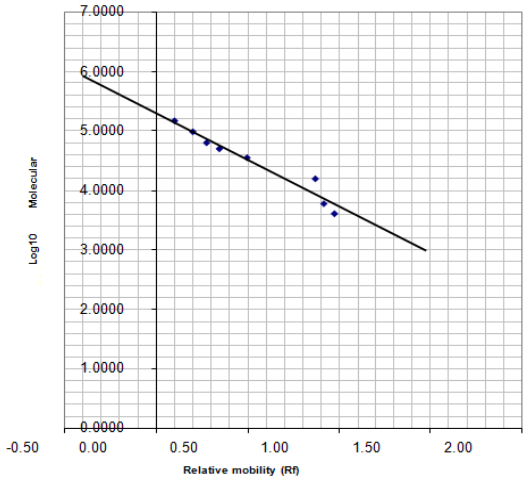
The interactive effect between temperature and δ-endotoxin concentration was statistically significant for *Bt* 44 but not for *Bt* 48. However, the tested isolates were significantly different from the control of the dose/temperature interactive effect on larval mortality. This study hypothesized no difference in the relative toxicity at different concentrations and temperatures of δ-endotoxins from local *Bt* isolates of *C. artellus*. This hypothesis was thus rejected as these effects were found to be significant. It might indicate that the tested isolates *Bt* 44 and *Bt* 48 appeared to have δ-endotoxins whose toxicity was more susceptible to temperature effects than the standard reference *Bta* with *Bt* 48 appearing the more vulnerable. The study, therefore, indicated that δ-endotoxins from tested isolates might be better suited for use in high and low-temperature regions of Kenyan maize adaptation zones. However, field tests would be needed to investigate these findings further.

Characterization of Cry proteins in *Bt* isolates

SDS-PAGE

The theoretical gel exclusion limit was 197000 Daltons determined from the y-intercepted (5.2944) of the gel calibration curve (Figure 9). Analysis of native *Bt* δ-endotoxin suspensions revealed a major protein band of M_r approximately 130 kDa in *Bt* 44 (lanes 6, 7, and 11), *Bt* 48 (lane 12), and *Bta* (lanes 4 and 9) (dark arrow) that was absent in *Bt* 51 (lanes 5 and 10). However, all these isolates revealed numerous minor molecular weight protein bands between ~ 98-35 kDa (Figure 10).

The trypsin activated δ-endotoxins from *Bt* 44 (Lanes 1 and 5), *Bt* 48 (Lanes 2 and 6), and *Bta* (Lane 5) were composed of a major protein band at M_r ~ 70 kDa (arrow A, Fig.13). However, trypsin digests of δ-endotoxins from Bt 51 (Lane 3 and 8) revealed a distinct protein band of M_r ~ 64 kDa (arrow B), as shown in Figure 11.



Equation: y =-1.5705x + 5.2944; R^2 = 0.9476

Figure 9. Gel calibration curve showing a plot of Log_{10} molecular weight against relative mobility of the standard marker proteins.

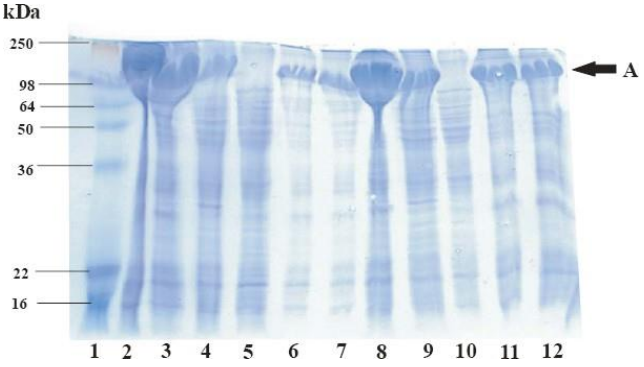


Figure 10. The 12 % SDS-PAGE gel of δ-endotoxins from local *Bt* samples. Lane 1: molecular weight markers. Lane 2: *Bt* 44 (30 µL). Lane 3: *Bt* 48 (30 µL). Lane 4: *Bta* (30 µL). Lane 5: *Bt* 51 (30 µL). Lane 6: *Bt* 44 (15 µL). Lane 7: *Bt* 44 (15 µL). Lane 8: *Bt* 48 (15 µL). Lane 9: *Bta* (15 µL). Lane 10: *Bt* 51 (15 µL). Lane 11: *Bt* 44 (20 µL). Lane 12: *Bt* 48 (20 µL). Arrow (A) indicates a significant protein band position.

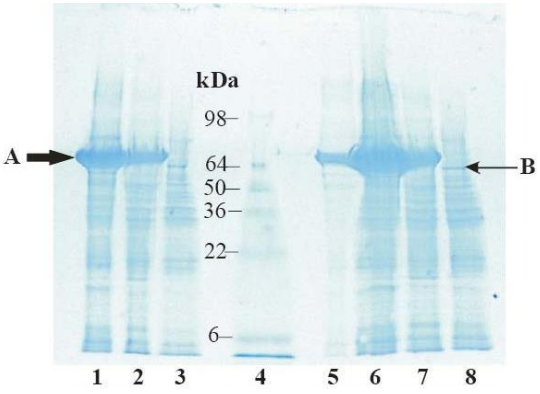


Figure 11. The 12 % SDS-PAGE gel of trypsin activated δ-endotoxins from local *Bt* samples. Lane 1: *Bt* 44 (20 µL). Lane 2: *Bt* 48 (20 µL). Lane 3: *Bt* 51 (20 µL). Lane 4: molecular weight marker. Lane 5: *Bta* (10 µL). Lane 6: *Bt* 44 (30 µL). Lane 7: *Bt* 48 (30 µL). Lane 8: *Bt* 51 (30 µL). Major protein bands (arrows A and B)

Liquid chromatography

The HPLC equipment used had a sensitivity of ±0.02 minutes peak deviation and the limit of detection in the order of 10^{-6} g of injected sample. The elution pattern of trypsin-digested δ-endotoxins from *Bt* 44 revealed 11 peaks resolved into four distinct peaks; A, B, C, and D (dotted lines at the base of each, Figure 12). Peak B consisted of a broad overlap of six peaks (2-7), while peak D, had three peaks (9-11). Peak 3 showed the highest absorbance and the highest amount of protein, which was 21.5 x 10^{-3} peak height, 29.9 % of peak height, and a peak area of 85.3 x 10^{-1}, 46.3 % of peak area of the profile, respectively, at a retention time (t_R) of 2.8 minutes. Other distinctly resolved peaks were peak 1(t_R = 2.1 mins; peak area = 6.5 x 10^{-1}), peak 8 (t_R = 4.7 mins; peak area = 3.6 x 10^{-1}) and peak 10 (t_R = 6.2 mins; peak area = 16.6 x 10^{-1}) as shown in Table 6.

Table 6 shows the retention times of the eleven peaks, their peak area, and height percentages. The elution pattern of trypsin-digested δ-endotoxins from *Bt* 48 revealed 12 peaks resolved into four distinct peaks; A, B, C, and D (dotted lines at the base of each) shown in Figure 13. Peak B consisted of a broad overlap of seven peaks (2-8), while peak D, had three peaks (10-12). Peak 3 showed the highest absorbance and the highest amount of protein, which was 41.8 x 10^{-3} peak height, 19.0 % of peak height, and a peak area of 131.4 x 10^{-1}, 36.7 % of peak area of the profile, respectively, at the retention time of 2.8 minutes. Other distinctly resolved peaks were peak 1 (t_R = 2.2 mins; peak area = 21.5 x 10^{-1}), peak 9 (t_R = 3.9 mins; peak area = 11.6 x 10^{-1}) and peak 11 (t_R = 6.2 mins; peak area = 40.9 x 10^{-1}) as shown in Table 7.

Table 7 shows the retention times of the twelve peaks, their peak area, and height percentages. The elution pattern of the standard isolate *Bta* trypsin-digested δ-endotoxins showed nine peaks resolved into five distinct peaks; A, B, C, D, and E (dotted lines at the base of each, Figure 14). Peak E consisted of a broad overlap of three peaks (7-9). Peak 1 showed the highest absorbance and the highest amount of protein, which was 44.4 x 10^{-3} peak height, 41.1 % of peak height, and a peak area of 23.0 x 10^{-1}, 17.2 % of peak area of the profile, respectively, at a retention time of 2.3 minutes. Other resolved peaks were peak 2 (t_R = 2.8 mins; peak area = 25.4 x 10^{-1}), peak 5 (t_R = 3.6 mins; peak area = 3.3 x 10^{-1}), peak 6 (t_R = 3.7 mins; peak area = 12.8 x 10^{-1}) and peak 8 (t_R = 6.3 mins; peak area = 43.1 x 10^{-1}) as shown in Table 8.

Table 8 shows the retention times of the twelve peaks, their peak area, and height percentages. The chromatograms of the trypsin-digested δ-endotoxins of the local isolate *Bt* 44 (A) and standard isolate *Bta* were aligned and compared (Figure 15). Peaks of eluents observed at similar elution times trypsin-digested δ-endotoxins of both isolates (*Bt* isolate; peak number, peak area) were at 2.8 mins (A; 3, 85.3 x 10^{-1} and *Bta*; 2, 25.4 x 10^{-1}), 3.0 mins (A; 4, 35.5 x 10^{-1} and *Bta*; 3, 2.1 x 10^{-1}), 6.1 mins (A; 9, 2.3 x 10^{-1} and *Bta*; 7, 4.4 x 10^{-1}) and 6.5 mins (A; 11, 4.5 x 10^{-1} and *Bta*; 9, 17.7 x 10^{-1}) (Tables 7 and 8). Nevertheless, the elution patterns differed at peaks 1, 2, 5, 6, 7, 8, and 10 of A and peaks 1, 4, 5, 6, and 8 of *Bta*.

Table 6. Peak properties of RP-HPLC elution profile of trypsin-digestedmδ-endotoxins of *Bt* 44

Peak number	Retention time (t_R)	Peak height		Peak area	
	(minutes)	X 10^{-3}	%	X 10^{-1}	%
1	2.1	7.4	10.2	6.5	3.5
2	2.4	3.6	5.0	3.1	1.7
3	2.8	21.5	29.9	85.3	46.3
4	3.0	13.3	18.5	35.5	19.3
5	3.4	7.0	9.7	19.2	10.4
6	3.8	3.3	4.5	3.0	1.6
7	3.9	2.9	4.0	5.0	2.7
8	4.7	2.4	3.3	3.6	2.0
9	6.1	2.1	3.0	2.3	1.2
10	6.2	5.7	7.9	16.6	9.0
11	6.5	2.8	3.9	4.1	2.2
Totals		71.9	100.0	184.2	100.0

Table 7. Peak properties of RP-HPLC elution profile of trypsin-digested δ-endotoxins of *Bt* 48

Peak number	Retention time (t_R)	Peak height		Peak area	
	(minutes)	X 10^{-3}	%	X 10^{-1}	%
1	2.2	40.0	18.2	21.5	6.0
2	2.4	6.3	2.9	4.4	1.2
3	2.8	41.8	19.0	131.4	36.6
4	2.9	32.2	14.6	30.6	8.5
5	3.0	29.8	13.6	57.7	16.1
6	3.3	14.6	6.7	22.8	6.4
7	3.5	8.9	4.0	11.6	3.2
8	3.6	7.0	3.2	6.4	1.8
9	3.9	10.4	4.7	11.6	3.2
10	6.0	4.8	2.2	4.6	1.3
11	6.2	15.1	6.8	40.9	11.4
12	6.4	9.2	4.2	14.8	4.1
Totals		220.1	100.0	358.4	100.0

Table 8. Peak properties of RP-HPLC elution profile of trypsin-digested δ-endotoxins of *Bta*

Peak number	Retention time (t_R)	Peak height		Peak area	
	(minutes)	X 10^{-3}	%	X 10^{-1}	%
1	2.3	44.4	41.1	23.0	17.2
2	2.8	13.2	12.2	25.4	19.0
3	3.0	1.7	1.6	2.1	1.6
4	3.3	1.9	1.8	2.0	1.5
5	3.5	3.8	3.5	3.3	2.5
6	3.7	12.1	11.2	12.8	9.6
7	6.1	4.7	4.3	4.4	3.3
8	6.3	15.7	14.4	43.1	32.2
9	6.6	10.6	9.8	17.7	13.2
Totals		108.1	100.0	133.7	100.0

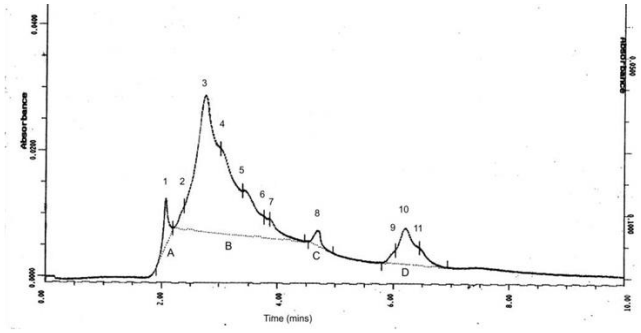


Figure 12. The RP-HPLC elution profile of trypsin-digested δ-endotoxins of *Bt* 44

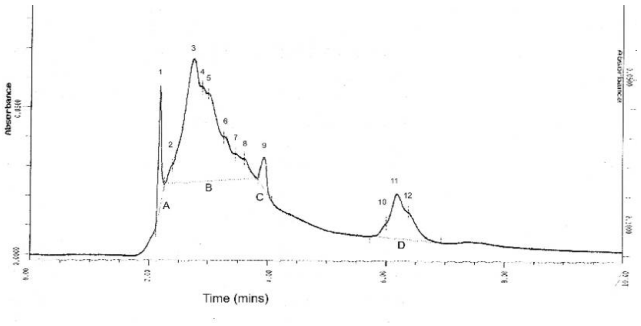


Figure 13. The RP-HPLC elution profile of trypsin-digested δ-endotoxins of *Bt* 48

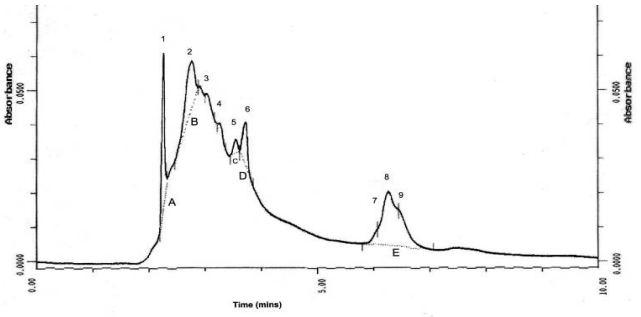


Figure 14. The RP-HPLC elution profile of trypsin-digested δ-endotoxins of *Bta*

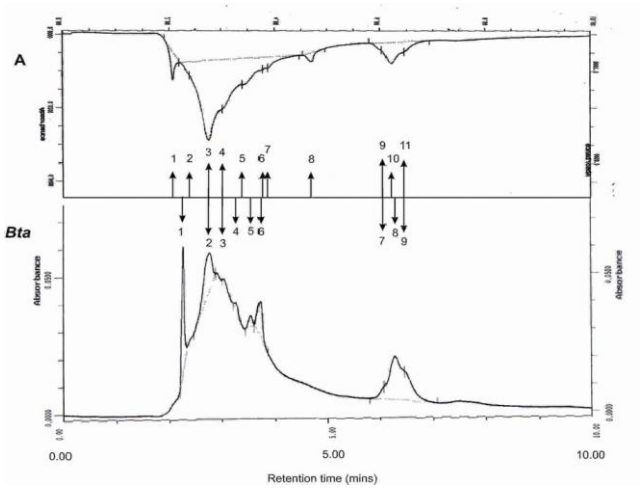


Figure 15. Comparison of the RP-HPLC elution profiles of trypsin-digested δ-endotoxins of *Bt* 44 (A) and *Bta*

The chromatograms of the trypsin digests of δ-endotoxins from the two local isolates were aligned and compared (Figures 16-17), with A representing that of *Bt* 44 and B that of *Bt* 48. Peaks of eluents observed at similar elution times for both isolates (*Bt* isolate; peak number, peak area) were 2.4 mins (A; 2, 3.1 x 10^{-1} and B; 2, 4.4 x 10^{-1}), 2.8 mins (A; 3, 85.3 x 10^{-1} and B; 3, 131.4 x 10^{-1})3.0 mins (A; 4, 35.5 x 10^{-1} and B; 5, 57.7 x 10^{-1}) and 6.2 mins (A; 10, 16.6 x 10^{-1} and B; 11, 40.9 x 10^{-1}). However, the elution patterns differed at peaks 1, 5, 6, 8, 9, and 11 of A and peaks 1, 4, 6, 7, 8, 10, and 12 of B.

ELISA

The ELISA technique had a limit of detection of 5 ng/mL. The contents of the microtitre plate testing for Cry1 proteins turned yellow for positive proteins. In contrast, Cry2 proteins turned blue with the intensity of the colors corresponding to absorbance values and thus relative Cry protein concentration. The positive control ratio (PC ratio) for each sample was calculated from mean absorbance values using the mean absorbance for the positive control as 0.393 for Cry1 and 0.886 for Cry2, with the latter having produced a more considerable background (Table 9).

The results revealed that the isolates *Bt* 44, *Bt* 48, and *Bta* contained Cry1 proteins while none contained Cry2 protein. The quantity of Cry1 proteins reduced the order of *Bt* 44 > *Bt* 48 > *Bta* as indicated by the PC ratio values of 4.529, 3.997, and 3.573, respectively. The isolate *Bt* 51 did not have either Cry1 proteins or Cry2 proteins in its crystals, indicating PC ratio < 0.5 and PC ratio < 1.0 for the proteins, respectively. The local isolates tested did not differ among themselves in Cry protein content, nor did they differ from the positive control (*Bta*) in terms of Cry1 and Cry2 protein content. However, they differed from the negative control (*Bt* 51) in terms of Cry1 though they were not different in terms of Cry2 content.

Polymerase Chain Reaction

The more efficacious local isolate (*Bt* 44) was analyzed alongside two positive and one negative reference isolates (*Bta*, *Btk*, and *Bt* 51, respectively). The local *Bt* isolate (lane 6; *Bt* 44) yielded a reliable amplification product of M_r = 277 bp with universal primers for the gene encoding Cry1 protein, similar to the positive standards *Btk* and *Bta* (lanes 3 and 4) respectively indicating the presence of *cry1* gene (Figure 18).

Table 9. Mean absorbance and PC ratio values for the sandwich ELISA test of δ-endotoxins

Sample	Cry1		Cry2	
	Mean absorbance	PC ratio *	Mean absorbance	PC ratio *
Bt 44	1.780	4.529**	0.129	0.146
Bt 48	1.571	3.997**	0.099	0.112
Bt 51	0.003	0.008	0.053	0.060
Bta	1.404	3.573**	0.396	0.447

Note:

$$\text{* PC ratio} = \frac{\text{mean absorbance of sample}}{\text{mean absorbance of positive control}}$$

** The *Bt* samples positive for Cry proteins tested

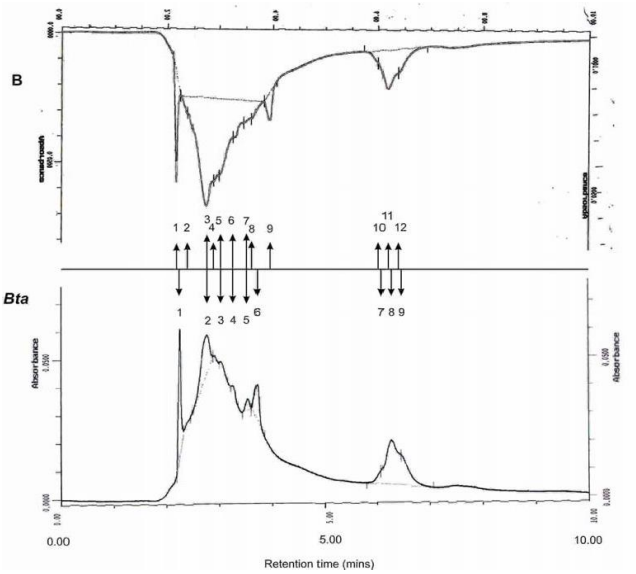


Figure 16. Comparison of the RP-HPLC elution profiles of trypsin-digested δ-endotoxins of *Bt* 48 (B) and *Bta*

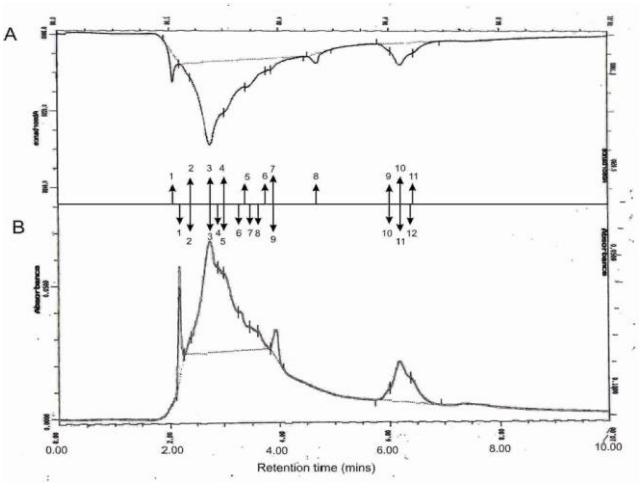


Figure 17. Comparison of the RP-HPLC elution profiles of trypsin-digested δ-endotoxins of *Bt* 44 (A) and *Bt* 48 (B)

The negative control local isolate (lane 5: *Bt* 51) gave a similar result as the negative control (lane 7: water), indicating the absence of the *cry1* gene. However, these two lanes 5 and 7 revealed a faint band (M_r = 277 bp), which may be false bands due to the high number of PCR cycles (35 cycles).

The local *Bt* isolate (lane 6: *Bt* 44) yielded a strong amplification product band of M_r = 689 bp with universal primers for *cry2* gene, similar to the positive standards *Btk* and *Bta* (lanes 3 and 4) respectively indicating the presence of the gene encoding Cry2 protein (Figure 19). The negative control isolate (lane 5: *Bt* 51) gave a similar result as the negative control (lane 7: water), indicating the absence of the *cry2* gene.

The PCR analysis using specific *cry2Aa1* primers for the amplified product of M_r = 498 bp revealed a strong band for *Bt* 44 and the positive controls (*Btk* and *Bta*) (Figure 20). A weak amplified product band of M_r = 546 bp was observed for *Btk* and *Bt* 44 but not for *Bta using cry2Ab2 primers.*However, analysis using specific *cry2Ac* primers for an amplified product expected at M_r = 725 bp yielded non-specific bands for isolates (*Btk*, *Bta*, and *Bt* 44) and no band for *Bt* 51 (NC) and water controls (data not shown). These results suggest that the presence or absence of the gene encoding Cry2Ac protein was not confirmed for *Bt* 44 and the standard reference isolates.

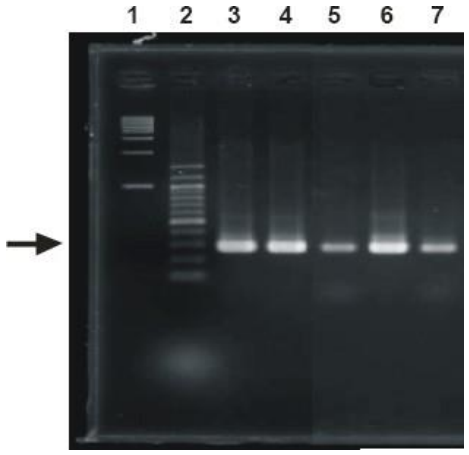


Figure 18. The 1.5 % agarose gel of PCR products amplified from the *Bt* isolates with *cry1* universal primers: Lane 1, high molecular weight marker (>1200 bp); Lane 2, low molecular weight marker (<1500 bp); Lane 3 and 4, *Bt kurstaki* HD-1 and *Bt aizawai* HD-133 respectively, positive controls; Lane 5, *Bt* 51; Lane 6, *Bt* 44; Lane 7, negative control. Note: Bold arrow showing the position of M_r of expected PCR product (277 bp)

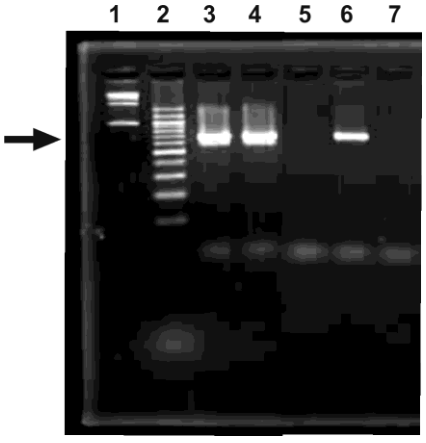


Figure 19. The 1.5 % agarose gel of PCR products amplified from the *Bt* isolates with *cry2* universal primers: Lane 1, high molecular weight marker (>1200 bp); Lane 2, low molecular weight marker (<1500 bp); Lane 3 and 4, *Bt kurstaki* HD1 and *Bt aizawai* HD133 respectively, positive controls; Lane 5, *Bt* 51; Lane 6, *Bt* 44; Lane 7, negative control. Note: Bold arrow showing the position of Mr of expected PCR product (698 bp)

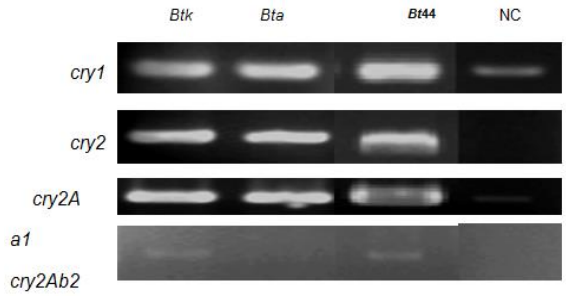


Figure 20. The 1.5 % agarose gel summary of PCR products amplified from the *Bt* isolates with *cry1* and *cry2* universal primers and *cry2Aa1* and *cry2Ab2* specific primers

Table 10. Summary of distribution of profile of genes encoding Cry proteins as analyzed by PCR

***cry* gene**	***Bt* isolate**			
	Btk	***Bta***	***Bt* 44**	***Bt* 51 (NC)**
cry1	+	+	+	-
cry2	+	+	+	-
cry2Aa1	+	+	+	-
cry2Ab2	+	-	+	-
cry2Ac	Ns	Ns	Ns	-
cry3	+	-	Ns	+

Note: +: Presence of gene encoding Cry protein, -: Absence of gene encoding Cry protein, Ns: Non-specific bands detected

Table 10 showed the presence and/or absence of genes encoding the Cry proteins tested. The local isolate *Bt* 44 contained *cry1* and *cry2* genes, which were absent in *Bt* 51 (NC). The two standard reference isolates (*Btk* and *Bta*) were mostly similar except for the *cry2Ab2* gene that was present in *Btk* but absent in *Bta*. Thus, these results showed that *Bt* 44 might be more similar to *Btk* than *Bta* for the tested genes, while *Bt* 51 announced uniqueness with the primers used not detecting any of the *cry* genes tested. A further test showed the presence of *the cry3* gene, which was identified in the *Btk* and *Bt* 51, and was absent in *Bta* and with non-specific bands in *Bt* 44 (data not shown).

This study employed a four-stage protein characterization strategy to investigate the presence or absence of Cry1 and Cry2 proteinous δ-endotoxins from *Bt* isolates with significant activity against *C. partellus*. Gel electrophoresis, ELISA, and HPLC techniques were used to investigate the δ-endotoxins at protein level while PCR was used to investigate the *cry* genes present at a molecular level.

The SDS-PAGE technique investigated the molecular mass of the δ-endotoxins present in the isolates. The results using the native proteins revealed that δ-endotoxins of *Bt* 44 and *Bt* 48 had a major protein band of M_r ~ 130 kDa characteristic of the protoxins of lepidopteran specific δ-endotoxins. This protoxin appeared to yield a trypsin resistant protein M_r ~ 70 kDa after trypsin digestion. Similar findings have been reported in a process where alkaline solubilization at high pH preceded enzymatic cleavage (Dulmage 1993; Hickle and Fitch 1990). Such a sequential and slow enzymatic digestion using commercial trypsin removes approximately 600 amino acids residues from the C-terminus and the first 28 N-terminal residues of the protoxin, producing a protease-resistant toxin core (Lightwood et al. 2000) does not result to over digestion as observed using the gut juice of *Choristoneura fumiferana* in the Eastern spruce budworm (Bah et al. 2004).

Several minor bands of molecular weight around ~ 98-35 kDa were also seen on both samples, probably due to crystal-associated endogenous proteolytic degradation processes. This observation was similar to that of *Bta* in all aspects but distinct from *Bt* 51 (negative control due to 0 % larval mortality observed during bioassay screening), which lacked the 130-kDa band. However, *Bt* 51 revealed a distinct band starting of M_r ~ 80 kDa, which appeared to be digested to a protein of M_r ~ 64 kDa with a series of other lower molecular weight proteinous products. Such numerous smaller molecular weight fragments are a common observation after a proteolytic digestion process (Hickle and Fitch 1990). However, the protein-banding pattern was not discriminatory enough within *Bt* isolates tested and could not reveal minute differences in the protein contents in the crystal inclusions necessitating further characterization.

The trypsin digests were further analyzed using RP-HPLC, which revealed that although the two uncharacterized *Bt* isolates may appear to have similar protein content, some differences revealed by different peaks. Due to their smaller size, the proteolysis products of the proteins were used because larger protoxins tend to undergo self-adsorption in the analytical column due to their larger molecular weights making elution difficult (Hickle and Fitch 1990). Five peaks from *Bt* 44 and *Bt* 48 trypsin-digested δ-endotoxins eluted at similar retention times while their percentage peak areas showed tiny differences, probably representing related proteins. However, the 1st peak for each isolate differed in their elution time and abundance. This observation could either be associated with concentration difference or a slight difference in hydrophobicity of different Cry proteins in the same Cry class (Cry1). Further, peak 8 for *Bt* 44 was unique for the isolates tested.

The chromatogram results made it possible to compare the protein content of the δ-endotoxins of the *Bt* isolates. The differing peaks from each strain may well represent different cry proteins in each isolate or even existence of novel Cry proteins. The two native isolates appeared more similar to each other (*Bt* 44, 11 peaks; *Bt* 48, 12 peaks) but distinctly different from the standard strain *Bta* (9 peaks). Furthermore, five peaks from both isolates eluted at the same time, with the rest showing minor differences. The δ-endotoxins from both *Bt* 44 (A) and *Bt* 48 (B) appeared to have four proteins, similar to those of the standard. Nevertheless, while *Bt* 44 showed peaks of proteins eluted both earlier and later as with the standard, *Bt* 48 showed related proteins eluted only in retention times below 3.5 minutes. The peaks observed could not be associated with any particular Cry protein nor their specific quantities determined, as protein standards were not used.

The ELISA analysis was used to evaluate the presence and relative abundance of Cry1 and Cry2 proteins. The findings detected only Cry1 proteins in the isolates tested. These results did not differ qualitatively from the standard *Bta* used. In the order of quantity, the amount of Cry1 proteins reduced as follows, *Bt* 44 > *Bt* 48 > *Bta*, as was validated by the PC ratio values of 4.529, 3.997, and 3.573, respectively. The isolate *Bt* 51, which served as the negative control, did not have either Cry1 proteins or Cry2 proteins in its crystals. The findings from the ELISA analysis corroborate those obtained from SDS-PAGE, where the major protein band (M_r ~ 130 kDa) observed with electrophoresis may be due to the presence of Cry1 proteins.

Furthermore, the relative amounts of the Cry1 protein in the isolates did correlate with the toxicity of the strains against *C. partellus* observed with the bioassay. The higher toxicity of *Bt* 44 may be due to the higher quantity of Cry1 proteins therein. It may indicate that that the two tested isolates may be expressing similar proteins in many aspects but only differ in expression levels of the Cry proteins. However, although the standard strain *Bta* appeared to have the least amount of Cry1 protein, it exhibited significantly higher larval mortality than the tested isolates. Indeed, a common trypsin-digested protein eluted at 2.8 minutes and appeared to be least in δ-endotoxins of the standard, *Bta*, while most in those of *Bt* 44.

As for the standard, *Bta* showed a unique chromatographic peak at a retention time of 6.3 minutes, which was the most abundant of its constituent proteins, as shown by its high percentage peak area of 32.2 %. Still, the trypsin digests from the local isolates showed peaks that only slightly deviated in height and domain from that of the standard. The results may be due to the standard expressing Cry1 proteins different from those expressed by the isolates tested. Masson et al. (1998) reported that the standard used (*Bta* HD-133) formed a proteinous crystal containing Cry1Ab, Cry1C, and Cry1D composed at 60 %, 37 %, and 3 % respectively and that the toxicity of *Bta* δ-endotoxins against *Mamestra configuration* Walker (bertha armyworm) was associated to Cry1Ab and Cry1C and not Cry1D.

A molecular approach using PCR was used to determine the genes present in the isolates tested. In this aspect, we tested only the more toxic *Bt* 44 because it appeared similar primarily to *Bt* 48 from the earlier tests carried out. The results revealed that *Bt* 44 contained *cry1* and *cry2* genes that produced strong bands at 277 bp for *cry1* and 689 bp for *cry2* when using universal primers. It contained *cry2Aa1* and *cry2Ab2* genes as well when specific primers were used. Although these results differed from those of the standard *Bta* in that the *cry2Ab2* was missing, *Bt* 44 was similar to the other standard isolate used (*Btk*) for the genes tested. Further, *Bt* 44 differed entirely from the negative standard (*Bt* 51) in that; the *cry1* and *cry2* genes tested were found to be absent in *Bt* 51. Also, *Bt* 51 was found to contain the *cry3* gene; thus, it may explain why *Bt* 51 showed no toxicity against *C. partellus* in the bioassay screening, as it did not include the lepidopteran-specific δ-endotoxins.

From this study, it appears that *Bt* 44 contains *cry1* and *cry2* genes, but only Cry1 proteins were expressed. Perhaps, the *cry2* gene being silent or its expression levels were below the detection limit of the ELISA technique used (Adugna and Mefsin 2008). Further, the use of universal primers for *cry1* genes may not reveal the specific *cry1* genes present, nor can they detect the presence of novel *cry1* genes. Therefore, it necessitates performing tests using specific *cry1* primers to determine the actual *cry1* gene present therein at least at the tertiary classification level, where differences in toxicity are distinguishable among proteins (Van Frankenhuyzen 2009). Another approach that may be used to reveal the specific identity of the δ-endotoxins expressed would be to sequence those proteins and compare them with a data bank of known Cry proteins.

Although none analytical technique sufficed in the detailed and complete characterization of the unknown native Cry proteins, each of the methods chosen contributed vital information about the δ-endotoxins tested. The analyses were largely qualitative due to the lack of appropriate standard proteins, and the results obtained were only relatively quantitative.

The challenge of appropriate standards as exacerbated by the necessity to use many pure compounds of the same Cry protein class, because the analyte was a mixture of closely related proteins, which were yet to be known in the *Bt* isolates tested. Furthermore, obtaining these standards was either expensive, or the sources (usually private multinational companies) carefully controlled their access. However, each of these techniques can be used quantitatively either when used individually or when combined with other methods, for example, SDS-PAGE-densitometry (Crespo et al. 2008) and HPLC-MS (Nakayama 2001). Indeed, the use of internal toxin standards needs to be promoted to increase confidence in comparisons between laboratories, insect colonies, and toxin preparations (Van Frankenhuyzen 2009). The phenomena are due to the reference standards would permit researchers to calibrate toxin protein estimates, by using the ratio of the known toxin protein content of the standard (based on mg of protein) to toxin protein content acquired with their method of choice.

This study had hypothesized that the relative toxicity of δ-endotoxins from local *Bt* isolates on *C. partellus* does not depend on the nature of the crystal protein. The results showed that the crystal inclusions of the two isolates (*Bt* 44 and *Bt* 48) with the highest toxicity against the pest contained Cry1 and Cry2 proteins while crystals from *Bt* 51, which caused 0 % larval mortality, did not contain Cry1 and/or Cry2 proteins. Besides, *Bt* 51 contained *a cry3* gene, which was absent in both *Bt* 44 and *Bt* 48 (data not shown). It explains why *Bt* 51 exhibited significant efficacy against the larger grain borer *P. truncatus* observed in a previous study by Mwathi (2007). These results, therefore, indicated that Cry1 and Cry2 proteins and not Cry3 were the toxins responsible for the mortality of *C. partellus*; therefore, the hypothesis was thus rejected.

Conclusions

The *B. thuringiensis* (Berliner) δ-endotoxins offer an array of advantages over conventional pest control approaches, including reduced reliance on chemical pesticides, increased crop yields, and reduced cases of aflatoxin poisoning from pest-damaged maize, a severe problem in Kenya. The conclusions drawn from this study were: (i) The yield of total protein in the crystal inclusions was 3.268 ± 0.627 mg/mL of culture broth with a protein purity level of 54.8 ± 15.3 %. This information may be useful when considering the mass production of the toxin. (ii) Two local *Bt* isolates (*Bt* 44 and *Bt* 48) had δ-endotoxins that showed significant insecticidal activity against neonate *C. partellus* larvae. They caused larval mortality of 62.6 (Bt 44) and 64.8 % (*Bt* 48) after 72 h. (iii) The isolate *Bt* 51 showed no insecticidal activity (0% mortality) against neonate *C. partellus* larvae. (iv) The effect of concentration of δ-endotoxins from *Bt* 44 and *Bt* 48 on larval mortality was significant, with LC_{50} values of 52.3 and 42.0 µg/mL, respectively. (v) Larval mortality due to δ-endotoxins from *Bt* 44 and *Bt* 48 was significantly higher at 24°C and 31°C and only differed considerably from the standard (*Bta*) at 27°C. These two local isolates might, therefore, be better suited for domestic use where temperatures are generally higher compared to the temperate regions, as they did not show loss of insecticidal activities at these elevated temperatures (31°C). (vi) The interaction effect between δ-endotoxin concentration and temperature on larval mortality was significant for *Bt* 48 but not significant for *Bt* 44. These results indicated that different levels and temperatures of endotoxins from *Bt* 44 and *Bt* 48 against *C. partellus* affect their relative toxicity. (vii) The major protein component on the endotoxins from *Bt* 44 and *Bt* 48 had a molecular weight of M_r ~ 130 kDa, which was digested to a trypsin-resistant core of M_r ~ 70 kDa. (viii) The isolates *Bt* 44 and *Bt* 48 expressed Cry1 proteins but not Cry2 proteins, although they both contained *cry1* and *cry2* genes. The isolate *Bt* 51 did not express Cry1 or Cry2 proteins; neither did it include *cry1* or *cry2* genes. (ix) Besides, only *Bt* 51 did not show any insecticidal activity on *C. partellus*. Furthermore, *Bt* 51 contained *a cry3* gene, which was absent in *Bt* 44 and *Bt* 48, which explains why *Bt* 51 showed significant efficacy against *P. truncatus* (Coleoptera). These results suggest that the toxicity of *Bt* δ-endotoxins from *Bt* 44, *Bt* 48, and *Bt* 51 depended on the nature of Cry proteins. (x) Reversed-Phase-HPLC chromatograms demonstrated five similar peaks in the trypsin-digested δ-endotoxins from *Bt* 44 and *Bt* 48 and one unique peak in *Bt* 44. These results revealed that the isolates *Bt* 44 and *Bt* 48 are mostly similar but not identical and thus might contain distinct quantities of related Cry proteins, other Cry proteins that were not tested, and probably novel Cry proteins.

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Investigation of the hemostatic effects of freeze-dried extracts of selected Kenyan plants

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Abstract. *Makunyi EG, Bukachi F, Waweru P. 2019. Investigation of the hemostatic effects of freeze-dried extracts of selected Kenyan plants. Biofarmasi J Nat Prod Biochem 17: 39-46.* This study aims to investigate the effect and mechanism of action of freeze-dried extracts of *Tridax procumbens*, *Terminalia brownii*, *Euphorbia tirucalli*, and *Asphillia africana* on hemostasis. Freeze-dried extract of the selected plants was prepared, and the dose was determined for the study. Twelve male New Zealand white rabbits were randomly allocated to two groups (control and test). Blood was collected under standard procedures. Duke's method was used for the bleeding time, while the capillary method was used for clotting time. ACL Elitepro machine was used to do prothrombin time and activated partial thromboplastin time. Thromboelastography was done for the most potent extracts. Data were analyzed using an independent t-test, and results were presented as mean± standard error of means. Differences were considered to be significant if $P < 0.05$. The results showed that the percentage yield of the extract was; *Tridax procumbens* (0.8%), *Terminalia brownii* (0.5%), *Euphorbia tirucalli* (0.2%), and *Aspillia pluriseta* (1.3%). Bleeding and clotting time: The bleeding time was reduced by freeze-dried leaf extract of *Tridax procumbens* ($p = 0.0068$) and freeze-dried bark extract of *Terminalia brownii* ($p=0.0068$). Freeze-dried leaf extract of *Asphillia africana* increased the bleeding time ($p=0.01$). The clotting time was reduced by freeze-dried leaf extract of *Tridax procumbens* ($p = 0.038$), the freeze-dried bark extract of *Terminalia brownii* ($p=0.043$), and freeze-dried stem extract of *Euphorbia tirucalli* ($p=0.01$). Prothrombin and activated partial thromboplastin time: The prothrombin time was reduced by freeze-dried leaf extract of *Tridax procumbens* ($p = 0.004$), the freeze-dried bark extract of *Terminalia brownii* ($p<0.001$) and Freeze-dried stem extract of *Euphorbia tirucalli* ($p=0.001$). Activated partial thromboplastin time was reduced by freeze-dried leaf extract of *Tridax procumbens* ($p< 0.001$), the freeze-dried bark extract of *Terminalia brownii* ($p<0.001$), and by freeze-dried stem extract of *Euphorbia tirucalli* ($p<0.001$). The results for thromboelastography showed that four parameters of thromboelastography were tested. Freeze-dried leaf extract of *Tridax procumbens* reduced the r time ($p =0.04$), k time ($p=0.04$) and maximum amplitude ($p =0.026$) but increased the alpha angle ($p =0.01$). The freeze-dried bark extract of *Terminalia brownii* did not have statistically significant differences in thromboelastography variables.

Keywords: *Asphillia africana*, *Euphorbia tirucalli*, freeze-dried extract, hemostatic, *Terminalia brownii*, *Tridax procumbens*

INTRODUCTION

One in seven deaths is associated with traumatic injury, and about a quarter of all trauma admissions present with coagulopathy (Mathew and Richard 2010). It is estimated that more than 1.24 million people die annually due to road traffic accidents globally. In addition, fifty million people suffer injuries from these road traffic accidents (NTSA 2016). In the United States of America, 40% of trauma fatalities are due to bleeding (Mathew and Richard 2010).

Hemorrhage (bleeding) causes thirty to forty percent of trauma mortality. It accounts for about fifty percent of death in the first 24 hours following the injuries. On admission, 25% to 35% of trauma patients present with coagulopathy, which is associated with a sevenfold increase in morbidity and mortality. Coagulopathy is a condition in which the blood's ability to clot is impaired (Brazzel 2013).

According to the World Health Organization (WHO), as many as 80% of people depend on traditional medicine for their primary health care needs (Essiett and Akpan 2013). Plants are an abundant natural source of potential new medicines. The approach to new drugs through natural products has proved the single most successful strategy for discovering new medicines (Jhample et al., 2009).

The use of herbal preparations for staunching blood flow and reducing the risk of blood disorders is prevalent worldwide (Cordier and Steenkamp 2012). Bleeding in rural setups can be caused by injuries or traditional circumcision. Many plants have been used to prevent bleeding. The plants are either chewed or crushed and then applied to the bleeding sites. Many plants are used, but this study chose the commonly used plants in Eastern Kenya.

The present study investigated the effect and mechanisms of action of *Tridax procumbens*, *Asphillia africana*, *Euphorbia tirucalli*, and *Terminalia brownii* that have been used traditionally to stop bleeding following cuts and after traditional circumcision by eastern Meru and Embu communities. This study used the leaves of *Tridax procumbens* and *Asphillia africana*, the bark of *Terminalia brownii*, and the stem of *Euphorbia tirucalli*. The aim of this study is to investigate the effects of freeze-dried extracts of *Tridax procumbens*, *Terminalia brownii*, *Euphorbia tirucalli*, and *Asphillia africana* on hemostasis.

MATERIALS AND METHODS

Extract preparation

The plants were collected and their identity verified at the University of Nairobi Herbarium, Department of Botany, School of Biological Sciences, and voucher specimens were deposited therein. The plants were air-dried, after which they were milled and then macerated with distilled water in a weight volume ratio of 1:4. The resulting suspension was filtered with cotton wool, after which Whatman's filter paper was used. The resulting filtrate was frozen using the Hot Point deep freezer. The frozen filtrate was freeze-dried at the International Centre for Insect Physiology and Ecology (ICIPE). The resulting freeze-dried extract was weighed and then stored in the deep freezer.

Animal preparation and welfare

Male adult Zealand white rabbits were locally obtained and used for the study. The male rabbits were used because they have almost constant hormonal levels, contrary to females. All the animals weighed 2.0-2.5 kg. They were housed in spacious cages in the animal house, Department of Medical Physiology, University of Nairobi. The room temperature was maintained between 15-25ºC and relative humidity of 45-65%, a regular 12 hours dark/12hours light cycle. The animals were handled humanely. The selection of rabbits as the experimental animal model ensured adequate blood volume was achieved. Adult male New Zealand white rabbits, 8-12 weeks old, weighing 2.0-2.5 kg and healthy, were included, while sick male rabbits were excluded. They were kept under standard laboratory conditions as recommended by The Federation of European Laboratory Animal Science Associations (FELASA) guidelines (Weiss et al. 2010).

Experimental protocol

Blood was collected using the standard method of bleeding the rabbit from the ear (Duke 1981). For prothrombin time, activated partial thromboplastin time, and thromboelastography, blood was collected in clean citrated bottles and tested within two hours.

The tests were performed using blood from six rabbits for each group (control and test groups). The dose of the freeze-dried extracts was determined using the titration method. The dose of freeze-dried extracts used in most tests was 10 mg/ml. The dosage for the freeze-dried extract of *Terminalia brownii* for thromboelastography was reduced to 2.5 mg/ml because doses of 10 mg, 7.5 mg, and 5 mg/ml were too potent that they only indicated a straight line on thromboelastography. Duke's method of bleeding time was used (Janzarik et al. 1988). The capillary clotting time method was used (Kumar et al., 2013). Prothrombin time and APTT were done at Kenyatta National Hospital Hematology laboratory. The freeze-dried extracts of *Tridax procumbens* and *Terminalia brownii*, which were the most potent, were evaluated in the thromboelastography stage.

For thromboelastography, 200 µl of sodium citrate was mixed with 1800 µl of rabbit's blood. The extract and rabbit blood were mixed at a ratio of 1:4, respectively, after which 360 µl of the mixture was loaded into a thromboelastography cup. The ratio is similar to the ratio of the reagents used in thromboelastography. Calcium chloride, 0.2 M, was added, and the test was run for one hour.

Data analysis and presentation

Data were entered into STATA Version 11 and were analyzed using independent t-tests. Results were expressed as means ± standard error of means (SEM). Differences were considered to be significant if $P < 0.05$.

RESULTS AND DISCUSSION

Extract yield

Table 1 shows the percentage yield of the extract, that is, *Tridax procumbens* leaf (0.8%), *Terminalia brownii* bark (0.5%), *Euphorbia tirucalli* stem (0.2%), and *Aspillia africana* leaf (1.3%).

Effect of Tridax procumbens *on bleeding time*

Figure 1 shows that the bleeding time is reduced by freeze-dried leaf extract of *Tridax procumbens* with statistically significant differences in the means (93.6±7.4(c) vs. 64.2±4.5 (t) seconds, P = 0.0068, t =3.39).

Effect of Terminalia brownii *on bleeding time*

The freeze-dried bark extract of *Terminalia brownii* reduced the bleeding time with a statistically significant difference in the means (100.3±7 (c) vs. 82.6 ±4.3 (t) seconds, p=0.0068, t=3.39) (Figure 2).

Effect of Asphillia africana *on bleeding time*

Freeze-dried leaf extract of *Aspillia africana* increased the bleeding time with a statistically significant difference in the means (107.8±10.5 (c) Vs. 152.8±9.2 (t) seconds, t=3.1 p=0.01) (Figure 3).

Effect of Tridax procumbens *on clotting time*

The result shows that the clotting time is reduced by freeze-dried leaf extract of Tridax procumbens with a statistically significant difference in means (88.7±7.8 (c) vs. 55.7±26.5 (t) seconds, t =2.45, p = 0.0338) (Figure 4).

Effect of Terminalia brownii *on clotting time*

Freeze-dried bark extract *Terminalia brownii* reduced the clotting time with a statistically significant difference in the means (88.5±8.7 (c) vs. 64.2±9.3 (t) seconds, t=1.9 p=0.043) (Figure 5).

Table 1. Extract yield

Plant	Weight of grounded product	Weight of extract obtained	Percentage yield
Tridax procumbens leaves	500 gm	4 gm	0.8
Terminalia brownii bark	2 kg	10 gm	0.5
Euphorbia tirucalli stem	1 kg	2 gm	0.2
Asphillia Africana leaves	300 gm	4 gm	1.3

Effect of Euphorbia tirucalli *on clotting time*

Freeze-dried stem extract of Euphorbia tirucalli reduced the clotting time with a statistically significant difference in means (88.5±8.7 (c) vs. 57±9.7 (t) seconds, t=2.4 p=0.01) (Figure 6).

Effect of Tridax procumbens *on prothrombin time*

The results show that the prothrombin time is reduced by freeze-dried leaf extract of *Tridax procumbens* with a statistically significant difference in the means (9.4±0.17 (c) vs. 5.3±0.17 (t) seconds, t =5.12, p = 0.004) (Figure 7).

Effect of Terminalia brownii *on prothrombin time*

The freeze-dried bark extract of *Terminalia brownii* reduced the prothrombin time with a statistically significant difference in the means (9.4±0.78 (c) vs. 3.8±0.38 (t) seconds, t=6.4, p<0.001) (Figure 8).

Effect of Euphorbia tirucalli *on prothrombin time*

Freeze-dried stem extract of *Euphorbia tirucalli* reduced the prothrombin time with a statistically significant difference in the means (5.8±0.17 (c) vs. 9.4±0.78 (t) seconds, t=4.6, p=0.001) (Figure 9).

Effect of Tridax procumbens *on Activated partial thromboplastin time*

The result showed that the APTT was reduced by freeze-dried leaf extract of *Tridax procumbens* with a statistically significant difference in the means (25.8±1.3 (c) vs. 8.3±1.6 (t) seconds, t =8.53, p< 0.001) (Figure 10).

Effect of Terminalia brownii *on activated partial thromboplastin time*

The freeze-dried bark extract of *Terminalia brownii* reduced the APTT with a statistically significant difference in the means of the treatment group (22.14±0.84 (c) vs. 4.2+0.48 (t) seconds, t=18, p<0.001) (Figure 11).

Effect of Euphorbia tirucalli *Activated partial thromboplastin time*

Freeze-dried stem extract of *Euphorbia tirucalli* decreased the APTT with a statistically significant difference in means (22.1±0.84 (c) vs. 5.7±0.31 (t), t=18, p<0.001) (Figure 12).

Effect of Tridax procumbens *on r- time*

The results indicate that freeze-dried leaf extract of *Tridax procumbens* decreased the time with a statistically significant difference in the means (6.2±1.6 (c) vs. 2.7±0.49 (t) minutes, t=2.08, p =0.04) (Figure 13).

Effect of Terminalia brownii *on r-time*

The freeze-dried bark extract of *Terminalia brownii* did not elicit a statistically significant difference in the means of r time (4.9±1.6 (c) vs. 5.3±0.9 (t) minutes, t=0.17 p=0.86) (Figure 14).

Effect of Tridax procumbens *on k-time*

The results indicate freeze-dried leaf extract of Tridax procumbens decreases the k time with a statistically significant difference in the means (3.7±1.1 (c) vs. 1.4±0.18 (t) minutes, p=0.04 t=2.03) (Figure 15).

Effect of Terminalia brownii *on k- time*

The freeze-dried bark extract of *Terminalia brownii* did not elicit a statistically significant difference in the means of k time (1.3±0.26 (c) vs. 2.2±0.6 (t) minutes, t=1.47, p=0.21) (Figure 16).

Effect of Tridax procumbens *on the alpha angle*

The results indicate freeze-dried leaf extract of *Tridax procumbens* increases the αlpha angle with a statistically significant difference in the means (43.3±6.9 (c) vs. 69.9±2.51 (t) degrees, t=3.65, p =0.01) (Figure 17).

Effect of Terminalia brownii *on the alpha angle*

The freeze-dried bark extract of *Terminalia brownii* did not elicit a statistically significant difference in the means of alpha angle (69.1±3.4 (c) vs. 51.3±10.8 (t) degrees, t=1.6 p=0.08) (Figure 18).

Effect of Tridax procumbens *on the maximum amplitude*

The results indicated freeze-dried leaf extract of *Tridax procumbens* increased the maximum amplitude with a statistically significant difference in the means (62.4±5.6 (c) vs. 34.8±7.6 (t) mm, t=3.65, p =0.026) (Figure 19).

Effect of Terminalia brownii *on the maximum amplitude*

The freeze-dried bark extract of Terminalia brownii did not elicit a statistically significant difference in the means of maximum amplitude (61.5±2.8 (c) vs. 46.9±9.7 (t) mm, t=1.44, p=0.19) (Figure 20).

Discussion

Coagulation requires complex interactions of cellular and molecular components that mainly involve platelets, plasma, and red blood cells (Hoffman and Monroe 2007). Initially, clotting involved intrinsic and extrinsic pathways with a common pathway at the end. Still, lately, it has been noted to be due to a balance between pro-coagulants and anti-coagulants (Hoffman and Monroe 2007). It involves the interaction of coagulation factors and platelets. Coagulation status can be measured using laboratory tests. The hemostatic effects of four Kenyan plant extracts (*Tridax procumbens*, *Terminalia brownii*, *Euphorbobia tirucalli*, and *Asphillia africana*) were elucidated using five laboratory tests; bleeding time, clotting time, prothrombin time, activated partial thromboplastin time (APTT) and thromboelastography. Bleeding time assesses the capillary integrity and platelet function. Clotting time measures the time taken to generate thrombin. Prothrombin time mainly measures the effect on the extrinsic pathway and is more sensitive to factor VII. Activated partial thromboplastin time evaluates the effect of the intrinsic pathway factors.

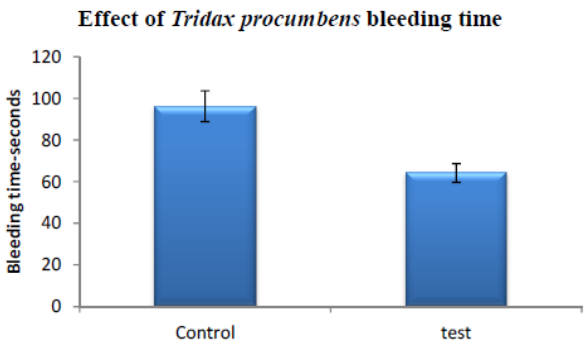

Figure 1. Effect of *Tridax procumbens* on bleeding time

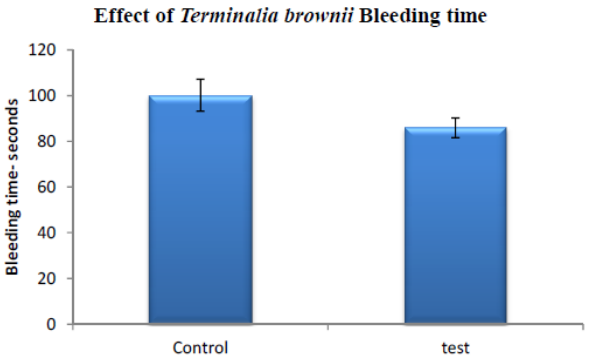

Figure 2. Effect of *Terminalia brownii* on bleeding time

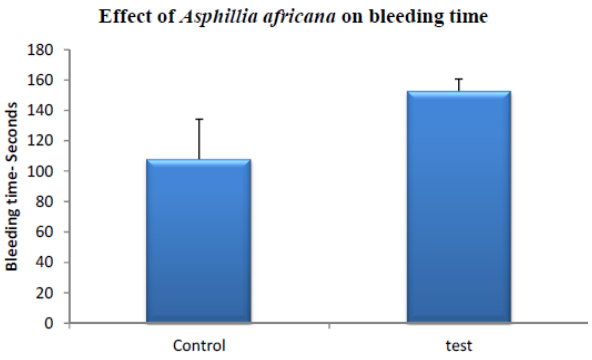

Figure 3. Effect of *Asphillia africana* on bleeding time

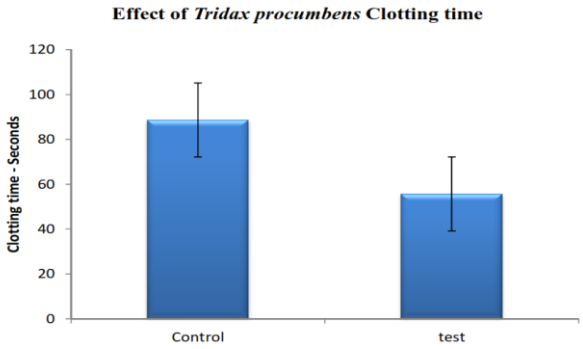

Figure 4. Effect of *Tridax procumbens* on clotting time

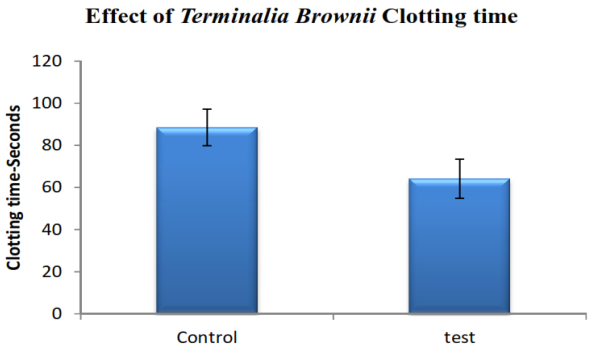

Figure 5. Effect of *Terminalia brownii* on clotting time

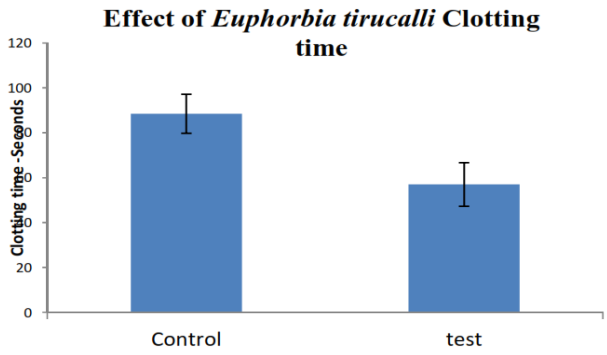

Figure 6. Effect of *Euphorbia tirucalli* on clotting time

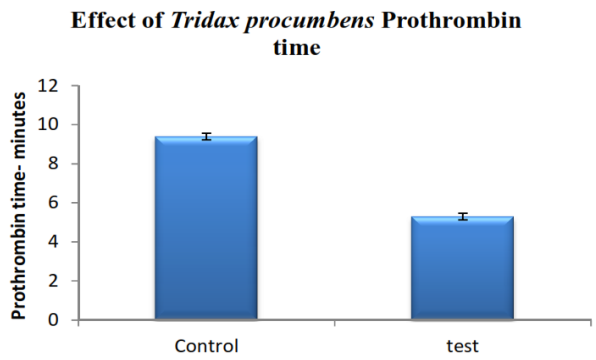

Figure 7. Effect of *Tridax procumbens* on prothrombin time

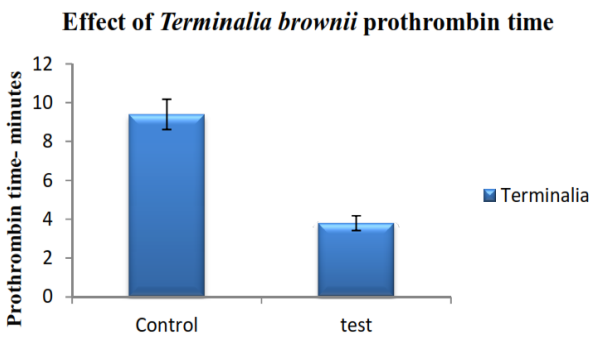

Figure 8. Effect of *Terminalia brownii* on prothrombin time

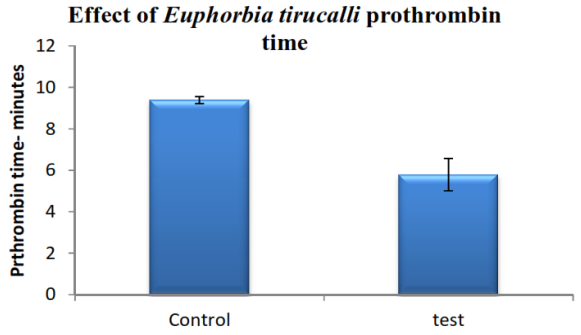

Figure 9. Effect of *Euphorbia tirucalli* on prothrombin time

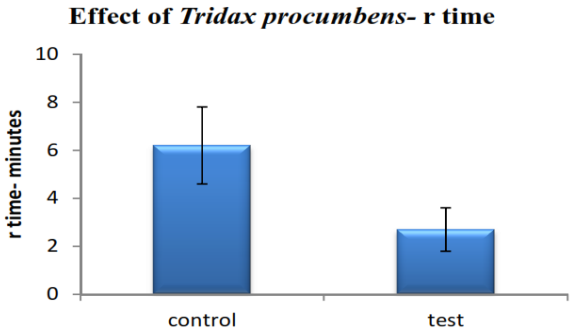

Figure 13. Effect of *Tridax procumbens* on r- time

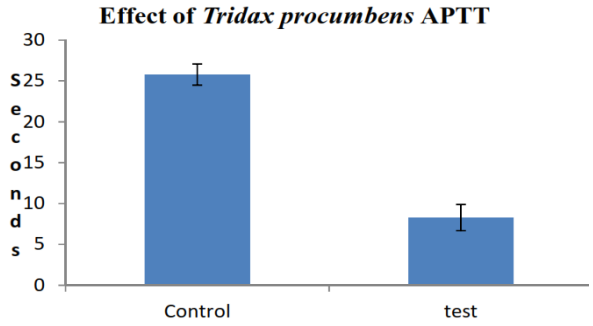

Figure 10. Effect of *Tridax procumbens* on Activated Partial Thromboplastin Time

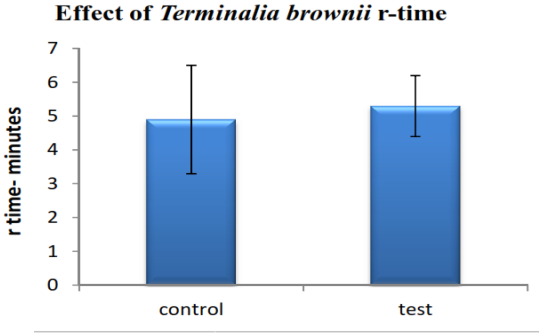

Figure 14. Effect of *Terminalia brownii* on r-time

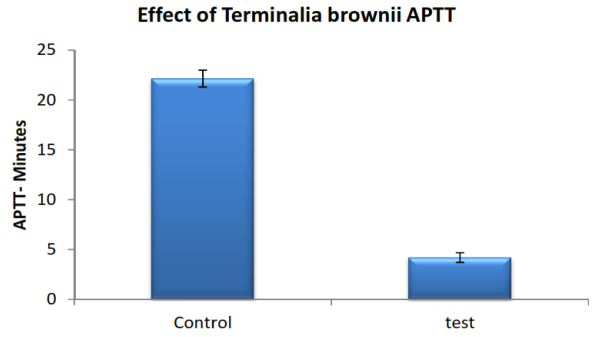

Figure 11. Effect of *Terminalia brownii* on Activated Partial Thromboplastin Time

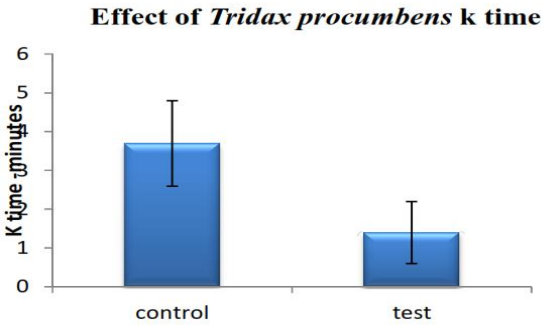

Figure 15. Effect of *Tridax procumbens* on K- time

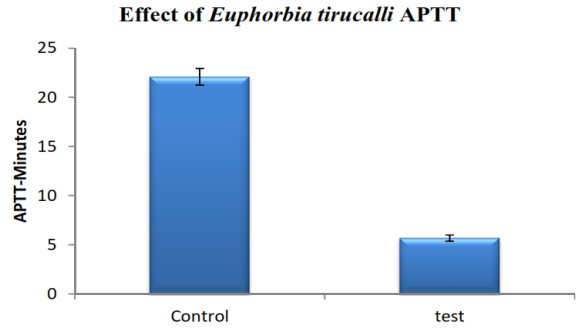

Figure 12. Effect of *Euphorbia tirucalli* Activated Partial Thromboplastin Time

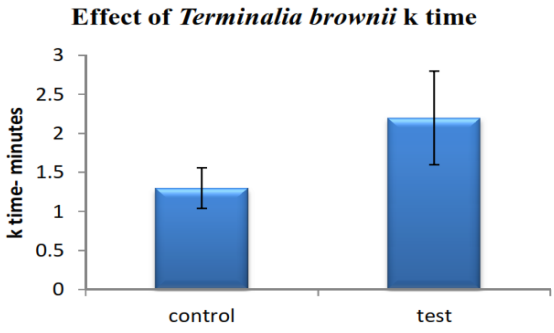

Figure 16. Effect of *Terminalia brownii* on k time

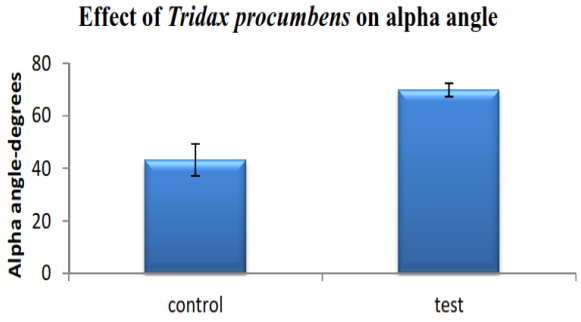

Figure 17. Effect of *Tridax procumbens* on the alpha angle

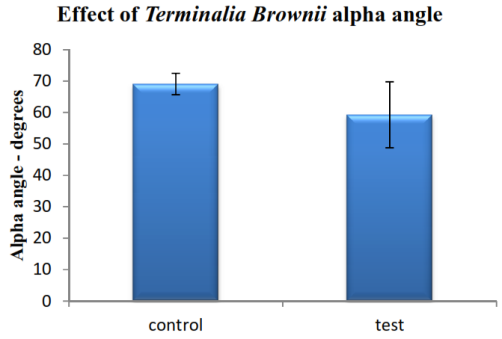

Figure 18. Effect of *Terminalia brownii* on the alpha angle

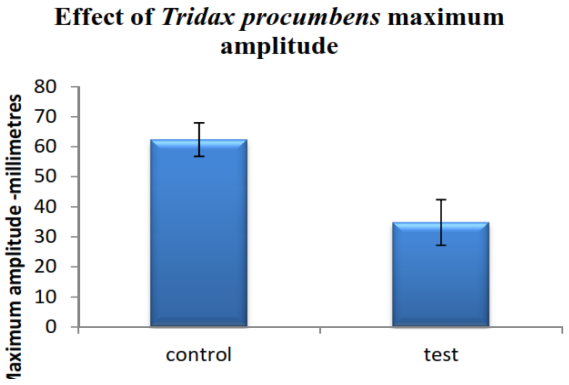

Figure 19. Effect of *Tridax procumbens* on the maximum amplitude

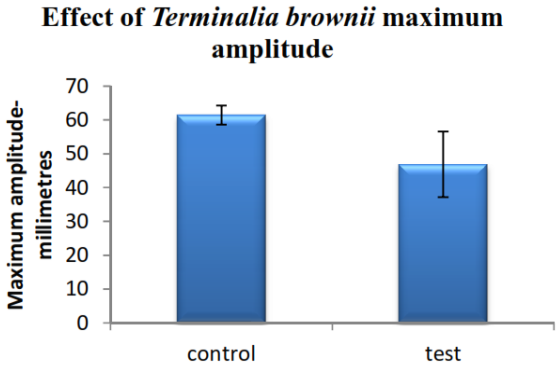

Figure 20. Effect of *Terminalia brownii* on the maximum amplitude

The freeze-dried leaf extract of *Tridax procumbens* significantly reduced the bleeding time (Fig.1). Ikese et al. (2015) confirm these findings, who found that a freeze-dried extract of *Tridax procumbens* significantly decreased bleeding time. The results are also similar to the ethanolic extracts of the same plant shown to reduce the bleeding time (Manjusha et al. 2014), which reflects platelet function (Kumar et al. 2013). This plant has potential activation effects on the platelets.

The freeze-dried bark extract of *Terminalia brownii* reduced the bleeding time (Figure 2). These are new findings on this plant that have previously not been reported. Bleeding time indicates the platelet function (Kumar et al. 2013), and therefore, this plant may have activation effects on the platelets.

The freeze-dried leaf extract of *Asphillia africana* increased the bleeding time (Figure 3). These findings were unexpected because other studies demonstrated that it reduces bleeding time. These findings are contrary to Okoli et al. (2007), which showed that methanol and hexane of *Asphillia africana* extract reduced bleeding time. Bleeding time indicates the platelet function (Kumar et al. 2013), and therefore, this plant may have an inhibitory effect on platelet function.

The freeze-dried leaf extract of Tridax procumbens significantly reduced the clotting time (Figure 4). This confirms the results of a similar study in which an aqueous extract reduced the clotting time (Ikese et al., 2015). The results are also identical to pet ether extract of the same plant, which significantly reduced the clotting time (Manjusha et al. 2014). The results are similar to those of Kale et al. (2008), which showed that ethanolic leaf extracts reduced the clotting time. Jhample et al. (2015) also demonstrated that the plant extract reduced the clotting time. Clotting time reflects the time to generate clotting factors, specifically thrombin, to form a clot (Hoffman and Monroe 2007). Therefore, this plant may have activation affect clotting factors that lead to thrombin formation.

The freeze-dried bark extract of Terminalia brownii reduced the clotting time (Figure 5). These are new findings on this plant that have previously not been reported. Clotting time reflects the time taken to generate clotting factors, specifically thrombin, to form a clot (Hoffman and Monroe 2007). Therefore, this plant could have activation effects on clotting factors that lead to thrombin formation.

The freeze-dried stem extract of *Euphorbobia tirucalli* reduced the clotting time (Figure 6). These are new findings on this plant that have previously not been reported. Clotting time reflects the time taken to generate clotting factors, specifically thrombin, to form a clot (Hoffman and Monroe 2007). Therefore, this plant may have some activation effects on clotting factors leading to thrombin formation.

The freeze-dried leaf extract of *Tridax procumbens* reduced the prothrombin time (Figure 7). These are new findings that have previously not been reported. Prothrombin time evaluates the extrinsic pathway (Hoffman and Monroe 2007), and therefore, this plant

extract may have some activation effects on the external pathway coagulation factor.

The freeze-dried bark extract of *Terminalia brownii* reduced the prothrombin time (Figure 8). These are new findings on this plant that have previously not been reported. Prothrombin time evaluates the extrinsic pathway (Hoffman and Monroe 2007), and therefore, this plant extract may have activation effects on the extrinsic pathway coagulation factor. The freeze-dried stem extract of *Euphorbobia tirucalli* significantly reduced the prothrombin time (Figure 9). These are new findings on this plant. Prothrombin time evaluates the extrinsic pathway (Hoffman and Monroe 2007), and therefore, this plant extract may have activation effects on the extrinsic pathway coagulation factor.

The freeze-dried leaf extract of *Tridax procumbens* reduced the activated partial thromboplastin time (Figure 10). These are new findings that have previously not been reported. Activated partial thromboplastin time evaluates the intrinsic pathway (Hoffman and Monroe 2007), and therefore, this plant extract has an activation effect on intrinsic pathway factors.

The freeze-dried bark extract of *Terminalia brownii* reduced the activated partial thromboplastin time (Figure 11). These are new findings on this plant that have previously not been reported. Activated partial thromboplastin time evaluates the intrinsic pathway (Hoffman and Monroe 2007), and therefore, this plant extract has an activation effect on intrinsic pathway factors.

The freeze-dried stem extract of *Euphorbobia tirucalli* reduced the activated partial thromboplastin time (Figure 12). These are new findings on this plant that have previously not been reported. Activated partial thromboplastin evaluates the intrinsic pathway (Hoffman and Monroe 2007), and therefore, this plant extract has an activation effect on intrinsic pathway factors.

Freeze-dried extracts of *Terminalia brownii* and *Tridax procumbens* were noted to be most potent among the group and were used during thromboelastography (Figure 12-20). Thromboelastography generated four variables; r, k times, alpha angle, and maximum amplitude. The r time evaluated the time of initiation of a clot of 2 mm amplitude. At 'initiation,' the Tissue Factor binds to circulating FVIIa and acts with FV to generate FIXa and FXa. The k- time evaluated the time taken for clot amplitude of 2 mm to reach 20 mm. This involves the interaction of clotting factors and platelets. During the 'amplification' of a clot, thrombin triggers reactions on the surface of activated platelets, where more FVIIa is produced. Thrombin activates co-factors FV and FVIII. The alpha angle measured the speed of clot strengthening. This mainly involves the interaction of fibrin and platelets. The maximum amplitude measures the ultimate clot strength. This includes mostly platelet function. In the hypercoagulable state, the r and k times decrease, and the maximum amplitude increase during the alpha angle.

The freeze-dried leaf extract of *Tridax procumbens* significantly reduced the r-time, k-time, and maximum clot amplitude but increased the α angle. These are novel findings that have previously not been reported. Thus, the plant extract acts on the clotting factors mainly in the extrinsic pathway and does not have much effect on the platelets. Factor VII is the main factor in the initiation of clot formation in the extrinsic pathway (Hoffman and Monroe 2007).

Freeze-dried bark extract at a dose of 2.5 mg/ml of *Terminalia brownii* increased the r-time, k-time, and maximum amplitude. Still, it decreased the alpha angle, though none of the changes were statistically significant. Doses of 10 mg/ml, 7.5 mg/ml, and 5 mg/mg caused rapid coagulation that indicated a straight line on thromboelastography. The lower dose produced opposite results may be due to the disproportional interaction of the extract molecules and the coagulation factors. This is the first time this study has been done on this plant. The limitation of the study was that the platelet aggregation test that would have indicated the effect of *Tridax procumbens* on coagulation was not possible because of time and logistical considerations.

In conclusion, freeze-dried leaf extracts of *Tridax procumbens* and freeze-dried bark extract of *Terminalia brownii* have some activation effect on the platelets. They have a stimulatory effect on the capillary muscles. Freeze-dried extract of *Asphillia africana* has an anti-coagulant impact contrary to traditional perceptions of it being pro-coagulant. Freeze-dried leaf extract of *Tridax procumbens*, freeze-dried stem extract of *Euphorbia tirucalli*, and freeze-dried bark extract of *Terminalia brownii* have some activation effects on both extrinsic and intrinsic coagulation pathways. Freeze-dried leaf extract of *Tridax procumbens* has more effect on factor VII, which is involved in the initiation of clot formation. *Tridax procumbens*, *Terminalia brownii*, and *Euphorbia tirucalli* are potential plants for developing drugs that can be used to reduce bleeding. The present research recommends further studies on *Tridax procumbens* to assess the active molecule in coagulation, which can further be evaluated for drug development. A platelet aggregation test for the freeze-dried extracts should also be carried out to verify the low platelet effect seen in thromboelastography.

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Antibacterial and antifungal activities of essential oil of Tawangmangu sweet orange (*Citrus sinensis*) peel at different altitudes

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Abstract. *Handayani ES, Nugraheni ER, Susilowati A. 2019. Antibacterial and antifungal activities of essential oil of Tawangmangu sweet orange (*Citrus sinensis*) peel at different altitudes. Biofarmasi J Nat Prod Biochem 17: 47-54. Staphylococcus aureus* and *Candida albicans* are human pathogenic microbes that cause skin infections. Essential oils are biologically active as antibacterial and antifungal. Environmental factors, e.g., temperature, lighting, and height, influence the synthesis of essential oils. This study aimed to determine the highest yield of essential oils of Tawangmangu sweet orange (*Citrus sinensis* L.) peel in different altitudes and to know the activity of essential oils of Tawangmangu sweet orange peel against bacteria *S. aureus* and fungi *C. albicans.* This study used essential oil of Tawangmangu sweet orange peel taken from four different altitudes of 1,000 ± 50; 1,200 ± 50; 1,400 ± 50, and 1,600 ± 50 m above sea level. First, the yield of essential oil was calculated then antibacterial and antifungal were tested using the Kirby Bauer method against *S. aureus* and *C. albicans*. Then, the diameter zones of inhibition were calculated. The results showed that Tawangmangu sweet orange peel at an altitude of 1,000, 1,200, 1,400, and 1,600 above sea level produced a different yield of essential oil. Tawangmangu sweet orange peel at an altitude of 1,600 m above sea level produced essential oil with the highest yield was 0.55%. Essential oil of Tawangmangu sweet orange peel at an altitude of 1,000, 1,200, 1,400, and 1,600 above sea level had different antimicrobial activity against bacteria *S. aureus* and fungi *C. albicans*. Essential oil at the altitude of 1,600 m asl in 100% concentration had the greatest antimicrobial activity with a diameter zone of inhibition of 23 mm against S. *aureus* and a diameter zone of 19 mm against *C. albicans.*

Keywords: Antibacterial, antifungal**,** *Citrus sinensis*, essential oil, Tawangmangu sweet orange

INTRODUCTION

Diseases caused by microbial infections are common in the tropics, like Indonesia, because of the dusty air conditions and warm and moist temperatures so that microbes can thrive. *Staphylococcus aureus* is a pathogenic bacterium in humans and causes symptoms ranging from localized wounds to life-threatening infections (Isbandrio 1999). The localized infection causes boils, pimples, and impetigo. When bacteria enter the bloodstream, bacteria can spread to other organs, causing diseases like pneumonia, infection of heart valves, leading to heart failure (endocarditis), inflammation of the bones (osteomyelitis), and even lead-to-dead infection. In the case of food poisoning due to S. aureus contamination, diarrhea, vomiting, and dehydration could occur, in which the symptoms appear approximately 1-6 hours after consuming contaminated food (Levinson 2004; Stroppler 2008). The *S. aureus* bacterial infection can be spread by contact with pus from an infected wound; direct skin-to-skin contact with an infected person in which *S. aureus* produces tissue-damaging hyaluronidase; and direct contact with objects used by an infected person such as towels, clothes, and so on. *S. aureus* can infect any tissue or organ in the body and cause characteristic signs, namely inflammation, necrosis, and abscess formation (Majid 2005).

The fungus *Candida albicans* usually lives as a saprophyte in the oral cavity, intestines, and vagina. In healthy people, this fungus is not pathogenic, but when the immune system decreases, this fungus can turn into a pathogen by causing various complaints. In the vagina, this fungus can cause symptoms of vaginal discharge known as vaginal candidiasis (Soemiati and Elya 2002). There is no factual data regarding the overall prevalence of candidiasis in Indonesia. However, the frequency of vaginal candidiasis is quite high among other sexually transmitted diseases in Indonesia, which is 43%. In Indonesia, it was reported that 84% of AIDS patients who were treated at the RSCM until 2000 also suffered from candidiasis caused by the opportunistic fungus *C. albicans.*

Handling that is often done in general to control the microbial disease uses chemicals such as potassium permanganate ($KMnO_4$) and methylene blue, antibiotics, and vaccinations. Infectious diseases due to *S. aureus* and *C. albicans* are generally treated with antibiotics (Majid 2005). Since their initial discovery in 1928, antibiotics have made an effective and positive contribution to controlling microbial infections in humans and animals. However, along with its development and use, pathogenic microbes become resistant to antibiotics. Some types of *Staphylococcus* have become resistant to the antibiotic methicillin and others previously used to treat infections. For example, infections caused by *Methicillin Resistant Staphylococcus Aureus* (MRSA) are difficult to treat because most antibiotics are difficult to kill the bacteria (Fitri et al. 2017).

Given the evidence about the current use of antibiotics, which often causes resistance, it is necessary to conduct research on natural antibiotics contained in plants. One of the plants that can be used as natural antibiotics is sweet orange (*Citrus sinensis* L.). Sweet orange peel contains essential oils that can be used for treatment. Recently, essential oils have attracted worldwide attention because essential oils from several plants are biologically active as antibacterial and antifungal agents. Therefore, they can be used as preservatives in food and as natural antibiotics. In addition, essential oils can inhibit several types of harmful bacteria such as *Escherichia coli*, *Salmonella* sp., *S. aureus*, *Shigella*, and *Pasteurella* (Agusta 2000). Based on the research of Chanthaphon et al. (2008), the diameter of the inhibitory power of *Citrus hystrix* DC. peel essential oil against *S. aureus* was 11 mm, while that of *E. coli* was 8 mm. From the research of Gulay et al. (2009), it is known that the diameter of the inhibition of "Turkish Citrus peel oils" against *C. albicans* is 12 mm.

Sweet orange peel essential oil contains the monoterpene compounds group, namely limonene (91.6%), *α-pinene* (0.9%), *sabinene* (1.0%) and *myrcene* (1.3%); the sesquiterpene group, namely *α-copaene* (0.1%) and *β-caryophyllene* (0.1%); the aldehyde group includes *octanal* (1.4%), *decanal* (0.2%) and *geranial* (0.2%); alcohol groups i.e. linalool (0.4%), α-terpineol (0.1%) and geraniol (0.1%); ester groups, i.e., *geranyl acetate* (0.1%) and *neryl acetate* (0.1%) (Gulay et al. 2009). Essential oil from sweet orange peel is also a rich source of bioactive compounds such as coumarins, flavonoids, carotenes, terpenes, linalool, limonene, and pinene (Mondello et al. 2005). Recently, orange peel essential oil has been studied for its natural antioxidant and antimicrobial properties. The antioxidant and antimicrobial abilities are related to the bioactive compounds such as phenolics and terpenoids (Viuda-Martos et al. 2008).

The environment can affect the content and quality of essential oils produced as secondary metabolites of plants. Bruneton (1995) and Rosman et al. (2004) stated that environmental factors could affect the yield of secondary metabolites. These environmental factors include air temperature, lighting (intensity of sunlight), lighting duration, and altitude where it grows (Ketaren 1987). Research on the effect of plant growth on the content and chemical content of essential oils has been carried out. Agustina et al. (2009) reported that the essential oil of *Cinnamomum burmannii* (Nees &Th. Nees) (cinnamon) obtained from 3 different growing sites had different chemical components. Arniputri et al. (2007) also reported that the essential oil of *Temu Kunci* taken at different heights of growing places showed differences in the main components that make up the essential oil. In connection with the indications that sweet orange peel has antimicrobial power, it is necessary to research the antibacterial and antifungal activity of the essential oil of Tawangmangu sweet orange (*Citrus sinensis* L.) grown at different altitudes.

The aims of this study were: (i) to determine the highest yield of essential oil of Tawangmangu sweet orange peel (*C. sinensis*), which grew at an altitude of 1,000 m asl, 1,200 m asl, 1,400 m asl, and 1,600 m asl. (ii) to determine the inhibitory activity of essential oil of Tawangmangu sweet orange peel (*C. sinensis*) growing at an altitude of 1,000 m asl, 1,200 m asl, 1,400 m asl, and 1,600 m asl against *S. aureus* and *C. albicans* bacteria.

MATERIALS AND METHODS

The materials used in this study were Tawangmangu sweet orange peel, as much as 450 grams for each altitude, *S. aureus* isolates, NA (Nutrien Agar) media, chloramphenicol, *C. albicans* isolates, PDA (Potato Dextrose Agar) media, ketoconazole and DMSO.

Sampling

Tawangmangu sweet orange peel samples were taken from Tawangmangu Sub-district, Karanganyar District, Central Java, Indonesia at different altitudes, namely 1,000 ± 50 m above sea level (Tawangmangu Village), 1,200 ± 50 m above sea level (Kalisoro Village), 1,400 ± 50 m above sea level (Blumbang Village) and 1,600 ± 50 m above sea level (Gondosuli Village). Four hundred fifty grams of Tawangmangu sweet orange peel from 3 different trees were taken from each altitude. Then the essential oil was distilled using Stahl distillation.

Essential oil distillation

Essential oil distillation was carried out using a Stahl distillation apparatus. Tawangmangu sweet orange peel was cut into small pieces and then put into a two-necked round bottom pumpkin. A total of 150 grams of Tawangmangu sweet orange peel was distilled using a Stahl device with 500 mL of distilled water at 95°C. The distillation process of Tawangmangu sweet orange peel essential oil was carried out in three repetitions. Furthermore, the resulting essential oil can be seen in the measuring cup in the Stahl tool. Data on sample weight and volume of essential oil will be obtained from the measuring cup, and then the yield will be determined. The obtained yield of essential oil was assumed to be 100% concentration. Based on Ashok et al. (2011) the yield of essential oils is stated as follows:

R = V/B X 100 %

Note: R = essential oil yield (%), V = essential oil volume (mL), B = Weight of sweet orange peel sample (grams)

The essential oil was put in a closed flakon and covered with aluminum foil. Then, the obtained essential oil was separated from the distilled water using anhydrate sodium.

Making of microbial growth curves and standard curves

Microbial growth curves were made using the turbidimetric method using a spectrophotometer. First, 1 ose of cultured *S. aureus* and *C. albicans* were transferred to an Erlenmeyer flask containing 10 mL of NB medium for bacteria and PDB medium for fungi. Then it was incubated in an incubator shaker at a speed of 120 rpm at 37°C for 24 hours. After 24 hours, every 5 mL of the

microbial suspension was taken and then mixed into 95 mL of NB and PDB media in a larger Erlenmeyer flask and homogenized for 5 minutes. Then, the NB and PDB media were prepared as blanks, and 3 mL of the media was put into a cuvette tube. Finally, 3 mL of microbial suspension was put in another cuvette to measure the absorbance value every 2 hours for bacteria and 4 hours for fungi for 24 hours at a wavelength of 530 nm. From the OD value obtained at each age of growth, a growth curve can be made that shows the relationship between the magnitude of the OD value and the age of the microbe so that the logarithmic growth phase of the test microbe can be known.

The relationship between OD and the number of microbes per mL was obtained by constructing a standard curve and determining the OD and plate count plot. One ose of microbes was grown in 10 mL of NB media and PDB media, and then it was shaken in an incubator shaker at a speed of 120 rpm for 24 hours. After 24 hours, a serial dilution of 5 tubes containing NB and PDB media was carried out.

Five milliliters of each microbial culture was added to 5 mL of NB and PDB media, respectively, and it was expressed as the first dilution /PI (10^{-1}). Then the PI culture was vortexed, and then 5 mL of this culture was added to 5 ml of new NB and PDB media and expressed as PII (10^{-2}). Next, PII was vortexed, and 5 mL of it was taken and added to 5 mL NB and PDB and expressed as PIII (10-^{3}). And so on until the PV (10^{-5}) was created. Furthermore, the absorbance value of each test tube containing the media and microbial culture was calculated using a spectrophotometer at a wavelength of 530 nm. Based on the experimental results, this wavelength can show the highest absorbance value in the medium used, surpassing other wavelengths.

After knowing the OD value, a serial dilution of 7 test tubes containing 9 mL of 0.85% physiological salt was carried out in the 10^{-3} (PIII). A total of 1 mL of the microbial culture from the PIII tube was put into 9 mL of 0.85% physiological saline, and it was expressed as the first dilution/EI (10^{-1}).

Then the EI culture (10^{-1}) was vortexed, and then 1 mL of it was taken and added to 9 mL of new 0.85% physiological salt and expressed as EII (10^{-2}). Next, EII was vortexed, and then 1 mL of it was taken and added to 9 mL of new 0.85% physiological saline and then expressed as EII (10-^{3}). And so on, until EVII (10^{-7}) was created. Then, 100 µL of the microbial culture was added from each dilution. Next, NA and PDA media were added and poured on a pour plate, and then it was incubated for 24 hours at 37ºC. The calculated number of bacterial colonies after incubation was a colony with a total of 30-300 colonies. The number of microbes in a test tube containing NB and PDB media is obtained in each tube. A standard curve can be obtained with a regression value of $y = bx + a$ with y = value of absorbance and x = number of microbes.

Antibacterial and antifungal activity testing

The antibacterial and antifungal activity of Tawangmangu sweet orange peel essential oil was tested using the paper disc method (Kirby Bauer). First, 15 mL of NA media for bacteria and PDA media for fungi were put into a petri dish until solid. The suspension of *S.aureus* and *C. albicans* bacteria in the log phase was taken with a cotton swab and swapped over NA media for bacteria and PDA media for fungi evenly. Next, 6 mm diameter filter paper spheres were dripped with 5 µL of essential oil samples at four concentrations (100%, 75%, 50%, and 25%) which had been dissolved in DMSO solution for each essential oil at different altitudes of 1,000 ± 50 m asl, 1,200 ± 50 m asl, 1,400 ± 50 m asl, and 1,600 ± 50 m asl. With sterile tweezers, the filter paper circles were placed on top of the NA and PDA media inoculated with the test microbes. Press the disc paper gently to ensure it adheres to the media. The same was done for chloramphenicol (antibacterial) and ketoconazole (antifungal) as positive controls and DMSO as negative controls. The culture was stored in the refrigerator for ± 2 hours so that the diffusion process went well. After that, all the petri dishes were incubated for 24 hours at 37ºC, and the Inhibitory Power Diameter (IPD) was measured, which was shown by the clear zone around the paper disc with a caliper. This test was repeated three times.

Data analysis

Essential oil distillation using Stahl distillation produced essential oil yield data with the assumption of 100% concentration. The essential oil yield data were statistically analyzed using one-way analysis of variance (ANOVA) with the help of the Statistical Product and Service Solutions (SPSS) 17 analysis tool.

Antibacterial and antifungal tests were carried out using the Kirby Bauer method to obtain the Inhibitory Power Diameter (IPD) of bacterial and fungal growth. Then the average Inhibitory Power Diameter of the antibacterial and antifungal activity test results was statistically analyzed using a two-way analysis of variance (ANOVA) with the help of the Statistical Product and Service Solutions (SPSS) 17 analysis tool.

RESULTS AND DISCUSSION

Essential oil distillation of Tawangmangu sweet orange peel

Essential oils have been widely used in the health and beauty fields as aromatherapy, relaxation, and a mixture of cosmetic ingredients. In addition, it is also used in the food sector as a flavoring agent. Tawangmangu sweet orange peel is a source of essential oils. This essential oil can be obtained by distillation, pressing, and solvent extraction. In this study, the extraction process of sweet orange peel essential oil was carried out by distillation using a Stahl distillation apparatus. The working procedure of Stahl distillation is the distilled material is in direct contact with boiling water so that hydrodiffusion or water penetration occurs in plant tissues. The water vapor causes the oil glands to rupture so that the essential oil can be carried by the water vapor and then cooled in the condenser to obtain a solution of essential oils. In this study, the essential oil

produced was in the form of a clear yellow liquid with a distinctive smell of Tawangmangu sweet orange peel, as shown in Figure 1.

One hundred fifty grams of Tawangmangu sweet orange peel from different altitudes produced essential oils with different volumes and levels. It is presented in Table 1. The results of ANOVA on the yield of essential oil of Tawangmangu sweet orange peel growing at different altitudes showed a significant difference ($P< 0.05$).

Table 1 shows an increase in the volume of Tawangmangu sweet orange peel essential oil according to the increase in the height of the sampling site. Tawangmangu sweet orange peel taken at an altitude of 1600 m above sea level produces the highest essential oil volume with 0.83 mL. This result is in accordance with the research of Ebrahimi et al. (2011) about the effect of temperature on the essential oil of chamomile. Chamomile grown at the lowest temperature (12ºC) produced the largest volume of essential oil compared to those grown at 15ºC, 20ºC, and 25ºC. According to Fitter and Hay (1991), the temperature is one of the plant stress factors. Therefore, the location with an altitude range of ± 200 meters will affect the environmental conditions, as listed in Table 2.

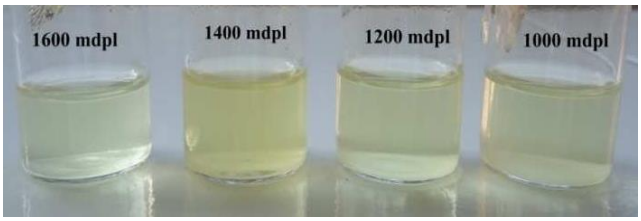

Figure 1. Essential oil of Tawangmangu sweet orange peel (*Citrus sinensis* L.) from different altitudes

Table 1. Volume and yield of Tawangmangu sweet orange peel (*C. sinensis*) essential oil from different altitudes

Altitude (m asl)	Sweet orange peel weight (gram)	The volume of essential oil (mL)	The yield of essential oil (%)
1000	150	0.23	0.15[a]
1200	150	0.40	0.26[b]
1400	150	0.46	0.30[c]
1600	150	0.83	0.55[d]

Note: Different letter notations in the same column based on the DMRT test with a 5% confidence level show a significant difference ($p>0.05$)

Table 2. Environmental parameters at each altitude

Parameter	Altitude (m asl)			
	1000	1200	1400	1600
Air temperature (ºC)	35-38	35-38	27-30	23-28
Light intensity (lux)	920 x 100	1014 x 100	1086 x 100	1197 x 100
Humidity (%)	50	55	64	67
Soil moisture (%)	3	3.5	4.9	5.3
Soil pH	7	7	6.5	6.5

The increase in altitude will cause a decrease in soil temperature and pH and an increase in light intensity and air humidity. The cold environmental conditions affect the formation of secondary metabolites in Tawangmangu sweet orange, especially in forming essential oils. The formation of the essential oil is the response of the Tawangmangu sweet orange plant to temperature stress in its surrounding environment. The lower the temperature, the more essential oils produced by the Tawangmangu sweet orange plant.

Growth curve and standard curve of *S. aureus* bacteria

The purpose of making the growth curve of *S. aureus* is to determine the pattern and time of achieving the logarithmic phase. The logarithmic phase (log phase) is a phase of the rapid growth of bacterial cells because the nutritional needs and ideal conditions for growth are optimally met.

On the growth curve in Figure 2, it can be seen that the logarithmic (log) phase of *S. aureus* bacteria occurred at 0 to 12 hours, and the stationary phase occurred at 12 to 18 hours. Therefore, the age of the *S. aureus* bacteria used for the antibacterial test was the age of the 8th hour with an OD value of 2.50. A standard curve was made to determine the equation in calculating the number of bacterial cells to be used in the antibacterial test. From the standard curve, a regression equation was obtained so that the number of bacteria tested at an OD of 2.50 could be determined as 7.3 x 108 cfu/mL.

Growth curve and standard curve of the fungus *C. albicans*

The purpose of making a growth curve for *C. albicans* is to determine the pattern and timing of *C. albicans* experiencing good and optimal growth by dividing rapidly. On the growth curve of *C. albicans* shown in Figure 3, it can be seen that *C. albicans* has good growth at 0 to 12 hours. *C. albicans* experienced good growth with increased cells, so the 8th hour was determined for an antifungal test at OD 2.00 with the number of fungal spores as much as 1.7 x 108 cfu/mL determined from the standard curve.

Antibacterial and antifungal activity of sweet orange peel (*C. sinensis*) Tawangmangu essential oil

The results of testing the antibacterial and antifungal activity of Tawangmangu sweet orange peel essential oil at each altitude at various concentrations using the Kirby Bauer method showed inhibition of microbial growth, which was indicated by the Inhibitory Power Diameter (IPD). IPD is a clear zone that indicates the inhibition of microbial growth around the paper disc, and a cloudy color on the media indicates the presence of microbial growth, which can be seen in Figures 4 and 5.

According to Ganjar (2006), antimicrobial strength is classified into 3, namely strong activity if it produces IPD more than 8 mm, moderate activity if it produces 7-8 mm IPD, and weak activity if it has IPD less than 7 mm. The essential oil of Tawangmangu sweet orange peel from an altitude of 1,600 m asl and 1,200 m asl can be said to have strong antimicrobial activity against *S. aureus* and *C. albicans* bacteria because it can inhibit microbial growth with an IPD of more than 8 mm, at the lowest concentration. On the other hand, the essential oil of

Tawangmangu sweet orange peel from an altitude of 1,400 m asl has weak antimicrobial activity against *S. aureus* and *C. albicans* bacteria. At the smallest concentration, it inhibits microbial growth with an IPD of less than 8 mm. The essential oil of Tawangmangu sweet orange peel from an altitude of 1,000 m asl has weak antimicrobial activity in inhibiting *S. aureus* bacteria. Still, it is strong in inhibiting *C. albicans* fungi.

The results of ANOVA (Appendix 6 and Appendix 7) showed that Tawangmangu sweet orange peel essential oil at each altitude with different concentrations had a significant effect ($p<0.05$) on the growth of *S. aureus* and *C. albicans*. It means that the concentration of Tawangmangu sweet orange peel essential oil affects the size of IPD against *S. aureus* and *C. albicans*. Furthermore, the height of the growing site also had a significant effect on the size of IPD on the growth of *S. aureus* and *C. albicans.*

The antibacterial activity of Tawangmangu sweet orange peel essential oil obtained from each altitude showed significant differences. It meant that there were differences in antibacterial activity at each altitude. For example, the antifungal activity of Tawangmangu sweet orange peel essential oil obtained from an altitude of 1,400 m asl and 1,600 m asl showed significantly different results. It indicated a difference in antifungal activity at an altitude of 1,600 m asl and 1,400 m asl compared to the other 2 altitudes. In comparison, at an altitude of 1,000 m asl and 1,200 m asl, there was no difference in antifungal activity because the ANOVA results showed no significant difference ($p>0.05$) presented in Table 3 and Table 4.

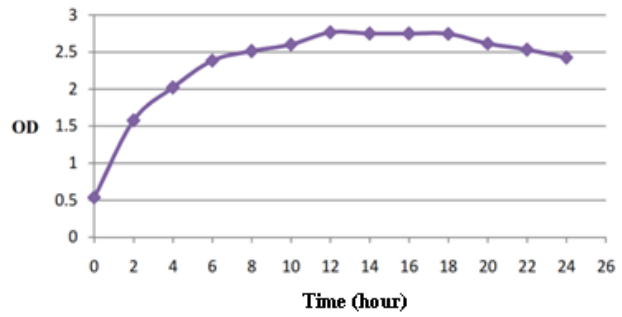

Figure 2. Bacterial growth curve *S. aureus*

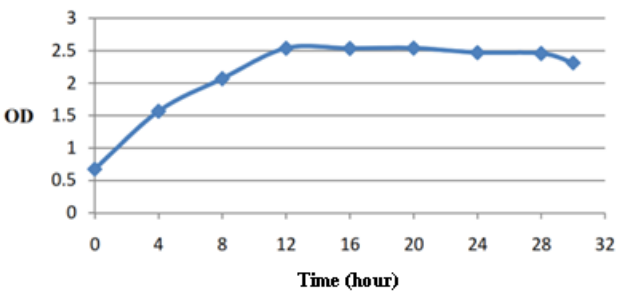

Figure 3. Growth curve of *C. albicans*

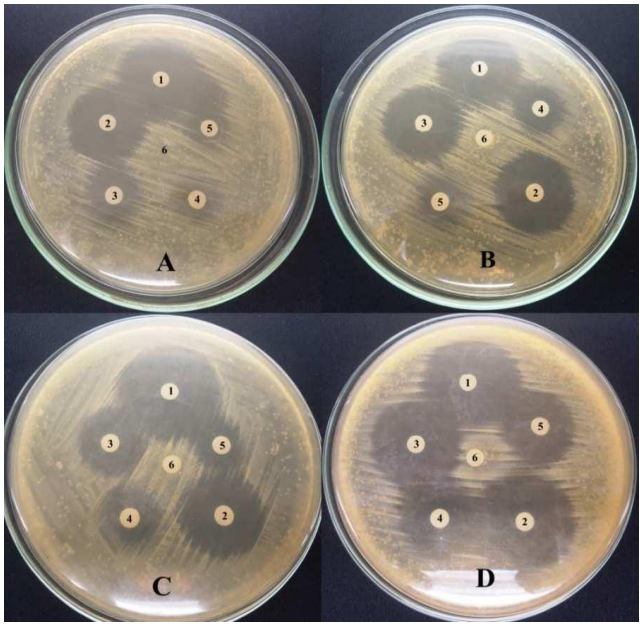

Figure 4. Inhibitory power diameter of essential oil of Tawangmangu sweet orange peel obtained from an altitude of 1,000 m asl (A), 1,200 m asl (B), 1,400 m asl (C), 1,600 m asl (D) with chloramphenicol (1), concentration 100% (2), 75 % (3), 50 % (4), 25 % (5), DMSO (6) against *S. aureus*

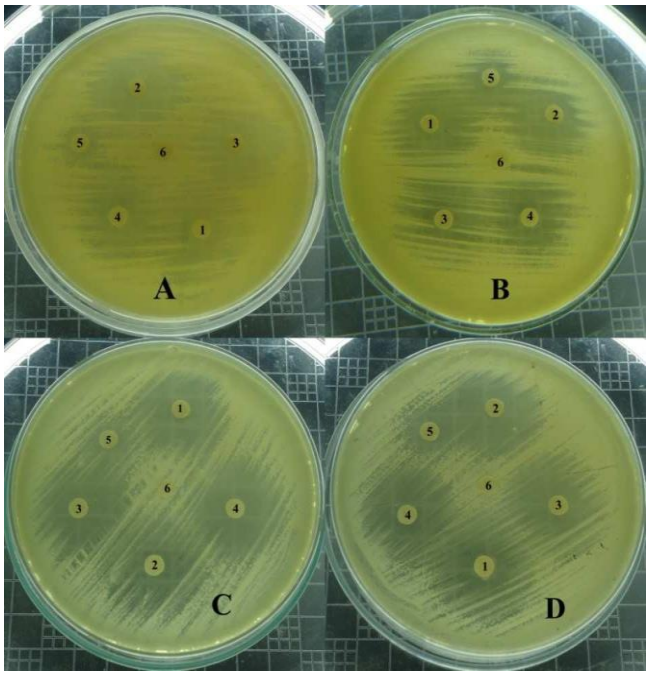

Figure 5. Inhibitory power diameter of essential oil of Tawangmangu sweet orange peel obtained from an altitude of 1000 m asl (A), 1200 m asl (B), 1400 m asl (C), 1600 m asl (D) with ketoconazole (1), concentration 100% (2), 75 % (3), 50 % (4), 25 % (5), DMSO (6) against *C. albicans*

Table 3. Inhibitory power diameter of Tawangmangu sweet orange peel (*C. sinensis*) essential oil on the growth of *S. aureus* bacteria

Concentration (%)	IPD *(mm) at altitude (m asl)			
	1000	1200	1400	1600
100	18aa	18ab	18ac	23ad
75	16ba	14bb	11bc	20bd
50	10ca	12cb	9cc	14cd
25	6da	9db	5dc	12dd
Chloramphenicol 30 mg/mL		27e		
DMSO		0f		

Note: Different letter notations in the same column and row based on the DMRT test with a 95% confidence level showed a significant difference (p>0.05). *: The size of the Inhibitory Power Diameter has been reduced by the diameter of the 6 mm paper disc.

Table 4. Inhibitory power diameter of Tawangmangu sweet orange peel (*C. sinensis*) essential oil on the growth of *C. albicans* bacteria

Concentration (%)	IPD *(mm) at altitude (m asl)			
	1000	1200	1400	1600
100	18aa	18aa	18ab	19ac
75	11ba	12ba	17bb	17bc
50	11ca	10ca	13cb	15cc
25	10da	10da	6db	9dc
Ketoconazole 30 mg/mL		22e		
DMSO		0f		

Note: Different letter notations in the same column and row based on the DMRT test with a 95% confidence level showed a significant difference (p>0.05). *: The size of the Inhibitory Power Diameter has been reduced by the diameter of the 6 mm paper disc.

Tawangmangu sweet orange peel essential oil with concentrations of 100%, 75%, 50%, and 25% can inhibit the growth of *S. aureus* and *C. albicans* bacteria. It is in accordance with the research conducted by Roy et al. (2012). They conducted an antibacterial test of lemon peel essential oil against *S. aureus* bacteria with a concentration of 100% having an inhibitory diameter of 8 mm. Meanwhile, in the study of Mathur et al. (2011), sweet orange peel essential oil with a concentration of 100% was reported to inhibit *S. aureus* with an IPD of 12 mm. This study is also in accordance with the research of Jwanny et al. (2012), who tested the antifungal and antibacterial activity of sweet orange peel from Egypt. This research shows that sweet orange peel essential oil has strong antibacterial activity against *Bacillus subtilis, E. coli, Pseudomonas aeruginosa, Salmonella typhi, Proteus mirabilis, Candida tropicals, Saccharomyces cerevisiae* including *S. aureus* and *C. albicans*. It shows that sweet orange peel essential oil has a strong antibacterial and antifungal potential. Based on Table 3 and Table 4, a diagram depicting the IPD of Tawangmangu sweet orange peel essential oil was made, which is presented in Figure 6 and Figure 7.

In general, Figure 6 and Figure 7 showed that the decrease in the concentration of Tawangmangu sweet orange peel essential oil was in accordance with the decrease in the amount of IPD. The increasing concentration of Tawangmangu sweet orange peel essential oil causes more antimicrobial content, so microbial growth inhibition is greater. Still, an increase follows not all increases in concentration in IPD. For example, Figure 7 showed that the essential oil of sweet orange peel at an altitude of 1,000 m above sea level for concentrations of 75% and 50% had the same strong antifungal activity, i.e., 11 mm. On the other hand, Tawangmangu sweet orange peel essential oil at an altitude of 1,200 m above sea level for concentrations of 50% and 25% had the same strong antifungal power as 10 mm. The possibility of an equally strong antifungal activity was indicated by the same large IPD, which appeared due to the difference in the rate of diffusion of the essential oil on the agar medium and the volatilization of the essential oil on the disc paper because it was left out in the air for too long.

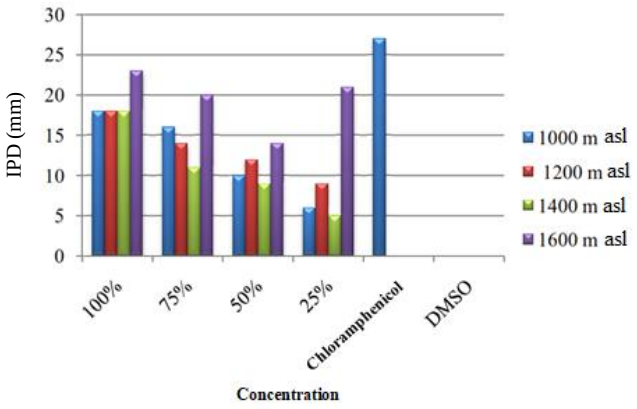

Figure 6. Inhibitory diameter of Tawangmangu sweet orange peel essential oil obtained from each altitude against *S. aureus*

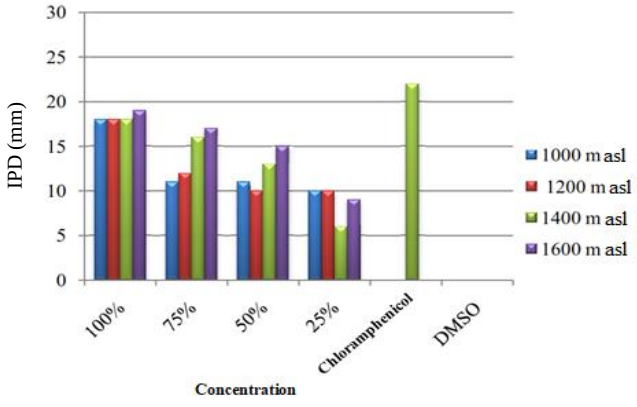

Figure 7. Inhibitory power diameter of Tawangmangu sweet orange peel essential oil obtained from each altitude against *C. albicans*

Nursal and Juwita (2006) stated that phenolic compounds, terpenoids, and flavonoids are secondary metabolic products of plants that actively inhibit the growth of bacteria and fungi. According to Lota in Vasudeva and Sharma (2012), the three main elements in orange peel essential oil are generally limonene, terpinene, and linalyl acetate. Jwanny et al. (2012) reported that the essential oil of sweet orange peel contains ascorbic acid, which causes a sour taste. In addition, this essential oil also contains bioactive compounds such as phenolic compounds, alkaloids, flavonoids (especially flavone glycosides), and limonene which is a terpenoid derivative and the most abundant. Essential oil from sweet orange peel is also reported to be a rich source of bioactive compounds such as saponins, tannins, coumarins, flavonoids, carotenes, terpenes, linalool, limonene, alkaloids, and pinene (Mondello et al. 2005; Ashok et al. 2011). Soković et al. (2007) reported that *C. aurantium* and *C. limon* essential oils contained the largest secondary metabolite compound, limonene, respectively 90.01% and 59.68%. In this study, limonene from *C. aurantium* and *C. limon* showed positive results in inhibiting S. *aureus* bacteria with IPD of 14 mm and 16 mm, respectively. In the study of Siddique et al. (2011), it was known that *C. aurantium* essential oil contained 98.17% limonene, tested for antibacterial, and showed positive results in inhibiting *S. aureus* with an IPD of 12 mm.

The working procedure of essential oils in inhibiting the growth or killing of microbes is by interfering with forming membranes and/or cell walls. The compounds contained in Tawangmangu sweet orange peel essential oil inhibit bacteria by damaging the peptidoglycan (protein) structure in the cell wall through protein denaturation. The *S. aureus* is a Gram-positive bacterium with a cell wall structure with a thick peptidoglycan layer and a thin layer of teichoic acid. Gram-positive bacteria are more sensitive to essential oils than Gram-negative bacteria due to their different outer membrane structure (Chanthaphon et al. 2008). Gram-positive bacteria do not have a lipopolysaccharide layer that protects the membrane, causing essential oils to damage the porin protein more easily, causing cell lysis (Mulyani et al. 2009). Flavonoids can inhibit bacterial growth by damaging the cell walls and cytoplasmic membranes of bacteria and preventing bacterial division so that bacteria cannot grow (Robinson 1995).

The cell wall of *C. albicans* contains 6-25% protein, the rest is carbohydrates (80-90%) and fat (1-7%), and the outer part of the wall is covered with a layer of fibrils in the form of fibers (Chaffin et al. 1998). Non-polar compounds can induce changes in the membrane permeability of *C. albicans* through the interaction between the active site of the compound and the active site of the cell membrane, especially the cholesterol and ergosterol parts. This interaction results in changes in permeability and causes the cell membrane to become unstable, causing fungal cell death (Kusumaningtyas et al. 2008). In addition, alkaloids work by inhibiting the biosynthesis of nucleic acids (Mc-Charty et al. 1992).

Flavonoids have antifungal activity in *C. albicans* by interfering with the formation of pseudohyphae during the pathogenesis process and denaturing proteins so that they can damage cell membranes that are irreversible (cannot be repaired) (Cushnie and Lamb 2005). The mechanism of action of inhibition of antibacterial and antifungal compounds in Tawangmangu sweet orange peel is not known for certain because to find out how these antibacterial and antifungal compounds work, and molecular research is needed. According to Jawetz et al. (2001), the combined activity of several antibacterial compounds can be more effective than the work of each compound. On the other hand, the combined working activity of several antibacterial and antifungal compounds can be less effective than the work of each compound. Judging from the antibacterial and antifungal activity of the active compound of Tawangmangu sweet orange peel essential oil, the inhibition of the growth of S. aureus and C. albicans bacteria may be carried out by all active compounds or only one active compound. It is uncertain and requires further research.

The research can be concluded: (i) Sweet orange peel (*C. sinensis*) Tawangmangu growing at an altitude of 1,000 m asl, 1,200 m asl, 1,400 m asl, and 1,600 m asl produced different yields of essential oils. Tawangmangu sweet orange peel, which grows at an altitude of 1,600 m asl, produces essential oils with the largest yield of 0.55%. (ii) Sweet orange peel essential oil (*C. sinensis*) Tawangmangu at an altitude of 1,000 m asl, 1,200 m asl, 1,400 m asl, and 1,600 m asl had different antimicrobial activities against *S. aureus* and *C. albicans*. Tawangmangu sweet orange peel essential oil at an altitude of 1,600 with a concentration of 100% had the greatest antimicrobial activity with an Inhibitory Power Diameter of 23 mm against *S. aureus* and an Inhibitory Power Diameter of 19 mm against *C. albicans*.

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