Bioteknologi

Biotechnological Studies

| Bioteknologi | vol. 15 | no. 2 | November 2018|
| ISSN: 0216-6887 | E-ISSN: 2301-8658 |

Botryococcus braunii. photo by Robin Matthews

Bioteknologi
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| Bioteknologi | vol. 15 | no. 2 | November 2018 | ISSN: 0216-6887 | E-ISSN: 2301-8658 |

Published semiannually **PRINTED IN INDONESIA**

9 772301 865305

Bioteknologi
Biotechnological Studies

| Bioteknologi | vol. 15 | no. 2 | November 2018 |

ONLINE
http://smujo.id/bbs

p-ISSN
0216-6887

e-ISSN
2301-8658

PUBLISHER
Society for Indonesian Biodiversity

CO-PUBLISHER
Universitas Sebelas Maret, Surakarta, Indonesia

OFFICE ADDRESS
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PERIOD OF ISSUANCE
May, November

Society for Indonesian Biodiversity

Universitas Sebelas Maret
Surakarta, Indonesia

Published by Smujo International for The Society for Indonesian Biodiversity and Sebelas Maret University, Surakarta, Indonesia

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Book:

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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]

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BIOTEKNOLOGI
Volume 15, Number 2, November 2018
Pages: 51-54

ISSN: 0216-6887
E-ISSN: 2301-8658
DOI: 10.13057/biotek/c150201

Cattle urine as a low-cost medium for accelerate growth, biomass productivity, and lipid production of *Botryococcus braunii*: Future energy

ZULFA HIDAYATI♥, ANNISA NUR AROFAH, JIHAN FAKHIRA
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Manuscript received: 10 September 2018. Revision accepted: 9 October 2018.

Abstract. *Hidayati Z, Arofah AN, Fakhira J. 2018. Cattle urine as a low-cost medium for accelerate growth, biomass productivity, and lipid production of* Botryococcus braunii*: future energy. Bioteknologi 15: 51-54.* The increasing necessities of global energy can trigger an energy crisis. Microalgae is a unicellular microorganism which has potency as bioenergy, such as biofuel, and efficient alternative to substitute fossil resources. *Botryococcus braunii* is a microalgae that has a high lipid concentration of around 25-80%, and it is this characteristic which shows its potency to produce biofuel. The research purpose was to utilize cattle urine as an alternative medium for growth of *B. braunii*. Some of the components in cattle urine are 95% water, urea, 2.5% mineral salt, hormone, enzyme, 69% nitrogen, sulfur, and magnesium. The components of cattle urine can substitute macronutrient and micronutrient in others synthesis medium. This research was designed by using Random Design Complete (RAL) with seven variables: control culture *B. braunii* (medium macronutrient+ micronutrient Johnson); cultures *B. braunii* with 5%, 7.5%, 10%, 12.5%, 15%, and 17.5% concentration of cattle urine+micronutrient Johnson. The methods of this research were divided into three steps: preparation, testing, and analysis of biomass also lipid extraction. The data from the observations of biomass were analyzed with linear regression equation. All of data was analyzed in Microsoft Excel. The results showed in culture *B. braunii* with a 12.5% of concentration cattle urine, that it can accelerate the growth of cell *B. braunii* being maximal which production 1.425 mg/L biomass in days 12. Additionally, *B. braunii* culture with 12.5% of concentration cattle urine can accelerate biomass productivity of *B. brauni* which production 9.018 g/L biomass. Whereas with just added cattle urine as medium is not given significantly to lipid production.

Keywords: Biofuel, *Botryococcus braunii*, cattle urine

INTRODUCTION

Energy is one of the most important sectors for life. The increasing necessities of global energy can trigger an energy crisis. In Indonesia, the necessities of global energy will increase from 144 billion Tones Oil Equivalent (TOE) to 1.049 billion TOE in 2050, with growth quickly 5.7% per year (Nurzaman et al. 2015). Microalgae is a unicellular photosynthetic organism that has great potential in the biotechnology industry, especially as a source of renewable energy (Ermavitalini et al. 2017). A renewable energy resource is energy that unlimitedly available in nature (Festus and Ogoegbunam 2015). The provider of using microalgae as an alternative is because microalgae have a short life cycle, capability to synthesized high lipid, and can survive in extreme conditions (Carvalho et al. 2011 and Gumbira 2016). Although production of biodiesel from microalgae still lacks industrial applicability because it requires a higher cost than using fossil (Ermavitalini et al. 2017).

Botryococcus braunii is one species of microalgae that has potency to produce biofuel. *B. braunii* is a unicellular and green microalga that exist in lakes, river, salty, and marine water. The cell size of *B. braunii* ± 15-20µm, and they live in colonies, and are non-motile (Tasic 2016). The chlorophyll of *B. braunii* consists of chlorophyll a, b, and c that amount ±1.5-2.8% (Saputro 2015). The characteristic of this species is lipid, which amounts to 15-80% of the dry weight biomass (Asma et al. 2015). The high lipid productivity can be influenced by factors like light intensity, media, temperature, harvesting technique, culture technique, extraction technique, etc. Medium culture of *B. braunii* is like BG11, BBM-3N, Jaworski's medium, Johnson, etc.

Media composition can have a significant effect on the growth rate and the final concentration of microalgae. Microalgae are known to grow more abundantly in nutrient-rich mediums(Blair et al. 2013). The utilized medium synthetic in large scale can burden the cost production by 60-70% (Patmawati et al. 2014). So, a solution is needed to modify the medium, like by using cattle urine. Cattle urine is one of the alternative medium cultures for growing *B. braunii*. The contents of cattle urine can fulfill macronutrient and micronutrient medium synthetic. Cattle urine contains about 69% nitrogen and other micronutrients (Sharma and Rai 2015). Some of the components in cattle urine are 95% water, urea, 2.5% mineral salt, hormone, enzyme, 69% nitrogen, sulfur, and magnesium (Manalu 2010). The benefits of using cattle urine can be because it is low cost to substitute micronutrient of synthetic Johnson, and can decrease pollution, especially in surface water and on land.

This research aims to use cattle urine as an alternative medium to accelerate growth *B. braunii,* biomass productivity, and production of Lipid cell *B. braunii*.

MATERIALS AND METHODS

This research was conducted between April and July 2018 at the Laboratory of Microalgae, Biology Department Faculty of Mathematics and Natural Sciences Padjadjaran University and Central Laboratories Padjadjaran University, Sumecang, Indonesia. The optimization of concentration medium cattle urine in this research was utilized Random Design Complete (RAL) toward Johnson medium (Agustini 2012). The methods of this research are divided into:

Preparation

Tools and materials preparation

The tools that were used in this research were: reaction tube, analytic digital scales, plastic bottles 1L, Erlenmeyer, aerator, spectrophotometer UV-VIS 680nm, dry oven, centrifuge, and Soxhlet extraction. Whereas the materials that were used in this research were: culture of *Botryococcus braunii* from Algae Laboratories Padjadjaran University, Cattle urine was taken from Ciparanje Faculty of Animal Science Padjadjaran University, 70% alcohol, NaOH, EDTA, n-hexane solvent, and Johnson medium synthesis.

Preparation of starter culture Botryococcus braunii

Microalgae *Botryococcus braunii* was cultivated in plastic bottle amount 4L by used Johnson synthesis. The cultures gave 2500 lux light intensity, radiation period 24 L, at temperature 25-27^{O}C, and aeration condition 3 days.

Testing of medium cattle urine

Cattle urine was taken from Ciparanje Faculty of Animal Science Padjadjaran University. First, neutralization was done from alkali condition. The culture of *B. braunii* was then made. 21 plastic bottles were divided to be: 1 bottle as control and 6 bottles as treatment with 3 replications. The concentration of cattle urine was made 5%, 7.5%, 10%, 12.5%, 15%, and 17.5%. Cattle urine with the respective concentration was made in every bottle treatment, with also 1mL EDTA added. Culture *B. braunii* in aeration condition for 15 days, 2500 lux light intensity, at temperature 25-27^{O}C, and radiation period 24L.

Biomass analysis

The curve diagram of growth *B. braunii* was analyzed by absorbance measurement (Optical Density) every 24 hours with using scanning spectrophotometer UV-VIS 680 nm. 1 mL culture of *B. braunii* was taken by syringe, and measurement of biomass *B. braunii* was analyzed by centrifuge. 10 mL culture of *B. braunii* was taken by syringe in tube centrifugation. The process of centrifugation was for 15 minutes 3000 rpm, and then separated between supernatant and pellet, put the tube centrifugation in oven and dried for 24 hours at 45^{O}C. Formula that used for dry weight biomass (Susanto et al. 2012):

$$\text{Biomass: } \frac{\text{Dry weight biomass (g)}}{\text{Culture volume (L)}}$$

Extraction lipid

The method of lipid extraction was by using Soxhlet extraction. After 15 days, the culture of *B. braunii* which has harvesting, had 1 mL NaOH as flocculant added and kept in 24 hours. Biomass of *B. braunii* was filtered by fabric filter < 20 um, dried using oven during 18 hours in 45^{O}C, then dry biomass of *B. braunii* was extracted with Soxhlet technique and using n-hexane as a solvent (Al-Hothaly et al. 2015). The formula for measurement content lipid (Hamedi et al. 2016):

$$\text{Lipid content: } \frac{\text{Weight of extracted lipid}}{\text{Weight of dried biomass}} \times 100$$

Data analysis

Data from the observations of biomass were analyzed with linear regression equation that used a control culture of *B. braunii*. The results are related between absorbance (Optical Density) and gram/liter biomass *B. braunii*. All of data was analyzed by Microsoft Excel.

RESULTS AND DISCUSSION

The effect of cattle urine media on the growth and biomass productivity of *Botryococcus braunii*

The results from this research showed in every treatment of concentrations of cattle urine gave preference to increase growth cell *B. braunii* for 15 days. The produce of biomass in day 12 (exponential phase) culture that was not given treatment (control) only production 0.156 mg/L biomass. Cattle urine concentration of 5% can produce 0.632 mg/L biomass. Cattle urine concentration of 7.5% can produce 0.763 mg/L biomass. Cattle urine concentration of 10% can produce 0.829 mg/L biomass. Cattle urine concentration of 12.5% can produce 1.425 mg/L biomass. Cattle urine concentration of 15% can produce 1.248 mg/L biomass. With finally, cattle urine concentration of 17.5% can produce 1.309 mg/L biomass. The average in every treatment was presented in Table 1 and the curve of growth cell *B. braunii* was presented in Figure 1.

These results are similar to biomass productivity (absorbance value) in Figure 1. The culture of *B. braunii* was not given a treatment (control) only produced 1.554 g/L biomass. Cattle urine concentration of 5% can produce 3.850 g/L biomass. Cattle urine concentration of 7.5% can produce 4.572 g/L biomass. Cattle urine concentration of 10% can produce 4.841 g/L. Cattle urine concentration of 12.5% can produce 9.018 g/L biomass. Cattle urine concentration of 15% can produce 8.883 g/L. Finally, cattle urine concentration of 17.5% can produce 8.907 g/L biomass. These results are presented in Figure 2.

Table 1. The average of biomass (absorbance value) cell *Botryococcus braunii* during15 days culture.

Day	Average of biomass						
	Control	5%	7.50%	10%	12.50%	15%	17.50%
0	0.018	0.028	0.035	0.033	0.034	0.056	0.039
1	0.038	0.016	0.026	0.031	0.068	0.071	0.079
2	0.059	0.075	0.088	0.121	0.132	0.165	0.174
3	0.063	0.115	0.123	0.154	0.204	0.273	0.293
4	0.101	0.143	0.161	0.182	0.308	0.336	0.333
5	0.062	0.176	0.178	0.239	0.47	0.504	0.489
6	0.133	0.258	0.322	0.382	0.63	0.647	0.642
7	0.154	0.29	0.376	0.435	0.743	0.767	0.732
8	0.144	0.34	0.488	0.551	0.978	0.9	0.902
9	0.165	0.448	0.638	0.662	0.967	1.044	1.1017
10	0.153	0.488	0.642	0.719	1.215	1.107	1.13
11	0.155	0.576	0.73	0.8	0.8	1.177	1.213
12	0.156	0.632	0.763	0.829	1.425	1.248	1.309
13	0.163	0.543	0.53	0.51	1.249	1.231	1.238
14	0.149	0.506	0.407	0.423	1.118	1.185	1.18
15	0.141	0.398	0.455	0.294	1.084	1.107	1.08

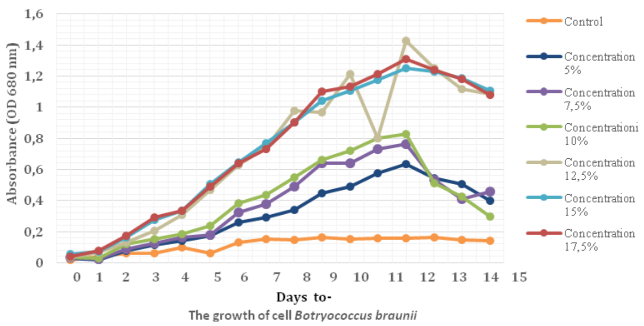

Figure.1 The growth of cell *Botryococcus braunii* using cattle urine as medium for 15 days.

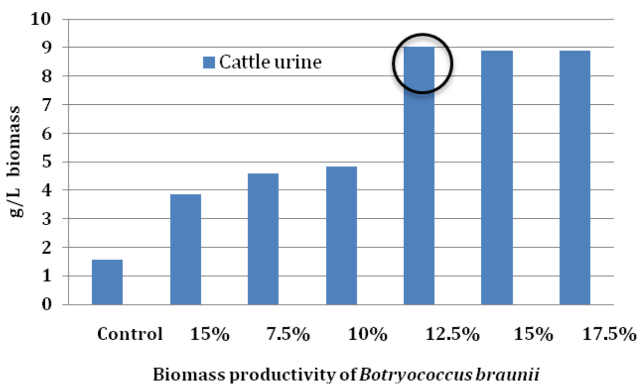

Figure 2. Biomass productivity of *Botryococcus braunii* with using cattle urine as medium.

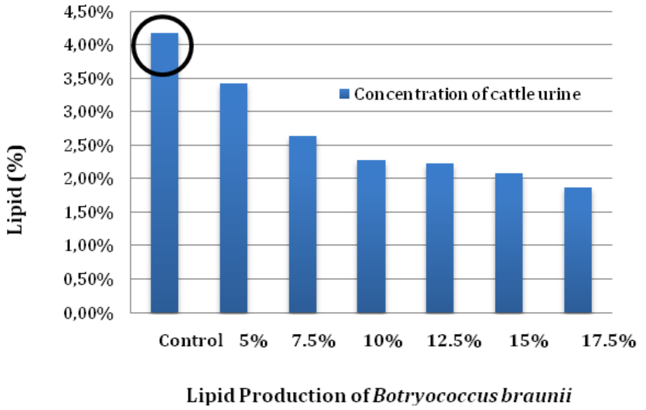

Figure 3. Lipid production of *Botryococcus braunii* with using cattle urine as medium.

The effect of media cattle urine on lipid production of *Botryococcus braunii*

This research showed that cattle urine can affect lipid production of cell *B. braunii*. Although, with some concentrations cattle urine not significantly affecting lipid production. The results from lipid extraction showed that culture of control *B. braunii* can more produce 4.17% lipid than using cattle urine (treatment). Cattle urine concentration of 5% can produce 3.43% lipid. Cattle urine concentration of 7.5% can produce 2.63% lipid. Cattle urine concentration of 10% can produce 2.28% lipid. Cattle urine concentration of 12.5% can produce 2.23% lipid. With cattle urine concentration of 17.5% producing 1.67% lipid. The results are presented in Figure 3.

Discussion

Algae growth was evaluated daily by optical density (OD) measurements at 680nm. The growth cell of *B. braunii* begins with lag phase; in lag phase cell *B. braunii* still adapts toward culture medium. In this research, the lag phase occurs in 0-3rd day for all treatment; the long duration of lag phase is because of the length culture as inoculum which was too long (Fog and Thake 1987). The growth of cell *B. braunii* which was showed by absorbance value, which increases between the low distance and it can show metabolism of cells *B. braunii*. The lag phase also showed that the cleavage of the cell was very low and it can make the cell in 4-9th day the culture *B. braunii* had a log/exponential phase. In exponential phase, the cell *B. braunii* was cleaved very quickly, so the cell can grow so fast. In 10-11th day it had a decreasing phase, this was because the cell *B. braunii* began to cleave slowly. While the next day it was still growing in increase and 13-15thday had a stationary phase, followed by decrease in growth. In this research, the culture with treatment (give cattle urine) can give effect to growth and biomass productivity of cell *B. braunii*. Algae growth was described to follow in five different phases: divided to lag or acclimatization phase, log growth phase, declining growth phase, stationary phase, and death (lysis) phase (Moazami et al. 2012).

The results showed that the culture with cattle urine concentration of 12.5% can accelerate growth cell *B.*

braunii by 1.425 mg/L in 12th day and can accelerate biomass productivity which produces 9.018 g/L biomass. Concentration of 12.5% of cattle urine has more nutrients than other concentrations. The cell of *B. braunii* still has tolerated with concentration of 12.5% cattle urine (Sharma and Rai 2015). If nutrients are added too much it can be toxic and result in the death of the cell of *B. braunii* (Gumbira 2016). But it was different with the result of lipid extraction that showed in culture non-treatment (control) that can produce high lipid amounts of 4.17% rather than culture that was given the treatment (used cattle urine). The culture that used cattle urine did not give significant effect to lipid production of cell *B. braunii*. The culture with low concentration of cattle urine can produce higher lipids, than culture that used a high concentration of cattle urine. The availability 69% of N in cattle urine can increase to form protein than lipid (Manalu 2010), so the culture that added cattle urine only produced a few lipids, because the cell *B. braunii* had formed protein more than formed lipid. Biomass concentration increased, with an increasing concentration of nitrogen in medium culture (Amin et al. 2013). Besides, this research was influenced by some factors. This research suggests to continue observing the effects of using cattle urine in other species of microalgae and considering the factors are like nutrient, pH, light intensity, temperature, salinity, harvesting technique, extraction technique, and etc. (Juneja et al. 2013). To prove that cattle urine can give effect for accelerate growth, biomass productivity, and can increase lipid production, future research must try in other species of microalgae.

ACKNOWLEDGEMENTS

Thank you for Indonesian Ministry of Riset, Technologie and High Education, Department of Biology, Padjadjaran University, and Dr. Joko Kusmoro, as supervisor of this research.

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BIOTEKNOLOGI
Volume 15, Number 2, November 2018
Pages: 55-69

ISSN: 0216-6887
E-ISSN: 2301-8658
DOI: 10.13057/biofar/c150202

Anticancer, antioxidant and antimicrobial screening of extracts from selected medicinal plants from Oshikoto, Namibia

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[2]Department of Molecular Biology and Biotechnology, Faculty of Science, University of Dar es Salaam. Uvumbuzi Rd., Mwalimu JK Nyerere campus University of Dar es Salaam. P.O. Box 35179, Dar es Salaam, Tanzania

Manuscript received: 6 July 2018. Revision accepted: 5 October 2018.

Abstract. *Ilonga SK, Kandawa-Schulz M, El-Sayed Lofty H, Lyantagaye S. 2018. Anticancer, antioxidant and antimicrobial screening of extracts from selected medicinal plants from Oshikoto, Namibia. Bioteknologi 15: 55-69.* Plants have been explored for years to treat ailments such as headaches, stomach-ache, diarrhea, tumors, wounds, and sexually transmitted diseases. *Heliotropium ciliatum*, *Ziziphus mucronata*, and *Gnidia polycephala* are traditionally utilized to treat tumors and wound-related illnesses. Tumors and persistent wounds can be an indication of cancer. Microbial wound infections can lead to fatal consequences, if unattended. This study evaluates the anticancer, antioxidant, and antimicrobial potential of extracts of these three medicinal plants. The leaves (*Z. mucronata*) and the aerial parts (*G. polycephala* and *H. ciliatum*) were ground and subsequently extracted with hexane, dichloromethane, ethanol and methanol, and water. The Brine shrimp lethality test (BST), 3- (4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromine (MTT) assay and APOPercentage^{TM} flow cytometry assay were employed to evaluate the anticancer potential of the extracts, whereas the antioxidant activity was evaluated by 1,1-diphenyl-picrylhydrazyl (DPPH) assay evaluated. The antimicrobial potential of the plant extracts was evaluated using the broth microdilution method against eight wound pathogens: *Escherichia coli*, *Candida albicans*, *Clostridium tetani*, Methicillin-resistant *Staphylococcus aureus*, *Mycobacterium terrae*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis* and *Streptococcus A*. Methanol extracts of *Z. mucronata* and G. polycephala showed good antioxidant activity, compared to that of butylated hydroxyl toluene which served as control. High cytotoxicity is shown by dichloromethane and hexane extracts of *Z. mucronata* and *H. ciliatum*, as well as ethanol extracts of *H. ciliatum*, with LC50 values < 250 µg/mL. Meanwhile, water extracts showed the least cytotoxic activity. Dichloromethane extract of *Z. mucronata* also demonstrated a broad spectrum of antimicrobial activity, achieving MIC values ≤ 1 mg/mL against six out of eight tested microbes. The low toxicity of aqueous extracts of the three plants and the antimicrobial activity observed validates the use of these extracts in conventional medicine. Some extracts appear to be good sources of potential antioxidant and anticancer agents. However, further studies need to be conducted the isolation of potentially valuable drugs from these extracts.

Keywords: Anticancer, antioxidant, antimicrobial, plant extracts

INTRODUCTION

Since ancient times, plants have been a vital source of medicine. Early written reports on the utilization of plants as medicine appeared around 2600 BC when plants were used as medicine by Sumerians and Akkadians (Shoeb 2006). Since this finding, plants have been used to treat ailments such as toothaches, stomach aches, headaches, diarrhea, wounds, tumors, as well as sexually transmitted diseases (Van Wyk and Gericke 2000, Khaleeliah 2001; Von Koenen 2001; Wuyang 2008). Nevertheless, the potential of several plants as medicinal agents has not been fully characterized and established because most scientific studies carried out on plants only focused on specific diseases, thereby revealing a narrow spectrum of bioactive compounds. This limitation is often attributed to the limited resources, tedious work and time allocated for the research.

An example of this limitation is the screening of more than 35, 000 plants extract by the National Cancer Institute (NCI) of the United States in the 1960s which just targeted bioactive compounds for cancer. Mixtures with other medicinal abilities were left unexamined (Hostettmann et al. 1996). Screening of plants for medicinal purposes is crucial because plants are an indispensable source of lead and backbone compounds used in the synthesis of novel drugs in pharmaceutical industries (Potier et al. 1996). About 122 drugs were predicted to have been discovered through ethnobotanical leads of 94 plant species (Funnell et al. 2004). The screening of plant extracts by the NCI during the early 1960s shed light to the discovery of critical anticancer compounds such as Taxol, Camptothecin, and Vinblastine, which are used clinically in the treatment of cancer. Taxol, Camptothecin, and Vinblastine were isolated from extracts of *Camptotheca acuminata*, *Taxus brevifolia* and *Catharanthus roseus*, respectively (Sarkar et al. 1996). In Namibia, plants have been used among different indigenous communities to treat various diseases and ailments. However, the compounds responsible for the healing actions in most of the Namibian medicinal plants are not yet investigated due to the lack of scientific studies and exposure.

Cancer is a disease caused by uncontrolled cell growth (Boyd, 1995). These cells proliferate uncontrollably and, in most cases, form lumps or tumors, and consequently interf with the normal functioning of the cells. In a particular case such as leukemia, the cancerous cells cause cell deformation which results in interference with normal blood functions without forming lumps. Not all tumors are malignant. Cells of cancerous and non-cancerous tumors differ in their ability to relocate to other parts of the body and invade healthy cells (Medical News Today 2009). Uncontrolled cell proliferation is primarily due to oxidative cell damage due to the presence of free radicals. Free radicals are incorporated into the body from the environment via exposure to cigarette smoke, products of incomplete combustion, ultraviolet radiation and are also generated in the body during the normal process of food breakdown (Tsang 2009). The anticancer compounds help prevent or slow down the development of cancer by regulating cell division and by killing cancerous cells (Wall and Wani 1995; Shoeb 2006). Meanwhile, by neutralizing the free radicals and repairing the oxidatively damaged cells, antioxidants can help prevent cancer (NCI 2004).

The objectives of the study were (i) To make crude extracts from the leaves of *Z. mucronata* and aerial parts of *H. ciliatum* and *G. polycephala,* (ii) To assess the anticancer and antioxidant activities of the extracts *Z. mucronata, H. ciliatum,* and *G. polycephala,* (iii) To assess antimicrobial activity of extracts of *Z. mucronata*, *H. ciliatum* and *G. polycephala* against eight wound pathogens.

MATERIALS AND METHODS

Preparation and extraction of plant material

Samples collection and preparation

Plant samples were obtained from Oshikoto, a region in northern Namibia in February 2010. The samples were collected from the leaves of *Ziziphus mucronata* and aerial parts of *Gnidia polycephala* and *Heliotropium ciliatum* collected. The fresh samples were cut in small pieces and air-dried in ambient temperature, then ground using a Waring® commercial heavy-duty blender to form a powder. The ground samples were sealed in a plastic bag at room temperature until use. **Figure 1** displays a map of Namibia pointing the location of the Oshikoto area.

Extraction

Hot water extraction

Aqueous extracts were done based on the method described elsewhere (Li et al. 2008) with little modifications. Briefly, 20 g of the powdered plant's sample was mixed with 200 mL of distilled water in a conical flask and incubated at 70 °C for 2 hours in a water bath. The mixture was cooled to room temperature before being filtered using Whatman filter paper (No. 1). The extracts were concentrated by heating in a water bath. These extracts were placed in a fridge until use.

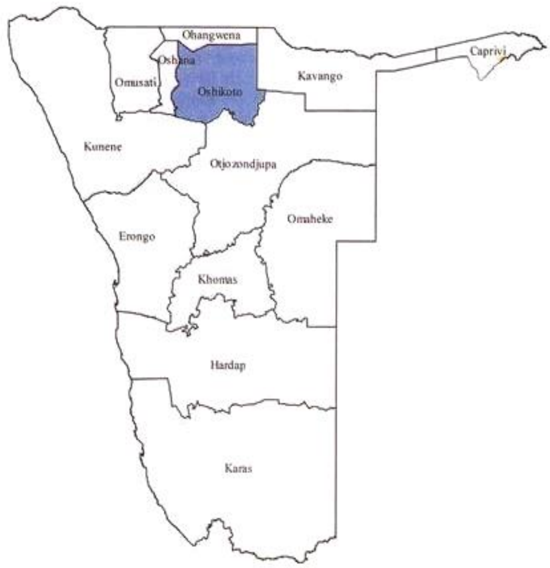


Figure 1. The map of Namibia pointing the Oshikoto are, the sample collection site (Cheikhyoussef et al. 2011)

Organic extraction (Method 1)

Method for organic removal was modified from Goze *et al*. (2009), with slight modifications. Briefly, plant materials were extracted sequentially using hexane, dichloromethane, ethanol, and methanol, in the order of increasing polarities. Fifty grams of the powdered plant samples were soaked in 200 mL of the extraction solvent for three days, then filtered through Whatman® filter paper. Extraction per solvent was only performed once, and the residues were air-dried before further extraction with the next solvent.

Organic extraction (Method 2)

Plant materials were subsequently extracted with hexane, dichloromethane, ethanol, and methanol in order of increasing polarities, as described above. Approximately 25-35 g of the dry powdered plant materials were obtained, with 150 mL of organic solvents in a Soxhlet apparatus for six hours each. The residues were also air-dried before extraction with the next solvent. All natural extracts were concentrated in a rotary evaporator and later left in the fume hood for a few days to dry off the excess solvent. The extracts were placed in a fridge until use.

Bioassays

Antioxidant bioassay – DPPH radical scavenging activity

DPPH radical-scavenging method measured the inhibition effects of the extract on a free radical DPPH (Goze et al. 2009). Two milliliters of different concentrations (25, 50, 75, 100 and 500 µg/mL) of the plant extracts in methanol was added into 2 mL of DPPH solution, shaken vigorously, and allowed to stand for 30 minutes before determining the absorbance with a UV spectrophotometer at 517 nm. One of the dissolving solvents (methanol), without plant extract, was applied as a negative control. Butylated hydroxyl toluene (BHT) was used as a positive control. The inhibition effects of the extract on free radical DPPH were expressed in the formula below:

% inhibition = [($A_{control}$- A_{sample})/$A_{control}$] × 100, where $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance of the sample.

Anticancer bioassay – Cytotoxicity assay using BST

The BST assay was carried out according to the previous method (Meyer et al. 1982) with some modifications (Silva et al. 2005). The shrimp were grown in a small tank that contained two compartments filled with artificial seawater. The shrimp eggs were placed in the covered chamber. A lamp was installed over the open side of the tank to attract hatched shrimps via perforations in the partition wall. The shrimp were grown for 48 hours until mature, now being called nauplii. All plant extracts were dissolved in 2 mL of DMSO. Artificial seawater made by dissolving 3.8 g of sea salt in 1 L of distilled water was added to mesh up 5 mL of total volume. Appropriate volumes of the resulting solution were transferred to tubes, in duplicate, with 5 mL of saline solution containing ten nauplii each to make the final sample concentrations of 24, 40, 80, 120 and 240 µg/mL. The negative control contained brine shrimp, artificial seawater and 0.6% DMSO, under the same conditions without the addition of plant extracts. The tubes were incubated under light for 24 hours, and the number of dead or surviving brine shrimps per tube was recorded. A graph depicting the percentage mortality against logarithm concentration was plotted using a computer program Microsoft Excel. The lethal level causing 50 % mortality (LC_{50}) was determined by taking the anti-logarithm of logarithm concentration corresponding to 50% mortality. An LC_{50} value higher than 1000 µg/mL were considered to represent an inactive extract.

Anticancer potential of the extracts against cancerous cell

Cell lines and cell culture

Small intestinal fetal tissue (H4), Colon adenocarcinoma (Caco-2), Chinese hamster ovary (CHO), non-tumorigenic immortalized human diploid fibroblasts (KMST-6), lung carcinoma (H157), and human cervical cancer (HeLa) cell lines were used to investigate the anticancer potential of the extracts of medicinal plants. Assays using Caco-2 and H4 cell lines were done at the Department of Microbiology, Biochemistry, Molecular Biology, and Biotechnology, University of Maribor, Slovenia. Meanwhile, the APOP*ercentag*e™ assays using CHO, H157, HeLa, and KMST-6 cell lines were performed at the Department of Biotechnology, Western Cape University, South Africa. The cells were seeded in 100 mL conical flasks in a selected medium, supplemented as listed in Table 1 and were incubated in a humidified 5% CO_2 incubator at 37ºC. After reaching ~90% confluency, they were disassociated with trypsin and cells counted before plating.

Cell trypsinization and subculturing

Cells were ready for trypsinization when they had reached ~90% confluency after 2-4 days of incubation. The cell monolayer was rinsed with 2 mL of a trypsin-EDTA solution, then incubated with 1 mL of trypsin to allow for cells detachment from the flask surface. Fresh media (9 ml) was added to stop, and the cell suspension was pooled by centrifugation at 800 rpm at 22 ºC for 5 minutes. The pellet was re-suspended in ten mL of fresh media with the addition of FBS (Table 1). One-fifth of the resuspended cells (2 mL) solution was seeded into a new culture 100 mL flask to which 8 mL of media was added for subculturing. The cells were then incubated at 37 ºC in the 5% CO_2 incubator. The remaining cells were seeded into plates and used for the experiments.

Cell counting

The trypan blue staining technique determined the cell viability. The cell suspension was mixed with 0.1% trypan blue at ratio 1:9 in an Eppendorf tube. A drop of this suspension was transferred to a sealed hematocytometer. The number of live cells inside the 25 squares was calculated under a light microscope, by following the rule of counting on only two sides of the square for cells trapped between the squares. For accuracy in calculations, the number of cells counted had to be between 10 and 25. If the cell number was more than 25 cells in the 25 squares, the cell solution was diluted with 4- (2-hydroxyethyl)-piperazine-1-ethanesulfonic acid - buffered saline solution (HEPES-BSS) and the cells were counted again. The cell concentration (the number of cells per mL of cell suspension) was calculated using the following formula:

Number of cells/mL cell suspension = number of cells counted ×10^6/25 squares

Cell plating

From the concentration of cells obtained from the step above, the cells were diluted to the desired concentration with fresh media. For the cell growth inhibition assay, the cells were seeded in 96-well plates at a density of 111 cells/mL and for the inhibitionMTT assay at 6 × 10^4 cells/mL. Cells were seeded in 12- well plates at a density of 2.5 × 10^4 cells/mL for the APOPercentage™ apoptosis assay. One-hundred µL of the cell suspension was delivered into each well using a multichannel pipette in 96-well plates. The cell suspension was transferred into a sterile petri dish which was shaken regularly before drawing out the cell suspension. For a 12-well plate, 1 mL of the cell suspension was poured into each well. The plates were incubated for 24 hours at 37 ºC in a 5% CO_2 incubator or until they reached 90% confluency before treatment with plant extracts.

Testing plant extracts for anticancer activity

Cell growth inhibition assay

Caco-2 and H4 cells suspensions were seeded into 96-well plates. The outlying wells of the plates, i.e., rows A and H and columns 1 and 12, were supplied with 100 µL of distilled water. The inner wells were provided with 100 µL of cell solution (Figure 2). The plates were incubated at 37 oC for 24 hours at 5% CO_2. The cells were treated with plant extracts on the next day, by replacing the media with fresh media containing plant extracts.

Table 1. Growth media and supplements for the cell lines used.

Cell line	Species	Media	Supplements
H4	Human	DMEM	5% Foetal Bovine Serum, 100 U/mL Penicillin, 100 µg /mL Streptomycin, 2 mM L-glutamine
Caco-2	Human	DMEM	5% Foetal Bovine Serum, 100 U/mL Penicillin, 100 µg /mL Streptomycin, 2 mM L-glutamine
CHO	Chinese Hamster	F-12Hams	10% Foetal Bovine Serum, 50 U/mL Penicillin, 50 µg /mL Streptomycin
H157	Human	DMEM	10% Foetal Bovine Serum, 50 U/mL Penicillin, 50 µg /mL Streptomycin
HeLa	Human	DMEM	10% Foetal Bovine Serum, 50 U/mL Penicillin, 50 µg /mL Streptomycin
KMST-6	Human	DMEM	10% Foetal Bovine Serum, 50 U/mL Penicillin, 50 µg /mL Streptomycin

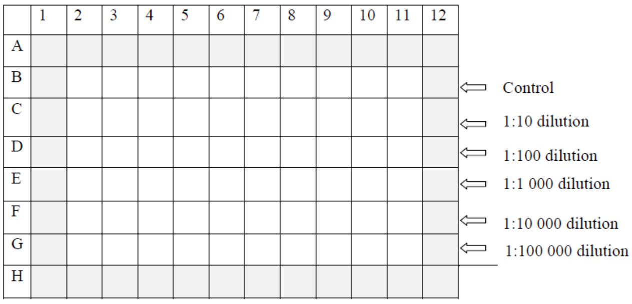


Figure 2. The treatment of cells in the 96-well plate for the growth inhibition assay.

The extracts were dissolved in DMSO to generate a stock solution of 50 mg/mL. Row B was filled with control solution. Eleven microliters of plant extract stock solution were supplied into well in row C, performing a 1: 10 serial dilution through to well in row G. The experiment was conducted in 5 replicates (Figure 2). The plates were incubated at 5% CO_2, 37 ºC for eight days, where cell growth was visible under a light microscope. After that, the media was removed, and the cells were treated with crystal violet for 5 minutes. Under running tap water, the crystal violet was washed off; then the plates were dried on a towel paper overnight. We counted the number of colonies observed in wells under the light microscope. The highest concentration, in which some selective toxicity against Caco-2 cells was observed (500 µg/mL), was used as the highest concentration in the MTT assay. Only extracts which exhibited selective toxicity towards Caco-2 cell lines were further used for the cytotoxicity (MTT) assay.

Cytotoxicity assay

Concerning the slow growth of Caco-2 cells, only H4 cells were used for this assay. Extracts that displayed selective toxicity against Caco-2 in the previous test were used in this assay. The cells were seeded and incubated as mentioned in the method section. Before the test, the cell monolayer was washed two times with 200 µL of sterile PBS. One hundred and twenty microliters of DMEM only supplemented with L-glutamine (no FBS, no indicator) was added.

Nine microliters of the extract stock solution (50 mg/mL) were mixed with 291 µL of DMEM (no phenol red) media to prepare working solution of 1.5 mg/mL. Row A served as a control. To row B, 60 µL of extract solution was applied (making the starting concentration of 500 µg/mL). A 1: 4 serial dilution was made through to wells in row H, always discarding 60 µL from the last well to afford a final volume of 120 µL per well. The experiment was done in triplicate, and the plates were incubated for 24 hours. The effects of DMSO as the extract dissolving solvent was also tested, which was treated similarly as the plant extracts.

After 24 hours, two sets of 40 µL of the incubation media from two wells were moved into sterile 96 well plates in a sequence corresponding to that of the original dish, and these were later used for the H_2O_2 and NO assay. The leftover media was discarded, and the cells were used for the MTT assay. The three tests were performed following the provided protocol as briefly described below.

MTT assay

First, the cell monolayer was rinsed with PBS. We added 220 µL of a mixture DMEM (only supplemented with L-glutamine) and 5 mg/mL MTT solution (at the ratio 10: 1). The plates were incubated at 5% CO_2 and 37 ºC for 5 hours to develop a purple formazan color. Then, the media was discarded, and the plates were air-dried on a towel paper overnight. One hundred microliters of 0.04% HCl in isopropanol was poured to each well to dissolve the formazan. The plates were shaken on a rotating shaker for 5 minutes, before being incubated at 5% CO_2 and 37 ºC for 20 minutes. The absorbance was read at 570 nm with background wavelength set at 630 nm.

NO assay

To the forty µL of the overnight incubation media, we added forty µL of Griess reagent. The plates were gently shaken on a shaker for 20 minutes, and the absorbance was read at 540 nm.

H_2O_2 assay

To the forty µL of the overnight media, forty µL of 0.01% H_2O_2 was added, followed by the addition of 100 µL of TMB-H_2O mixture (at ratio 1:1). The control was made by mixing 50 µL of 0.001% H_2O_2, 50 µL of 0.01% peroxidase and 100 µL mixture of 1:1 TMB and H_2O_2 in one well. The mixture was placed on a shaker for 20 minutes before measuring the absorbance at 450 nm.

APOPercentage™ assay (Flow cytometric analysis of apoptosis)

Preparation of extracts. Plant extracts (20 mg) were dissolved in 200 µL DMSO, then 800 µL of an appropriate media was mixed to make a stock solution of 20 mg/mL.

From the stock, 2.5 and 5.0 mg/mL of working solutions were prepared by diluting the stock solution with the media. The working solution was filtered through a filter paper with pore size: 0.45 µm.

APOPercentage™ assay. Chinese hamster ovary (CHO), human cervical cancer (HeLa), lung carcinoma (H157), and non-tumorigenic immortalized fibroblasts (KMST6) cell lines were utilized in this assay. When the cells in the culture flask had grown to 90% confluency, cells were trypsinized, and the pellet was resuspended in complete media to make a cell density of 2.5×10^4 cells per mL. These cells were seeded in 12-well tissue culture plates, 1 mL per well and incubated at 37°C for 24 hours.

Following this step, the media was replaced with working extract solution at 2.5 and 5.0 mg/mL. Complete media without plant extract served as a negative control, whereas 150 µM ceramide was used as a positive control. The cells were incubated again at 37 ºC and 5 % CO_2 for 24 hours. The negative control cells were applied to aid in adequately distinguishing healthy cells from apoptotic cells. Floating (apoptotic) cells were moved to 15 mL centrifuge tubes, and the adherent cells were trypsinized and added to tubes containing floating cells. The cells were centrifuged at 300 x *g* for 3 minutes to obtain a pellet, washed twice with PBS by centrifuging at 300 x *g* for 3 minutes for each wash. At the end of the last roll, the cell pellet was resuspended in the residual PBS added with 250 µL APOP*ercentage*™ dye (a 1: 160 dilution in complete media). The cells were incubated for 30 minutes. After that, 500 µL of PBS was added to the tube, and the cell mixture was spun down for 5 minutes at 300 x g to obtain a cell pellet. The pellet was rinsed one more time with PBS, resuspended in 400 µL of PBS and analyzed using a FASCan™ (Becton Dickson) instrument equipped with a 488 nm Argon Laser as a light source within an hour. The acquisition was made by setting forward scatter (FSC), and side scatters (SSC) on a log scale dot plot to differentiate the population of cells and debris. On a linear histogram dot plot, APOP*ercentage*™ (FL-3 channel) was calculated against the relative cell numbers. Negative control cells were applied to set the cells in the negative quadrant before measurement of all samples. A minimum of 10, 000 cells per sample were obtained and analyzed using CELLQUEST Pro software by setting the non-stained (untreated) cell population in the first quadrant (10^1) of the forward side scatter histogram dot plot and cells which appeared in the second (10^2) or the third quadrant (10^3) were regarded as APOP*ercentage* ™ positive (apoptotic/necrotic) cells.

Antimicrobial test

We assessed antimicrobial activity of the selected medicinal plants according to method established earlier with few modifications (Chingwaru et al. 2011). Briefly, *M. terrae*, *E. coli*, *Streptococcus* A, *P. aeruginosa*, C. tetani, MRSA and *S. epidermidis* were cultivated in nutrient broth (10 mL), and *C. albicans* in yeast extract peptone glucose (YEPG) broth (10 mL) for 24 hours at 37 oC. The optical density of the microbial suspension was determined at 600 nm, and the microbial suspension was diluted with media to yield microbial suspensions of 1× 103 colony forming unit (CFU)/mL for bacteria and 1× 103 CFU/mL for yeast, which was then used for the experiments. Before the addition of the microbe, the microbial suspension was shaken to distribute the microbes evenly.

Plant extracts were dissolved in DMSO to prepare a stock solution of 50 mg/mL. The extracts were diluted with nutrient broth for bacteria (or YEPG for *C. albicans*) to make a working solution at a concentration of 20 mg/mL for *E. coli* and *M. terrae*; and 5 mg/mL for all the other microbes. In a 96-well plate, forty µL of the appropriate media was filled into each well. To the first well, 40 µL of working extract solution was given and a 1:2 serial dilution was carried out through to the last well, always discarding 40 µL from the previous well. Twenty microliters of the bacterial suspension (1 ×105 CFU/mL) or yeast (1 ×103 CFU/mL) was added. The plates were incubated at 37 oC. Measurement of the microbial growth was done at 600 nm every hour within the first 10 hours and at the end of the experiment, i.e., at 24 hours. DMSO and appropriate growth media were used as negative control whereas gentamycin (4 mg/mL) and streptomycin (10 mg/mL) were utilized as a positive control. Data was set in triplicate, and the absorbances were expressed as mean ± standard deviation. The minimum inhibition concentration (MIC) was determined as the lowest concentration which inhibited the growth of the microbe.

RESULTS AND DISCUSSION

Natural products extraction

Depending on the method, some native plant products could be successfully extracted during the extraction process (Table 2). As described earlier, method 1 is room temperature extraction, and method 2 is the Soxhlet extraction. The weight of starting material used for these two extraction methods is about 50 g for method 1 and ranged between 25-35 g for method 2. All yields were showed as yield fraction, i.e., yield fraction = amount of extract/amount of starting material to allow for statistical comparison. The percentage yields were shown as means ± standard deviation and are shown in Table 2.A-D. Although the yield of the Soxhlet extraction was slightly higher than that obtained from room temperature extraction, the yield per extraction solvent were not statistically significant except for ethanol extract of *H. ciliatum* (t= -5.533, p=0.005), hexane extracts of *G. polycephala* (t= -4.708, p=0.009) and hexane and methanol extracts of *Z. mucronata*. The yield accumulation from the two extraction methods, however, were statistically significant, with p values of 0.001 (*Z. mucronata*), 0.004 (*G. polycephala*) and 0.005 (*H. ciliatum*). Since the yields per extraction solvent for all plants were not statistically significant with a few exceptions, only extracts of method one were used in the assays for this experiment. Codes of these extracts were shown in Table 3 and were referred to by these codes throughout this document.

Table 2. Comparative analysis of extraction yield of different extracts of the three medicinal plants using cold extraction by soaking for three days (for method 1) and Soxhlet extraction for six hours (for method 2). The yield per extraction solvent per plant are displayed in Table 2.A (*G. polycephala*), Table 2.B (*H. ciliatum*) and Table 2.C (*Z. mucronata*). Table 2.D presented the pooled yields per plant. * Values were the mean ± standard deviation of three replicates.

Extract	Percentage yield (%)	
	Method 1*	Method 2*
A. *G. polycephala*		
Hexane	1.90±0.31	3.42±0.47
Dichloromethane	2.27±0.25	2.95±0.55
Ethanol	2.51±1.09	4.14±0.47
Methanol	5.77±0.52	12.02±1.94
B. *H. ciliatum*		
Hexane	1.10±0.19	1.23±0.68
Dichloromethane	1.03±0.32	1.27±0.12
Ethanol	1.06±0.36	2.68±0.35
Methanol	2.70±0.12	4.26±1.16
C. *Z. mucronata*		
Hexane	1.50±0.10	3.35±0.36
Dichloromethane	1.57±0.16	1.88±0.86
Ethanol	4.98±0.09	14.2±0.16
Methanol	9.70±0.92	12.9±1.3

D. The pooled yields per plant

Plant	Cumulative percentage yield (%)	
	Method 1*	Method 2*
Z. mucronata	17.74±1.20	32.34±2.16
H. ciliatum	5.88±0.52	9.45±1.01
G. polycephala	12.45±1.44	22.53±2.64

Table 3. Codes assigned to hexane, dichloromethane, ethanol, methanol, and water extracts of *G. polycephala*, *Z. mucronata*, and *H. ciliatum*.

Plant		
	Hexane	Dichloromethane
Z. mucronata	ZH	ZD
G. polycephala	GH	GD
H. ciliatum	HH	HD

Table 4. IC50 values (µg/mL) for the DPPH scavenging activity.

Extract	IC_{50} (µg/mL)
BHT	29.92
GM	50.20
GE	140.68
GH	> 1000
HM	357.59
HE	343.81
ZM	45.19
ZE	82.68

Antioxidant activity: DPPH assay

The ethanol and methanol extracts of the three traditional medicinal plants were examined for their ability to scavenge DPPH radical. These extracts demonstrated dose-dependent DPPH scavenging activities. Methanol extracts tend to show higher antioxidant activity compared to ethanol extracts (Figure 3-5). The DPPH scavenging activity for the methanol extract of GM and ZM was comparable to the activity of the synthetic antioxidant BHT, although ZM showed slightly higher activity than that of GM (Figure 6). For all extracts, as indicated in Figure 6 and 7, *Z. mucronata* showed the highest DPPH scavenging activity, followed by *G. polycephala*, while *H. ciliatum* is showing the least activity. The concentration of the extracts that were able to scavenge at least 50% of the DPPH dye (IC50) was measured. ZM and GM showed the most active with low IC50 of 45.19 µg/mL and 50.20 µg/mL respectively. *Z. mucronata* exhibited the most DPPH radical scavenging activity among the three plants with both extracts showing IC50 < 85 µg/mL and *H. ciliatum* possessed the least DPPH radical scavenging activity with IC50 for both extracts > 300 µg/mL. The results are summarized in Table 4.

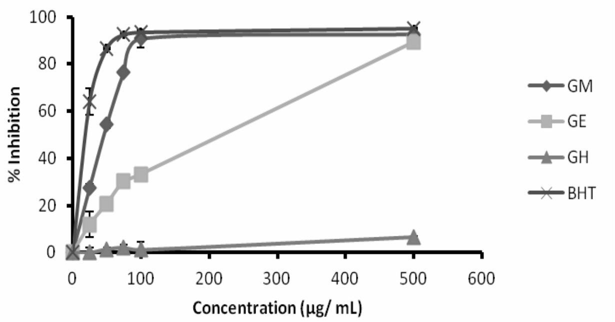


Figure 3. DPPH scavenging activity of GE, GM, and GH extracts as compared to that of BHT.

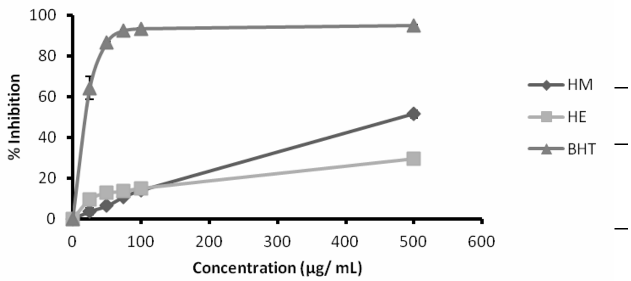


Figure 4. DPPH scavenging activity of HE and HM extracts as compared to that of BHT.

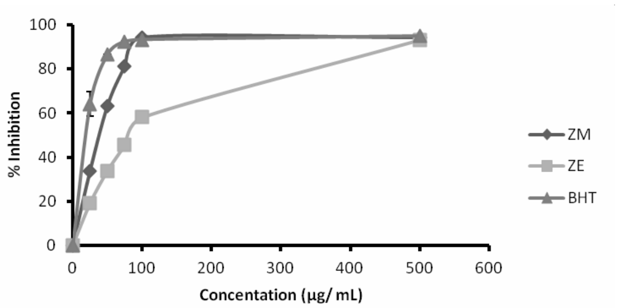


Figure 5. DPPH scavenging activity of ZE and ZM extracts as compared to that of BHT.

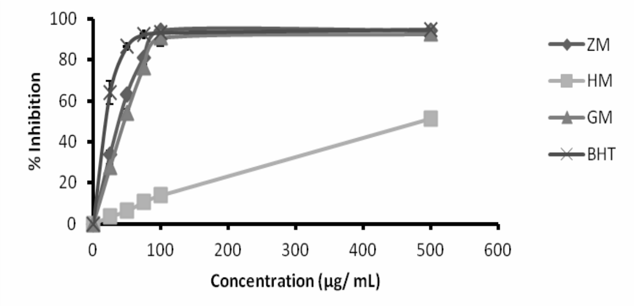


Figure 6. DPPH scavenging activity of methanol extracts of the three indigenous plants.

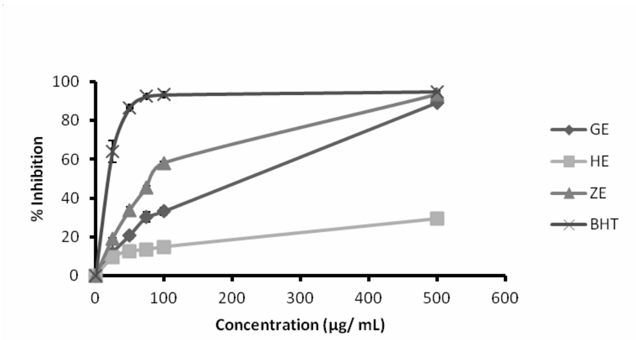


Figure 7. DPPH scavenging activity of ethanol extracts for the three indigenous plants.

Anticancer activity

Brine shrimp lethality test

None of ZM, ZW, ZE, and GM extracts were active, showing lethal concentration to at least 50 % (LC50) of the *A. salina* larvae at a concentration higher than 1000 µg/mL. An LC50 value of ≥ 1000 µg/mL was applied as inactive and extracts which showed this score were classified safe to the tested organism. *H. ciliatum* demonstrated the most toxicity with LC50 for the hexane, ethanol, and methanol extracts < 200 µg/mL. Other extracts that exhibited an LC50 < 200 µg/mL are hexane and dichloromethane extracts of *Z. mucronata*. Extracts of *G. polycephala* fell in the moderate toxicity range with LC50 values for ethanol, dichloromethane, and hexane extract all above 250 µg/mL (Table 5).

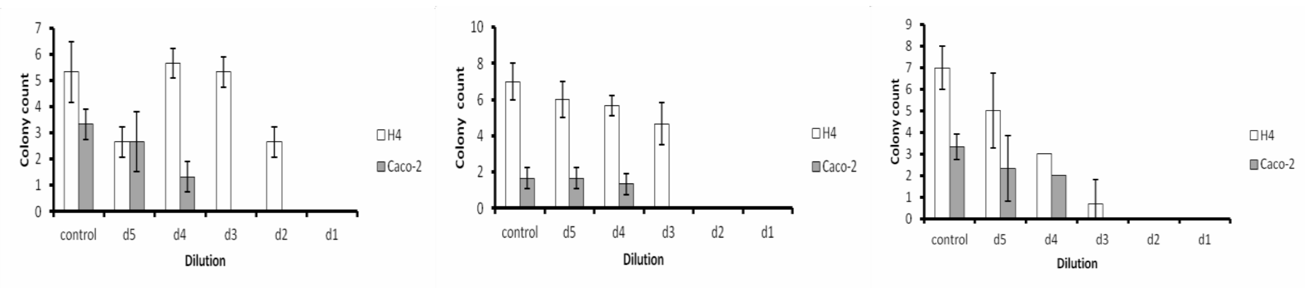
Table 5. Brine shrimp activity of extracts of the three selected medicinal plants. LC= Lethal concentration; LCL= Lower confidence limit; UCL= Upper confidence limit; *= No activity (mortality) up to the maximum tested concentration (240 µg/mL); # = LC50 ≥1000 µg/mL.

Extract	LC_{50} (µg/mL)	95% Fiducial Limit (µg/mL) LCL — UCL
ZW	#	#
ZM	#	#
ZE	*	*
ZD	165.64	44.50—616.43
ZH	198.54	119.04—331.12
GM	*	*
GE	264.46	48.39—1445.34
GD	590.85	181.862—442.95
GH	729.27	100.945—268.53
HM	169.71	51.52—558.99
HE	142.67	54.723—71.93
HD	230.27	40.863—76.46
HH	131.84	94.991—82.99

Cell growth inhibition assay

We identified GH, GE, GD, GM, ZD, ZE, ZM, HH, HE and HM after screening for selective activity against cancerous Caco-2 cells and unhealthy H4 cells. These extracts were used in the cytotoxicity assay. The influence of plant extracts on the proliferation of the two cell types is shown in Figure 8 below. The plant extracts dilutions were d1 is a 1:10 dilution, d2 is 1:100 dilution, d3 is 1:1000, d4 is 1:10 000, d5 is a 1: 100 000 dilution. The extracts which showed selected toxicity towards Caco-2 cells are listed in Table 6.

However, as the proliferation of any two different cell lines differs, it makes identification of which extracts are active against Caco-2 cells but not H4 cells, quite tricky. Also, it is difficult to keep the same concentration of diluted cell solution, and only some of the transferred cells survive and proliferate, thus, making it tough to compare entire colonies acquired with different cell lines. The spectrophotometric method is also not reliable as much of the absorbance recorded is somewhat because of the dye attached to the plate walls than the dye retained by the cell colonies.



A **B** **C**

Figure 8. The proliferation of H4 and Caco-2 cell lines against GE (A), ZD (B) and HH (C).

Table 6. Plant extracts which showed selective toxicity towards the Caco-2 cell line.

Plant	Hexane	Dichloro-methane	Ethanol	Methanol
Z. mucronata	✗	✓	✓	✓
G. polycephala	✓	✓	✓	✓
H. ciliatum	✓	✗	✓	✓

Table 8. Anti-proliferation activity of selected plant extracts against H4 cells.

Extract	IC_{50} (µg/mL)
GM	180.91
GE	264.04
GD	126.95
GH	160.37
HM	145.73
HE	116.80
HH	119.34
ZE	658.40
ZD	126.73

MTT assay

Chinese hamster ovary cells

Water extracts showed no activity at a concentration of 2.5 mg/mL, while at a concentration of 5 mg/mL, the proliferation of CHO cell lines declined by 25% for GW and 55% for ZW (see Figure 9).

H4 cells

Figure 10 illustrates plant extracts which exhibited anti-proliferation activity in a dose-dependent manner. IC50 was determined by plotting concentration against % cell survival. Table 7 shows HE and HH were the most active with IC50 of 116.80 µg/mL and 119.34 µg/mL respectively. Whereas, GE and ZE showed the least activity with IC50 values of 264.04 µg/mL and 658.40 µg/mL, respectively.

NO assay

Cells did not produce a significant amount of NO after treated with various concentrations of the plant extracts, except at the lowest dilution factor (Figure 11).

H_2O_2 assay

H4 cells treated with different levels of plant extracts did not produce a significant amount of H_2O_2 because the absorbances of cells treated with the extracts were like that of the DMSO (control), except at the highest concentration tested (Figure 12).

APOPercantageTM assay

This assay evaluates the apoptotic effect of extracted samples and to evaluate its dose-response activity. Apoptosis also is known as programmed cell death is a process characterized by cell shrinkage, membrane blebbing and nuclear condensation (Chinkwo 2005). The cells treated with plant extracts showed morphological changes such as cell shrinkage and disintegration, compared to the cells in control. Cells of the negative control were more intact and maintained their shape as compared to cells treated with an apoptotic agent, ceramide, thus reveals the apoptotic activity of some of the tested extracts. An example of cell morphology observations is illustrated in Figure 13.

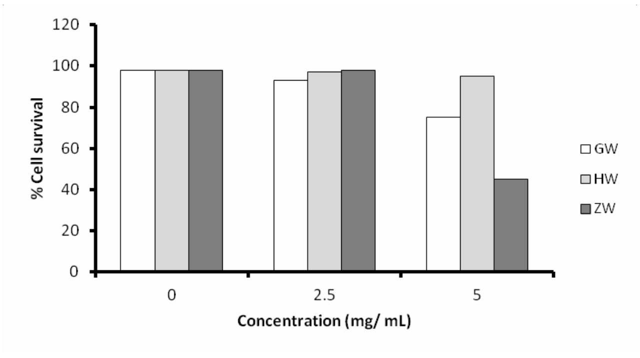
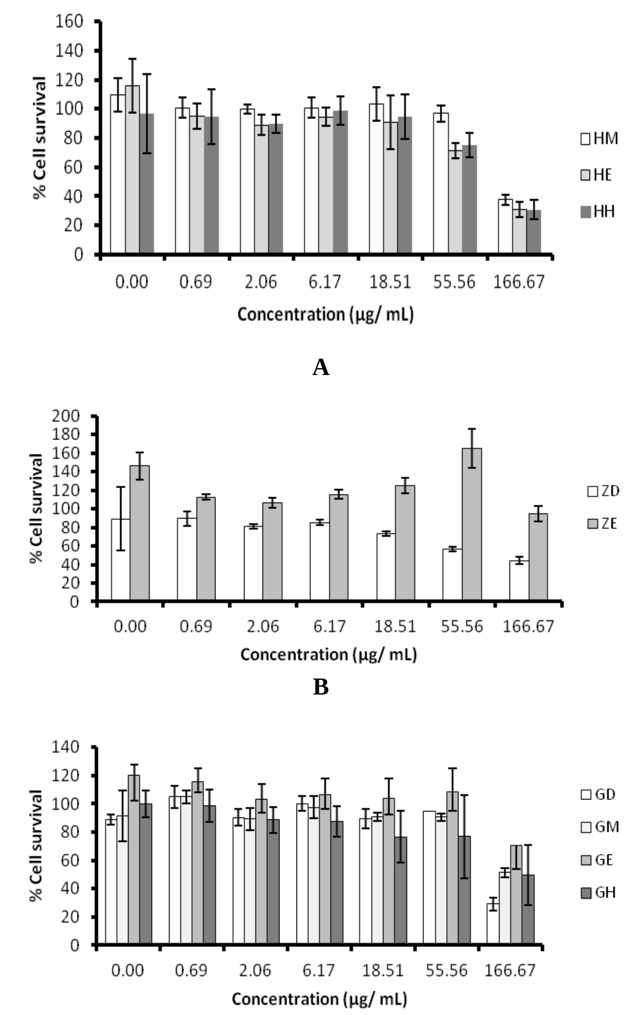
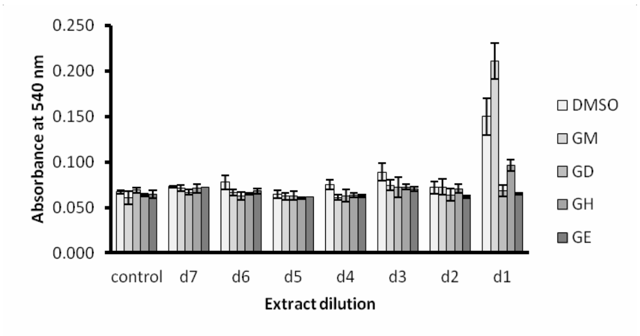


Figure 9. The effects of water extracts of selected traditional medicinal plants on the proliferation of CHO cells

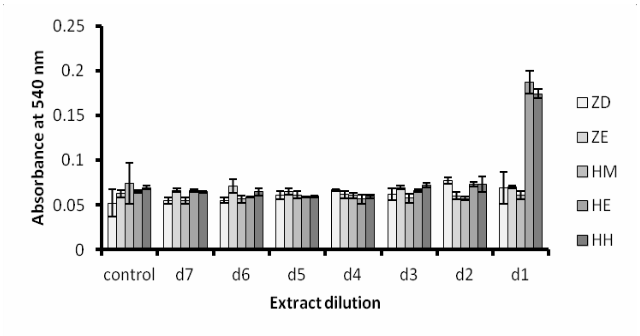


C

Figure 10. The effects of different extracts on the proliferation of H4 cells, *H. ciliatum* (A), *Z. ucronate* (B) and *G. polycephala* (C)

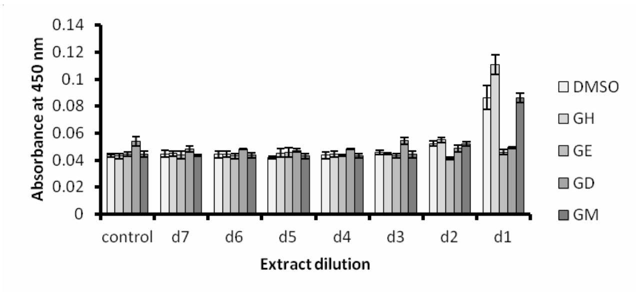


A

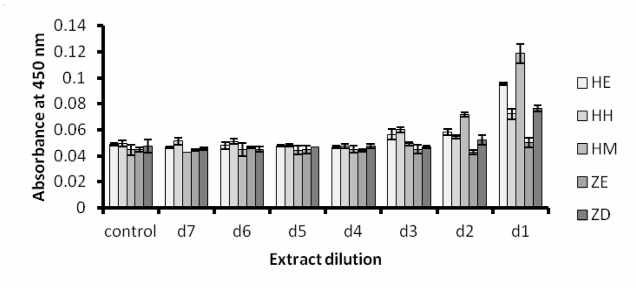


B

Figure 11. The release of NO by H4 cells treated with different levels of plant extracts, (A) extracts of *G. polycephala* and (B) *Z. ucronate* and *H. ciliatum.*



A



B

Figure 12. The release of H_2O_2 by H4 cells treated with different concentrations of plant extract, (A) *G. polycephala*, and (B) *Z. ucronate* and *H. ciliatum.*

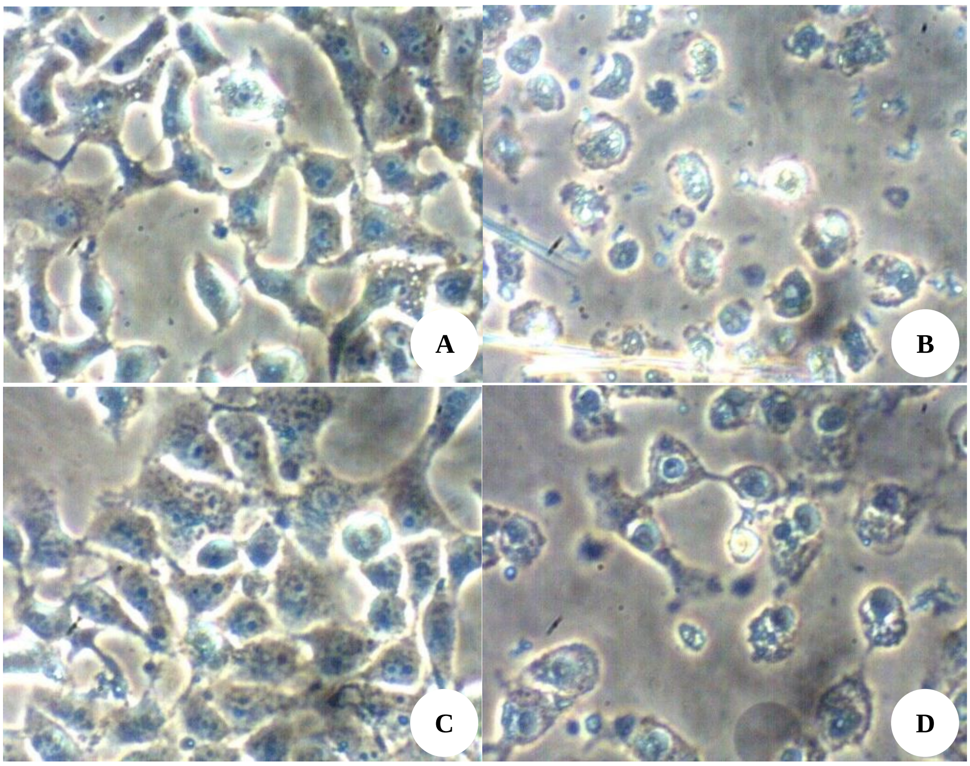


Figure 13. Morphology of HeLa cells treated with 2.5 mg/mL plant extracts. (A) Above, an untreated control, (B) cells treated with 150 µM ceramide, (C) cells treated with ZH and (D) cells treated with ZM.

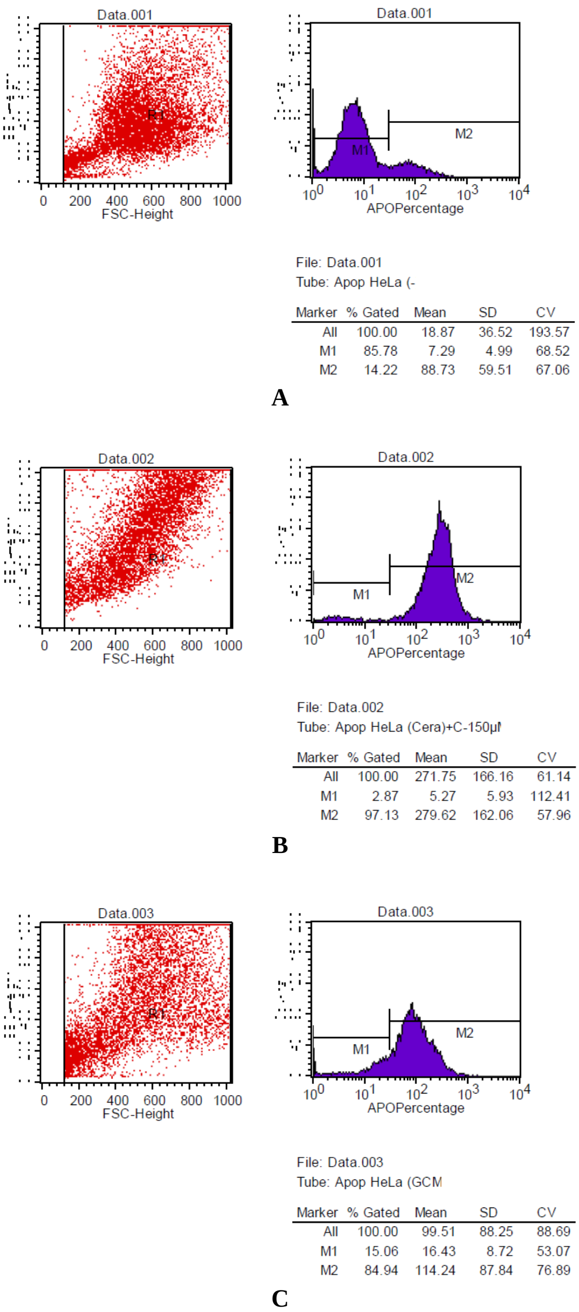


Figure 14. Forward and side scatter and histogram analysis of HeLa cells stained with APOPercentage™ dye. (A) Untreated cells/control, (B) cells treated with 150 µM ceramide and (C) cells treated with 2.5 mg/mL of GM.

As stated in the materials and methods, APOPercentage™ assay was done by setting forward scatter (FSC), and side scatters (SSC) on a log scale dot plot to differentiate a population of cells and debris. On a linear histogram dot plot, APOPercentageä (FL-3 channel) was examined against relative cell numbers (Figure 14).

Plant extracts commonly exhibited apoptotic activity in a dose-dependent manner and reacted differently towards different cell lines. HM was highly active at a 2.5 mg/mL against H157 and KMST-6 cells causing more than 90% cell death (99.15 % and 95.77 %, respectively) and even a higher percentage cell death was observed at 5.0 mg/mL (99.76 % and 99.06 % respectively). The percentage of cell death caused by GM ranged from 58.46 % against H157 and 61.54% against KMST-6. When the extract concentration was increased to 5.0 mg/mL (Figure 14-15), it showed increased cell death population by 78.52 % for H157 and 66.30 % for KMST-6. The LC50 for various extracts tested could not be determined because most extracts exhibited over than 50 % cell death at the lowest concentration examined (2.5 mg/mL). The water extracts for the three medicinal plants were the least active since they caused less than 10 % cell death at the highest level (Figure 15).

The results on the apoptotic activities of different extracts on HeLa cells revealed that, at a level of 2.5 mg/mL, all extracts (except GH, HD, and ZH) were highly apoptotic, killing more than 50 % of the cells, and some causing more than 90 % cell death. This activity is comparable to that of a conventional drug ceramide (150 µM) (Figure 16). The apoptotic percentage of GH, HD, and ZH were of 20.62 %, 42.25 %, and 11.06 % respectively.

Antimicrobial activity

The antimicrobial activity exhibited by the plant extracts depended on the dose and length of exposure, thus, enabling the determination of the MIC of each extract against the pathogens tested in this study. Also, the antimicrobial activities exhibited by different plant extracts were pathogen-specific as they showed different antimicrobial activity against different pathogens. The MIC values shown by different extracts against different pathogens are presented in Table 9.

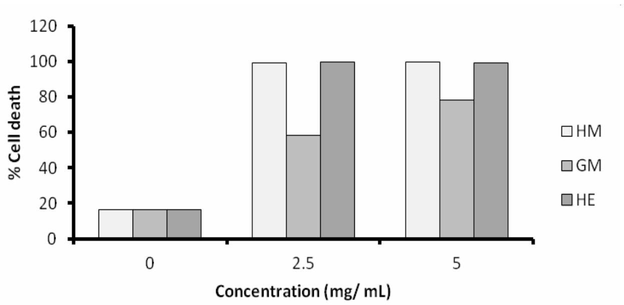


Figure 15. The effects of selected traditional medicinal plant extracts on H157 cells.

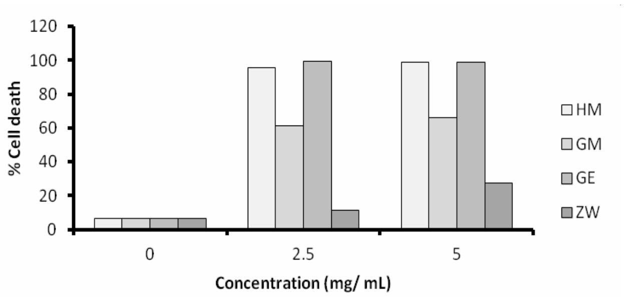


Figure 16. The effects of selected traditional medicinal plant extract on KMST-6.

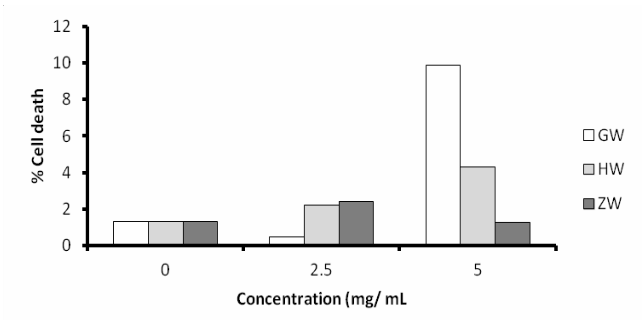


Figure 17. The effects of water extracts of traditional medicinal plants on the proliferation of CHO cells.

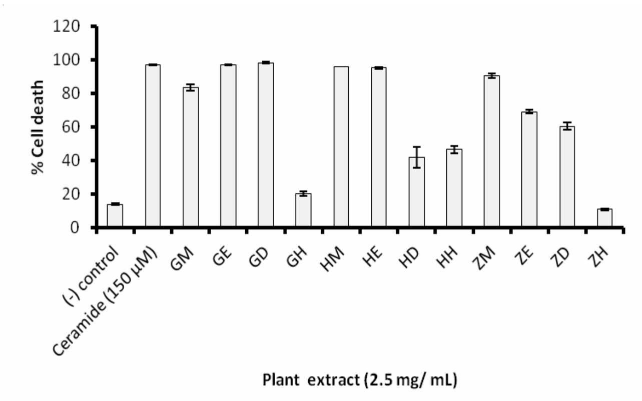


Figure 18. Apoptotic effects of different extracts on HeLa cells.

A comparison of the MIC of various plant extracts and those of the antibiotics (gentamycin, and streptomycin) for the different test organisms, demonstrated that the plant extracts were generally less active. Some extracts, however, showed stronger antimicrobial activity than the conventional drug streptomycin against some of the microorganisms tested. Plant extracts were less active against *M. terrae* than the two antibiotics. HH, HD and ZD (showing MIC < 0.156 mg/mL) exhibited the highest activity against *M. terrae* and GE showed the least activity against the same microbe (MIC = 20 mg/mL) compared to gentamycin and streptomycin (0.03125 and 0.07813 mg/mL). As compared to gentamycin and streptomycin, HM, and GD (MIC 5.0 mg/mL each) show similar activity compared to streptomycin. ZE and ZM (MIC 2.5 mg/mL each) were more effective than streptomycin. The remaining extracts were less active, exhibiting MIC ≥ 10.0 mg/mL.

All extracts showed better results against *P. aeruginosa*, except HD, HE and HH compared to streptomycin, with all exhibiting MIC values ≤ 1.25 mg/mL compared to 5.0 mg/mL recorded for streptomycin. The same is correct about the activity of the extracts against *S. aureus*, through some extracts activity was comparable to that of streptomycin. All extracts showed more potent antimicrobial activity than streptomycin against MRSA (MIC of ≤ 1.25 mg/mL and 2.5 mg/mL respectively). ZD showed better activity than gentamycin. All extracts demonstrated some antimicrobial activity against C. tetani and *S. epidermidis* with MIC values ≤ 2.5 mg/mL. Although, in all cases they were less effective than gentamycin and streptomycin MIC < 0.03125 and < 0.07813 mg/mL against C. tetani and *S. epidermidis*.

Extracts were grouped as being good, moderately good, moderate, or poor antimicrobial agent using the following criteria to ease interpretation (Gibbons 2004; Ríos and Recio 2005 in Suliman 2010):

MIC ≤ 1 mg/ml: good antimicrobial activity
MIC > 1mg/ml or < 4 mg/ml: moderately good antimicrobial activity
MIC = 4 mg/ml or < 6 mg/ml: moderate antimicrobial activity
MIC ≥ 6 mg/ml: poor antimicrobial activity

Table 9. The minimum inhibition concentrations (MIC, mg/mL) of different extracts of *Z. mucronata, H. ciliatum* and *G. polycephala* on different pathogens. Note: MT: M. terrae, PA: *P. aeruginosa*, SA: *Streptococcus* A, EC: *E. coli*, CA: *C. albicans,* MRSA: Methicillin-Resistant *Staphylococcus aureus*, CT: C. tetani, SE: *S. epidermidis.*

Extract	MT	EC	PA	SA	CA	MRSA	CT	SE
ZD	<0.156	10	0.625	0.625	>5.0	0.078	0.313	0.313
ZE	0.313	2.5	1.25	1.25	2.5	0.625	0.625	1.25
ZH	5.0	10.0	1.25	1.25	1.25	1.25	1.25	1.25
ZM	5.0	2,5	1.25	1.25	2.5	0,625	0.625	1.25
GD	2.5	5.0	0.313	0.313	2.5	0.313	1.25	0.313
GE	20.0	10.0	0.313	0.313	2.5	0.313	0.625	0.625
GH	1.25	10.0	0.625	0.625	2.5	0.625	0.625	1.25
GM	2.5	10.0	1.25	1.25	2.5	1.25	1.25	0.625
HD	<0.156	10.0	>5.0	1.25	1.25	1.25	1.25	0.313
HE	1.25	10.0	>5.0	1.25	1.25	0.625	1.25	0.625
HH	<0.156	>20	>5.0	1.25	1.25	1.25	1.25	2.5
HM	2.5	5.0	0.625	1.25	2.5	>5.0	1.25	0.625
Gentamycin	<0.0313	<0.0313	0.25	0.25	1.0	0.5	<0.0313	<0.0313
Streptomycin	<0.0781	5.0	5.0	1.25	5.0	2.5	<0.0781	<0.0781

ZD extract, for example, demonstrated fair antimicrobial activity against MRSA, *P. aeruginosa* and S. aureus, but poor antimicrobial activity against *E. coli*. ZE showed good antimicrobial activity against MRSA, *M. terrae* and *C. tetani* and moderately good antimicrobial activity against all other pathogens. ZH exhibited good antimicrobial activity against all pathogens except for M. terrae, and *E. coli* where it exhibited moderate and poor antimicrobial activity respectively. ZM displayed good antimicrobial activity against MRSA and C. tetani; moderately good antimicrobial activity against *C. albicans*, *S. epidermidis*, and *E. coli* and moderate activity against *M. terrae*. The antimicrobial activities of all extracts against all microorganisms tested were less effective than the conventional antibiotic gentamycin, except for ZD against MRSA (MIC 0.078 mg/mL).

Discussion

Extract yield

Soxhlet extraction method yielded slightly higher as compared to the cold extraction method, though not statistically different. The high yield obtained with the former method is attributed to the increase in temperature of the extraction solvent because the solubility of solute increases with increased temperature (Chang 1998). Nevertheless, the risk of destroying valuable substances increases with temperature.

Antioxidant activity (DPPH assay)

The DPPH assay assesses the antioxidant activity of the extracts. DPPH is a stable free radical, which receives an electron or hydrogen radical to form a stable diamagnetic molecule that is widely employed to investigate radical scavenging activity. An antioxidant will react with the DPPH radicals (Prasad et al. 2009). In this assay, *Z. mucronata* exhibited the most activity, followed by *G. polycephala* whereas *H. ciliatum* showed the lowest activity. Methanol extracts proved to be more potent antioxidants than the corresponding ethanol extracts in all cases (Figure 3-7). A pattern of increasing antioxidant activity with increasing polarity of the solvent has been studied (Goze et al. 2009). The polar methanol solvent easily polyphenols and phenolic compounds which are also polar. Polyphenols and phenolic compounds are great antioxidants (Goze et al. 2009). Flavonoids, a subclass of the class of the phenolic compound was tentatively identified from the leaves of *Z. mucronata*, which justifies the high antioxidant activity described for the methanol extract of *Z. mucronata* (Suliman 2010). The presence of carbon-carbon double bond, carbonyl, and free hydroxyl groups contribute to the free radical scavenging activities of scavengers since they can donate either hydrogen radical or an electron to other radicals. By doing so, the scavenger forms a more stable radical (Solomons and Fryhle 2008).

Anticancer activity

A few essays were employed in the assessment of the anticancer activity of the extracts of the three medicinal plants, namely: the BST, MTT, NO, H_2O_2 and APOPercentage™ assay. These widely used cytotoxic and anticancer assays employed in the preliminary examination of toxicity, detection of toxins and evaluating the anticancer potential of compounds or extracts. The assays are reasonably reliable, and the results obtained are an accurate reflection of the possibility of the test compounds as anticancer agents. The NO and H_2O_2 assays produced no significant activity from all the extracts tested in this study (Figure 11-12). These results suggest that no H_2O_2 were released during cell death or that these compounds were excreted in low amounts at the concentrations of extracts tested. We compared the performance of different extracts in the three anticancer assays: BST, MTT, and APOPercentage™ assays. ZD, HE and HM exhibited excellent anticancer activity in all the three experiments. HD, ZW, GW and HW extracts were inactive against cancer in all the three tests performed. GM, GD, ZM showed negative results in the BST test but positive results in the other two tests. ZE, GE only showed positive results in the APOPercentage™ assay. HH and ZH displayed positive results for the BST and MTT assay and adverse effects in the APOPercentage™ assay.

Water extracts of the three medicinal plants demonstrated low anticancer activity. The low cytotoxic potential of the aqueous extracts is of great significance for their conventional use in the treatment of various disorders other than cancer (Uddin et al. 2009). Aqueous extracts are administered in conventional medicine. Because they are of low toxicity, their use in traditional medicine is justified as the extracts can provide toxicity enough to cure a particular ailment but not high enough to intoxicate the cells.

Anticancer activity was observed for at least one of extracts of the three medicinal plants, in at least one anticancer test. Antioxidant activity was also reported for some of these extracts. Plant anticancer and antioxidant compounds are secondary metabolites, compounds which can be restricted to specific taxonomic groups be it, family, genus, or species according to (Balandrin et al.,1985). Although no published work could be referred for the three medicinal plants on the exact work done in this study, there have been reports on studies of other species in the same genus and on different topics regarding these plants. Biological activity and chemical composition on species belonging to the same group as the plants under study had been published. Assuming the production of secondary metabolites is restricted to the genus level, the conclusion concerning the observations made in this study can be drawn.

Most plant-based secondary metabolites are phenolic compounds, alkaloids, flavonoids, and tannins (Gupta et al. 2004; Wong et al. 2006; Uddin et al. 2009). These natural products possess large pharmacological properties including cytotoxic and cancer chemopreventive effects. Flavonoids, steroids, and triterpenoids particularly exert multiple biological effects due to their antioxidant and free radical scavenging abilities (Gupta et al. 2004). Reports have shown antioxidant and anticancer activity associated with a variety of classes such as polyphenols, flavonoids, and catechins (Uddin et al. 2009).

The literature supports the results of this study. Numerous alkaloids, flavonoids, and anthocyanins were isolated from *Z. ucronate* (Suliman 2010). Anticancer activity of *Z. jujube* against HepG2 cells has also been reported (Huang et al. 2007). Some triterpenoid acids isolated from *Ziziphus jujube* showed moderate anticancer activity against some cell models (HT-29, HepG-2, and NCI-H460) using the MTT assay (Guo et al. 2011). Pyrrolizidine alkaloids and indicine-n-oxide were reported for H. indicum and other Heliotropium species (Spjut, 1985; Velasco et al. 2005). Methanol extracts of *H. zeylanicum* also possessed anticancer and antioxidant activity against Ehrlich ascites carcinoma cells in Swiss albino mice in vivo (Kandasamy et al. 2005). Some Gnidia species contain diterpene esters and coumarins which possessed antitumor activity (van Wyk et al. 1997, van Wyk and Gericke 2000). All these works are in support of the findings of this study.

The exact mechanism behind the biological activity is not known, but speculations have been made. Polyphenolic compounds might inhibit cancer cells progression by xenobiotic metabolizing enzymes that alter metabolic activation of potential carcinogens. While some flavonoids could also change hormone production and inhibit aromatase to prevent the progression of cancer cells. In another theory, the mechanism of action of anticancer activity of phenolics could be done by disrupting cellular division during mitosis at the telophase stage. Phenolics reduce the amount of cellular protein, mitotic index, and formation colony during cell proliferation. The more the number of hydroxyl groups in the phenolics, the higher is the antioxidant activity. The presence of a 4-carbonyl group of the flavonoid molecule also plays roles to the anticancer activity. Also, the presence of 2, 3-double bond in the flavonoid molecules correlates with mitochondrial damage and cancer cell death (Prasad et al. 2009). Some extracts of traditional medicinal plants studied here have the potential to yield useful antioxidant and anticancer drugs. However, further studies need to be conducted the isolation of potentially valuable drugs from these extracts.

Antimicrobial activity

The method used here method entails two criteria: the first being the number of plant extracts that showed excellent antimicrobial activity, i.e., MIC value of ≤ 1 mg/mL and the second is the average MIC value obtained by the plant extracts against a specific pathogen (Table 10) (Suliman 2010). Seven extracts out of the twelve tested plant extracts showed excellent antimicrobial activity against MRSA and *S. epidermidis* (MIC value ≤ 1 mg/mL), making the two pathogens the most sensitive. *P. aeruginosa* and *C. tetani* were the second sensitive as good antimicrobial activity (MIC value ≤ 1 mg/mL) against these pathogens were acquired in five of the twelve extracts tested. *C. albicans* and *E. coli* were the least sensitive as no good antimicrobial activity was received against these pathogens by the plant extracts tested.

About the average MIC values, the lowest average MIC value (0.91 mg/mL) was demonstrated against *S. epidermidis*, followed by *C. tetani*, *Streptococcus* A and MRSA showing average MIC values of 0.96 mg/mL, 0.99 mg/mL, and 1.10 mg/mL respectively. *C. albicans*, *M. terrae*, and *E. coli* were the least sensitive (Table 10).

Extract that showed the best antimicrobial activity

We determined which extract exhibited the best antimicrobial activity against the microorganisms tested using the method described by Suliman (2010). Two criteria were adopted in this method: first, determining the lowest MIC value of each extract and the pathogen against which this MIC value. Second, determining the number of pathogens against which the extract obtained a MIC value of ≤ 1 mg/mL. The criteria summarize the pathogens against which the plant extracts obtained good antimicrobial activity.

ZD exhibited the lowest MIC value (0.078 mg/mL) among all extracts tested against MRSA. HD and HH acquired the second lowest MIC values (<0.156 mg/mL) against M. terrae. The next lowest MIC value (0.3125 mg/mL) was shown by ZE, GE, and GD against M. terrae, *Streptococcus* A, *P. aeruginosa*, MRSA or *S. epidermidis*. For all extracts, a MIC value ≤ 1 mg/mL was obtained against at least one of the pathogens, except for ZH extract which lowest MIC value was 1.25 mg/mL. This result makes many of the extracts screened good antimicrobial agents against pathogens.

About the second criterion which was the number of pathogens against which the plant extracts obtained MIC value ≤ 1 mg/mL, ZD possessed the broadest- spectrum activity, exhibiting good antimicrobial activity against six of the eight test pathogens. GE followed, obtaining a MIC value ≤ 1 mg/mL against five of the eight tested pathogens followed by GD and GH, which both exhibited good antimicrobial activity against half (four) of the tested pathogens. ZH was the least active, not obtaining a MIC value of ≤ 1 mg/mL against any of the tested pathogens (Table 11).

Table 10. Summary of antimicrobial activity of plant extracts against wound pathogens.

Pathogen	Number of extracts with MIC value ≤ 1 mg/mL	Average MIC value (mg/mL)
M. terrae	4	3.40
E. coli	0	8.75
P. aeruginosa	5	1.88
Streptococcus A	4	0.99
C. albicans	0	2.29
MRSA	7	1.10
C. tetani	5	0.96
S. epidermidis	7	0.91

Table 11. Lowest MIC values obtained per plant extracts and pathogens against which MIC values of ≤ 1 mg/mL were obtained.

Plant extract	Lowest MIC value (mg/mL)	Pathogen against which the lowest MIC value was obtained	Pathogens against which the extract obtained an MIC value of ≤ 1 mg/mL
ZD	0.078	MRSA	*M. terrae*, *P. aeruginosa*, *Streptococcus* A, *C. tetani*, *S. epidermidis*, MRSA
ZE	0.3125	*M. terrae*	*M. terrae*, *C. tetani*, MRSA
ZH	1.25	*P. aeruginosa*, *Streptococcus* A, *C. tetani*, *S. epidermidis*, MRSA, *C. albicans*	None
ZM	0.625	*C. tetani*, MRSA	*C. tetani*, MRSA
GD	0.3125	*P. aeruginosa*, *Streptococcus* A, *S. epidermidis*, MRSA	MRSA, *P. aeruginosa*, *Streptococcus* A, *S. epidermidis*
GE	0.3125	*P. aeruginosa*, *Streptococcus* A, MRSA	MRSA, *P. aeruginosa*, *Streptococcus* A, *C. tetani*, *S. epidermidis*
GH	0.625	*P. aeruginosa*, *Streptococcus* A, MRSA, *C. tetani*	*C. tetani*, *P. aeruginosa*, *Streptococcus* A, MRSA
GM	0.625	*S. epidermidis*	*S. epidermidis*
HD	<0.156	*M. terrae*	*S. epidermidis*, *M. terrae*
HE	0.625	MRSA, *S. epidermidis*	MRSA, *S. epidermidis*
HH	<0.156	*M. terrae*	*M. terrae*
HM	0.625	*P. aeruginosa*, *S. epidermidis*	*P. aeruginosa*, *S. epidermidis*

ZD, ZE, ZM, GD, GE, GH, GM, HD, HE, HH and HM all exhibited good antimicrobial activities against some of the pathogens tested. ZD, ZE, and ZM showed excellent antimicrobial activity against *M. terrae*, *P. aeruginosa*, *Streptococcus* A, *S. epidermidis*, *C. tetani* or MRSA. Antimicrobial activity showed by extracts of *Z. mucronata* was as expected since the antimicrobial activity of different extracts of *Z. mucronata* against different pathogens has been published. Good antimicrobial activity for methanol and acetone extract of both the leaves and bark of *Z. mucronata* against *S. aureus*, *Bacillus subtillis, Klebsiella pneumoniae* have been reported (MIC ≤ 6.25 mg/mL) (Mthethwa 2009). Antimicrobial activity of ethanol extracts of *Z. mucronata* against *S. aureus* and *E. coli* has also been reported (Adamu et al. 2004). Olajuyigbe and Afolayan (2011) said antimicrobial activity of methanolic extracts of the bark of *Z. mucronata* against *E. coli* and *S. aureus*.

The findings of the potent antimicrobial activity of some plant extracts in this study support the use of these plant extracts in traditional medicine. The medicinal plants studied here are all used in the treatment of wounds, and the antimicrobial activity reported for these plants supports the traditional use of these plants. The prevention of microbial wound infections is one of the critical factors in wound healing and wound management (Ayyanar and Ignacimuthu 2009; Davis and Perez 2009). However, more studies need to be carried out to confirm the role of the different natural products in the observed biological activity.

Conclusion

The yields per extraction of the two extraction methods were not significantly different, which indicates the preference of the cold extraction method to lower the risk of destroying valuable compounds. *Z. mucronata* showed high antioxidant activity that could be attributed to flavonoids, alkaloids, and terpenes, as reported earlier. High apoptotic activity was reported for *H. ciliatum*. Heliotropium species were said to possess toxic pyrrolizidine alkaloids and indicine-n-oxide. Antitumor activity has also been recorded in some Heliotropium species. Diterpene was found in Gnidia species, which could explain the toxicity observed for *G. polycephala*, though detail analysis is required to confirm this. Low toxicity was published for the water extracts of the three plants. Water extracts are commonly used in conventional medicine and the low toxicity observed here is of significance to their application in the treatment of other diseases and ailments other than cancer. The low toxicity could also indicate a low concentration of the active compound since crude extracts were analyzed. Antimicrobial activity of extracts against different wound pathogens is not surprising as these plants are also used in the treatment of wounds.

Nevertheless, many aspects of this research need to be further investigated to confirm the observed activity. The simple assays employed in this study gave good results about the potential of these plant extracts as antioxidant, anticancer and antimicrobial agents. More in-depth studies are necessary to confirm the activity of the promising extracts. These include the employment of other antioxidant assays such as the total antioxidant assay, whole phenolic assay, and the superoxide assay to mention a few and compare the antioxidant potential of the extracts through different methods. For the anticancer assay, numerous cell lines were used, some of which were only used against some of the extracts and not others. Also, for some tests such as the APOPercentage™ assay, the test concentrations were high, and as a result, it was not possible to determine the IC50 concentrations. In future, the use of fewer cell lines and instead the test of all plant extracts at different levels against the cell lines is suggested as this will enable the comparison of the activity of various extracts against a particular cell line and give their IC50 concentrations. An assay-guided fractionation and purification of the crude extracts are also recommended because this will provide detail information as to which fraction of the crude extract is active and the percentage activity as compared to the crude extract.

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BIOTEKNOLOGI
Volume 15, Number 2, November 2018
Pages: 70-83

ISSN: 0216-6887
E-ISSN: 2301-8658
DOI: 10.13057/biofar/c150103

Determination of bacterial composition, heavy metal pollution and physicochemical parameters of fishpond water in Abothuguchi Central, Meru County, Kenya

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Manuscript received: 27 July 2016. Revision accepted: 24 October 2018.

Abstract. *Laibu PK, Maingi J, Kebira A. 2018. Determination of bacterial composition, heavy metal contamination and physicochemical parameters of fishpond water in Abothuguchi Central, Meru County, Kenya. Bioteknologi 15: 70-83.* Humans have consumed fish as a supplementary source of proteins and as a source of income. Because fish perform all of their body functions in water, the quality of water is essential to their livelihood. Fish farmers have encountered losses due to the death and stunted growth of fish due to bacterial infections and diseases, among other causes. Bacteria found in fishpond water and their pathogenic effects differ with the quality of the pond water, along with the variation in the frequency with which water is changed in the ponds. Physico-chemical parameters of pond water and heavy metals influence the growth and productivity of fish. This study aimed at determining the contamination levels of both fecal and pathogenic bacteria; namely *Salmonella* spp., fecal *Streptococcus*, fecal coliforms *Pseudomonas* spp., *Vibrio cholerae* and *E. coli* in fishponds water. Additionally, the study examined the concentration of heavy metal contamination and variability of physicochemical parameters and their effects on fish. Isolation of fecal indicators and pathogens was carried out using standard laboratory methods. Some physicochemical parameters were measured in situ using a portable Universal multiline P4 WTW meter, while others were analyzed in the laboratory. The determination of the heavy metal presence and concentration in the water samples was carried out by employing of Flame Atomic Absorption Spectrometer. The results indicated that the pond water was heavily contaminated with fecal streptococci and fecal coliforms, and they varied significantly in the sites. Pearson correlation analysis showed a positive correlation between the prevalence of fecal streptococci and fecal coliforms. Potential pathogens such as *Vibrio* spp., *Salmonella* spp., *P. aeruginosa*, and *E.coli* were taken from the water samples with high population. Physico-chemical parameters, namely pH and dissolved oxygen, deviated from the permissible limits according to international standard. The study has shown that the fishpond water was highly contaminated with both fecal and pathogenic bacteria, with physicochemical parameters varying significantly. Heavy metals, except for iron, were within the recommended limits; hence, no significant contamination of the fishpond water. The study suggests the use of treated tap water, routine monitoring of fishpond water and sensitization of farmers on bacterial contamination of pond water. More studies with the aid of molecular techniques should be employed to characterize the bacteria. The findings of this study can, thereby, serve as an impetus to improve fish farming in Meru County, as a way of meeting the growing nutritional demands in the country.

Keywords: Heavy metal, physicochemical, pond water

INTRODUCTION

Fish can be considered an essential food and for many globally, is a main source of protein. Most developing countries consumed more than 30% of their animal protein from fish (Emikpe et al. 2011). Fish farming has previously not been well practiced in Meru County, though the area is an extensively agricultural zone. Now, fish farming is practiced at a small scale by farmers, organized groups and even institutions (Gitonga 2006). The demand for fish in Meru has increased; this has led to a growing interest in fish farming. Fish farming has faced several challenges of infections and disease occurrence resulting in stunted growth and even death of fish (Egbere et al. 2008). Successful pond management demands an understanding of the role of nutrients and other water quality parameters, as well as regular monitoring of environmental parameters within the pond's ecosystem. Water quality is often not prioritized in pond management, and this has led to common problems, such as noxious smells, excessive algal blooms, and subsequent death of fish. An understanding of primary water chemistry and other physical parameters is necessary to prevent these problems.

The primary sources of contamination in water supplies are pathogenic microbes, organic substances and inorganic chemicals, and heavy metal (Sosbey 2002). Treatment methods that are easy to use, effective, affordable, functional, and sustainable are necessary (Sosbey 2002). Enteric bacteria such as *Escherichia coli*, *Salmonella* spp. and *Vibrio* spp, Staphylococcus aureus, are likely to accumulate in fish that live in waters contaminated with human wastes, where sanitary standards are improper in the residential area or fishponds supplied with water from polluted rivers. Therefore, the microbial quality of farmed fish is primarily determined by the quality of water in which they are cultivated (Fafioye 2011).

Bacteria from genera *Aeromonas*, *Pseudomonas*, *Vibrio*, *Salmonella*, *Corynebacterium* and *Myxobacterium* cause infectious diseases in fish (Ampofo and Clerk 2010). *E. coli* is a common contaminant of food and water and a well-recognized foodborne pathogen (Dutta et al. 2010). Bacteria are known to cause disease, subsequently leading to a low production rate of fish.

Different sources of water utilized for fish farming affect bacterial invasion of fish in many ways, causing stunted growth or even death (Egbere et al. 2008). Most aquaculture practices favor disease occurrence. While high fish densities increase stress among stocks and the feeds provide an abundant substrate for microbial growth, the sub-optimal environment of poor water exchange predisposing infections (Ampofo and Clerk 2010). Physical and chemical properties of fishpond water are essential in the growth and productivity of fish. Fish dependency on water is crucial; thereby the source, volume, and the quality of physical-chemical parameters such as dissolved oxygen (DO), pH, temperature, conductivity, total alkalinity, total hardness, total solids, transparency values, carbon dioxide, nitrite-nitrogen, carbonates, sulphates, and ammonia are some of the salient factors to consider in relation to fish health (Fafioye 2011).

Agro-based industries (for example, paper, sugar, coffee, dairy, tea, and fish tanneries) discharge semi-treated effluents with high Biochemical Oxygen Demand (BOD) to the rivers (Nzomo 2005). Heavy metals like cadmium, zinc, mercury, chromium, cobalt, copper, nickel, manganese, iron, vanadium, and molybdenum cause heavy pollution particularly in the ponds, lakes and river systems in zones affected by effluents discharged from industries, sewage as well as agricultural drains (Ida 2012). The accumulation of these heavy metals in fish leads to the suppression of fish immunity; thus, allowing the normal pathogenic microbes to develop ulceration and possible bacteria in the bloodstream (Mutuku 2010).

Most of the fish consumed in Kenya come from the wild, although some through fish importation. The research of fish diseases is often hampered by poor understanding of the ecological intervention, involving interactions between pathogens-hosts in the aquatic ecosystem as well as the lack of knowledge on physiological features of fish (Nyaku et al. 2007). The art of artificial fish culturing has not been sufficiently developed. Furthermore, the physical, chemical, and biological environments of the fishponds that are being used for fish farming have not been adequately studied. Therefore, this work assessed the microbial contamination, heavy metal contamination and physicochemical properties of selected fishponds in Abothuguchi Central, Meru County, Kenya and to ensure the suitability of such ponds for artificial fish culture.

The objectives of this study were: (i) To determine the microbial quality and the level of pathogenic bacteria in pond water in Abothuguchi Central. (ii) To determine the concentration of heavy metal contamination of fishpond water in Abothuguchi Central. (iii) To assess the variability of physicochemical parameters of fishpond water in Abothuguchi Central.

MATERIALS AND METHODS

Study area

The study was done in Abothuguchi, Meru Central Sub-County, Meru County (Figure 1). The location was chosen due to its high number of fishponds which use a variety of sources of water. It lies on the Northeastern side of Mt. Kenya, and this is one of the geographical features that leads to the climatic patterns experienced in the region. Abothuguchi lies adjacent to the equator, within longitudes $37^{\circ}40'$E and latitude 0° $03'$N with an altitude around 5199 meters and this explains the cold temperatures experienced the whole year. The total population of Abothuguchi is 116,516 (KNBS 2009).

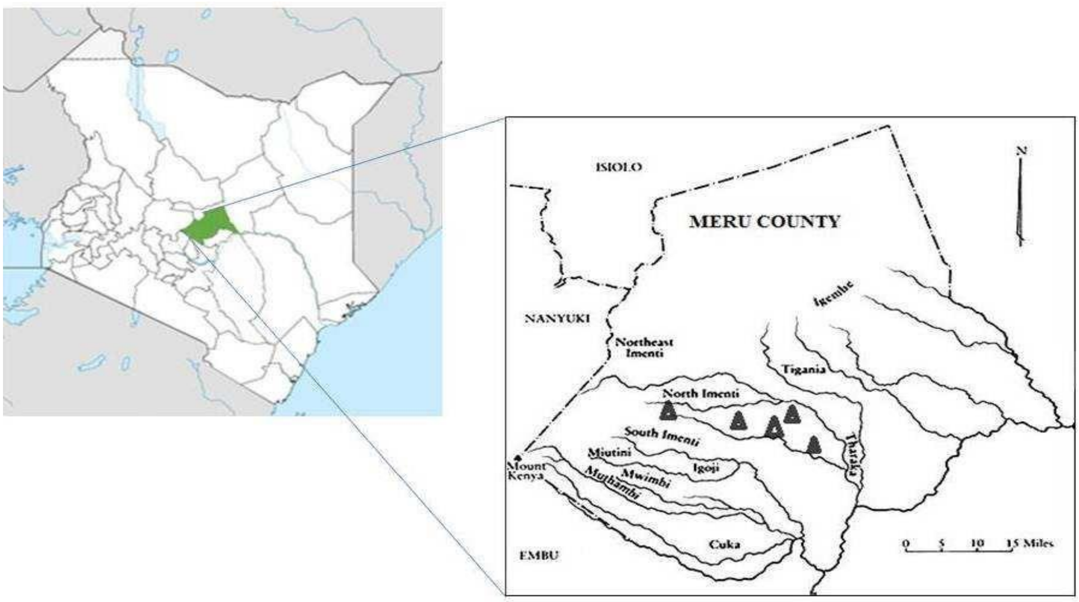


Figure 1. Map of Kenya showing Meru County from Google maps. Triangles show the sampling points.

Abothuguchi lies within the time zone of EAT (UTC+3), and it has many seasonal and permanent rivers; hence water scarcity is not a big problem in the area. The volcanic soils are rich in almost all the plant nutrients, especially in the high regions. The city experiences bimodal precipitation, whereby the short rains last between March and May while the long showers come between October and December. This pattern enables farmers to grow a wide range of crops for subsistence and commercial purposes. However, the kind of crops grown varies based on the ecological zones, which differ in terms of precipitation, temperature, and soils. Some of the plants grown in the highlands include cash crops such as coffee, sugar, tea, pyrethrum, and food crops such as maize, bananas, sugarcane, sorghum, yams, cassavas, and millet. Livestock reared include cattle, sheep, rabbits, chicken, and goats.

Sampling design and sample size

This study used a cross-sectional study design. We applied stratified sampling, in which the sampling zone was divided into five locations each approximately two kilometers apart. From each area, six sites (fishponds) were identified. The sampling mainly targeted different fishponds with different sources of water, geographic patterns, and economic activities. The first site (Mbwinjeru) had a river as the primary source of water. It is purely an agricultural zone, and located on the lower sides of Abothuguchi; hence it experiences higher temperatures as compared to the other sites. The second site was Kithirune (Kit), located in the marketplace whose source of water was mainly from taps. This place is quite cold due to its altitude, as compared to Mbwinjeru. The third site was Githongo, located within a market. The farmers use tap water, and Gitjongo is also cold because of its altitude. The fourth site was Muri which is next to Mt. Kenya forest, it is freezing due to its high altitude with the farmers using river water. The fifth site was Kianthumbi (Thu), which uses swamp water as the water source, and it is on a lower altitude.

The samples were taken from each fishpond twice between November 2012 and May 2013, during the wet and the dry season, to provide for seasonal variations. The sample size was determined using the equation by Fisher with the confidence interval of 95% and an error of 5% (Fisher 1998).

$$n=\frac{Z^2PqD}{d^2}$$

Where n= sample size, p= anticipated prevalence which was 3% (0.03) in this study, q= failure which was measured as (100%-3%) giving 97% (0.97), Z= is the appropriate value from normal distribution for the desired confidence level which was 1.96 in this study, d= allowable error (0.086) and D= design effect which was given a value of 2 since replication was done. Based on 3% prevalence and Z value of 1.96, the sample size was:

$$n=\frac{1.96^2\ (0.03\times0.97\times2)}{(0.086)^2}=30$$

Water samples were collected directly into 250 mL of pre-sterilized polypropylene (glass) bottles. The ponds were stirred before sample collection. The bottles were opened aseptically, held at their bases, and submerged to about 20 cm deep with the mouth facing upwards. Samples were collected by filling the bottles to the top to exclude air. A total of 30 samples were collected (Samie et al. 2011). The water samples were placed in ice cool box after which they were transported to Kenyatta University Microbiology laboratory for analysis. All the samples were analyzed within 24 hours of collection.

Bioassays

Detection of fecal streptococci (FS)

Azide dextrose broth can detect fecal streptococci in the fishpond waters. The single, double strength broths were inoculated with water samples (10 mL) using a pipette and incubated at 37 °C for 24 hours. Positive test which confirmed the presence of gas production and fermentation of sugars (indicated by the yellow color change of the Bromthymol blue) showed the presence of colonies on Kenner fecal (KF) (Mariita and Okemo 2009). The occurrences of pinpoint colonies from the slant were Gram stained to confirm the presence of FS. All red and pink colonies were counted (Mariita and Okemo 2009).

Detection of fecal coliforms

The presence of fecal coliforms in the water was carried out using the multiple-tube fermentation technique (APHA 2003). The steps include the likely, the confirmed and the completed tests. Each batch was inoculated with the sterile diluted water samples. In the possible test, three series of five tubes each containing 10 mL, 1 mL, and 0.1 mL portions of the sample was inoculated with sterilized lactose broth. Pure, sterile lactose broth was inoculated with sterilized distilled water and served as a control. Inoculated tubes were incubated at 37°C for 48 hours (Gyles 2007). Sterile loop transfers were created from all tubes showing acid and gas production to tryptose bile broth (EC Broth) and then incubated at 44°C for 24 hours. A positive reaction will produce gas in a fermentation tube within 24 hours or less.

The estimated number of fecal coliforms found in 100 mL was determined from a tabulated probability table using the corresponding results of various combinations of positive and negative reactions from each of the three batches (APHA 2003). For further confirmation, suspected positive samples from the tryptose broth were streaked on a plate of Eosin Methyl Blue (EMB) agar to give well-isolated colonies. Incubation was done at 37°C for 48 hours. Growth of the typical colonies on the plates was seen and a Gram staining performed. The completed test was done by picking two colonies that were fecal coliform, followed by transferring them to nutrient agar slopes and fermentation tubes containing brilliant green lactose broth. Incubation was carried out at 44.5°C for 48 hours. A Gram stain was conducted to confirm the completed test from the agar slope. Brilliant green lactose broth was also seen for gas production (Gyles 2007). Gas and turbidity in the tubes, color of metallic sheen or pink with dark center colonies on EMB agar indicated positive for fecal coliforms. Fecal coliform was identified from all isolates that produced gas at 44.5°C, stained Gram-negative and were non-spore forming and rod-shaped where the total counts calculated using a standard probability table (APHA 1992).

Detection of Salmonella

Salmonella detection was carried out in three successive phases. First, the selective enrichment was carried out using the tetrathionate broth base as outlined by APHA (2003). One milliliter of each sample from different sites was mixed well with 10 mL of tetrathionate broth, and the solution was incubated for 24 hours at 35 °C. For selective growth, pour plating method was carried out using 1 mL of the enriched with nutrient agar. Streaking was made from the same enriched samples on Deoxycholate Citrate (DCA) agar, Salmonella-Shigella (SS) agar and MacConkey agar (Andrews and Hammack 2003). The plates were incubated at 37 °C for 24 hours. Typical colonies look clear to pale pink on DCA agar, pink on SS agar and white on MacConkey agar. Enumeration of typical colonies was carried out using colony counter and Gram staining, before being confirmed by biochemical tests: TSI, urease tests, and motility based on the procedure described by Mariita and Okemo (2009).

Screening for Vibrio

Vibrio detection was conducted in three successive phases.

Enrichment in a non-selective medium. One mL of the samples were enriched in sterile alkaline peptone water, dispensed in 10 mL tubes, and incubated at 35 °C for 18 hours (HPA 2003).

Plating out on selective medium. The streaking of the enriched samples was performed on Thiosulfate citrate bile salts (TCBS) agar, then incubated at 35 °C for 24 hours. The existence of characteristic yellow colonies after streaking was suspected of being of *Vibrio cholerae* (Mariita and Okemo 2009).

Biochemical reactions. Biochemical tests such as TSI, Motility test, Citrate utilization test, urease test, and cytochrome oxidase test were applied to confirm gram staining (Mariita and Okemo 2009).

Detection of Escherichia coli

In a suspected case of *E. coli*, enrichment glucose peptone broth was used for inoculation of the samples, and the broth was incubated at 37 ºC for 24 hours before being sub-cultured onto Sorbitol MacConkey agar (Bopp et al. 1999). *Escherichia coli* were identified according to morphological and biochemical tests (motility-indole-urease test, methyl-red-voges-Proskauer (MRVP) test, and Simmons citrate utilization (Alam et al. 2010).

Detection of Pseudomonas

Isolation procedure and maintenance. Isolates were collected by using a membrane filter together with a selective growth medium. *Pseudomonas* spp. was examined using a qualitative method, after collecting 100 mL of pond water through 0.45 µm membranes, which were then pre-enriched in NKS Cetrimide plates (Sartorius AG, Germany) at 30°C for 24 hours. Enrichment was followed by *Pseudomonas* selective medium (Oxoid, CFC-SR103) at 30°C for 24 hours. Positive cultures were subcultured on Nutrient Agar (NA) (Difco) to isolate a single pure colony for identification (Hossain et al. 2006).

Morphological and biochemical characterization. Colony characteristics, including green pigment production, were evaluated on Acumedia and NA (Difco). The following classical tests characterized all isolates according to Bergey's Manual of Systematic Bacteriology (Palleroni 1984): Gram staining, cytochrome oxidase production, catalase production and the growth on MacConkey agar.

Biochemical tests--Oxidase test. Two to three drops of oxidase reagent were placed on a strip of paper. A moderate amount of the organism was taken and streaked on the wet surface of the paper with the use of a glass spreader. We avoided using a nichrome wire which could have given false positive results. The presence of *Pseudomonas* appears as deep purple coloration (Hossain et al. 2006).

Biochemical tests--Nitrate reduction. On nitrate broth, a Nitrate blood agar was dried at 37ºC for one hour after which the plate was seeded by stab inoculation with the sample; then it was incubated at 37ºC overnight. Formation of a sizeable greenish zone, which was because of the reduction of nitrate to nitrite, caused the alteration of the hemoglobin to methemoglobin due to bacterial growth confirmed the presence of *Pseudomonas*. The reduction of nitrate by bacteria was based on the presence of the bacteria which caused the decline of nitrates into nitrite, nitrous oxide, ammonia, and nitrogen gas. The nitrate broth was made by mixing nutrients broth, 5 g/liter KNO_3 or $NANO_3$. One tube of nitrate broth was inoculated. One milliliter of naphthylamine and 1 mL of sulphanilamide reagent were added to the tube cultures. Reduction of nitrates will show the appearance of red color within 80 seconds (Hossain et al. 2006).

Heavy metals quantification

The determination of the heavy metal presence and concentration in the water samples was done by using Flame Atomic Absorption Spectrometer (FAAS no VAA 350) and the contamination amount of manganese, lead, iron, zinc, and copper were analyzed.

Metal standard solutions

Metal powder (1 g) in a clean beaker was added with 20 mL concentrated nitric acid. The mixed solution was then transferred into a 1-liter flask and filled up to the mark with distilled water. The solution contained 1000 µg/mL of the specific metal, then it was kept in labeled plastic containers as a stock solution. Working solutions were diluted into varying concentrations of different metals, which were later used with FAAS (APHA 1992).

Physico-chemical parameters

Physical and chemical parameters that are water temperature, pH, salinity, DO, total alkalinity, transparency, sulfates, phosphates, nitrates, ammonia, and electrical conductivity were measured in situ using a portable Universal multiline P4 WTW (Wilheim, Germany) meter. The meter was calibrated and operated as per the manufacturer's instructions. At the sampling point, the measuring probes were lowered into the water and then allowed to settle for 1-2 minutes before the readings were taken (APHA 1992).

Data analysis

The data were analyzed by the Statistical Analysis System (SAS) computer software Version 9.3. Two-way ANOVA shows the interactions between the sites and the seasons. While, One-way ANOVA calculates the significant differences at P value $\leq$ 0.05. The p-value of <0.05 was considered significant. In cases where data was significantly different, Tukey`s Honest Significant Difference (HSD) test was used to separate the means. A correlation coefficient test was used to examine whether there was a relationship between fecal streptococci and fecal coliforms.

RESULTS AND DISCUSSION

Enumeration of fecal bacteria indicators and detection of pathogens

Fecal bacteria were quantified, while pathogenic bacteria were detected and confirmed using biochemical procedures.

Enumeration of fecal bacteria indicators

There was a significant interaction between season and site in determining the FS CFU/100 mL populations (Table 1). The FS CFU/ 100 mL dry season was not significantly different from the FS CFU/100 mL during the wet season. Nevertheless, their population was higher during the wet season. The FS CFU/100 mL differed significantly in the various sites. Streptococcal species displayed white colonies on streptococcal KF agar plates, cream colonies on nutrient agar plates, and these were confirmed by Gram stain (Figure 2-4).

Interaction between site and season in determining the MPN of fecal coliform in the fishpond water was not significant. The FC MPN /100 mL during the dry season remained similar from the MPN wet season (Table 1). The MPN varied considerably in the various sites (Table 1). The wet season indicated a higher population of FC. The FC MPN /100 differed significantly in the various sites when compared with the seasons. Also, the prevalence of FS from all sites exhibited a significant positive correlation with that of FC= 0.832 at P < 0.01 level).

According to one-way ANOVA, there was a significant difference in FS CFU/100 mL in all the sites during the dry and wet season (Table 2). Higher FS CFU/100 mL populations were shown during the wet season (W) in all locations as compared to the dry season (D) (Table 2). The wet season indicated a higher population of FS. The FS CFU/100 mL differed significantly in the various sites, with the highest population being observed in Mbwinjeru and the lowest in Kianthumbi (Table 2) in both dry and wet seasons. There was a significant difference in FC MPN in all the sites during the wet and dry season (Table 2). The highest FC MPN populations were seen during the wet season (Table 2). The FC MPN /100 differed significantly in the various locations with the highest population being observed in Mbwinjeru and the lowest in Kianthumbi (Table 2) in both dry and wet seasons.

Detection of pathogens

The presence of the potentially pathogenic bacteria in water samples from various water sources varied within the sampling sites. The following biochemical tests: Indole test, Simmons citrate agar, Indole test, oxidase test, urease test, motility on SIM media, the reaction on TSI, urease test and Gram stain confirmed pathogenic bacteria over the study period. From most of the sites, *Vibrio*, *Salmonella*, *Escherichia coli*, and *Pseudomonas* were mainly isolated although their abundance was different.

Table 1. Populations of Fecal streptococci and fecal coliform.

Treatment	Pop FS CFU/100 mL	Pop FC MPN/100mL
Season		
Wet season	575466.67 ± 90950.51a	150.533 ±17.1043a
Dry season	546833.33 ± 61069.36a	115.600 ± 22.0154a
Sites Githongo	768166.67 ± 84811.09 b	189.750 ± 40.2319ab
Kithirune	423333.33 ± 28954.86 c	139.917 ± 7.8889bc
Mbwinjeru	1079166.67 ±109533.85a	248.583 ±19.6740a
Muri	396750.00 ± 100441.31c	70.417 ± 9.3909cd
Kianthumbi	138333.33 ± 18701.54d	16.667± 2.6949d
P values		
Season	0.6043	0.0680
Site	0.0001	0.0001
Season*site	0.0001	0.9367

Note: Values (Means±SE) followed by a dissimilar letter (s) along the columns are significantly different at P≤0.05 (Tukey's HSD test). Pop-Population (Each column indicates the separation of means and the standard deviation). FC-fecal coliform density expressed in MPN/100 mL (most probable number) and FS-fecal streptococci density expressed in CFU/100 mL (colony forming unit).

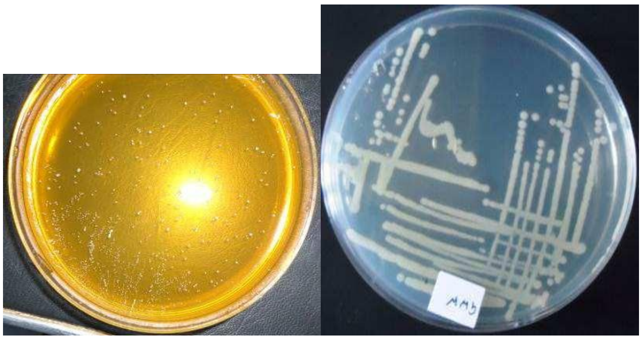
Table 2. Comparison of FS and FC populations during the wet and dry seasons.

Site	Pop FS CFU /100 mL	POP FC MPN/100 mL
Githongo (D)	640833.33±142575.46bc	170.833±81.497abc
Githongo (W)	895500.00±69538.36ab	208.667±18.3588ab
Kithirune D	393333.33±34896.67cd	126.833±3.590bcde
Kithirune W	453333.33±45946.83cd	153.000±13.873abcd
Mbwinjeru D	890000.00±74565.41ab	223.333±34.988ab
Mbwinjeru W	1268333.33±181427.98a	273.833±14.963a
Muri D	701666.67±84829.90bc	44.000±9.295cde
Muri W	918333.33±91851.43ab	96.833±4.743bcde
Kianthumbi D	108333.33±23863.04d	13.000±1.789e
Kianthumbi W	168333.33±24686.93d	20.333±4.835ed
P. value	0.0001	0.0001

Values (Means ±SE) followed by different letters along the columns are significantly different at P≤0.05. (W): wet season, (D): dry season.

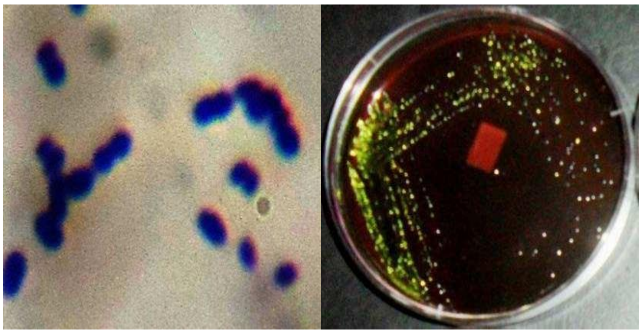
Salmonella species

Salmonella spp. were the most prevalent (40%), followed by *E. coli* (34%), *Pseudomonas aeruginosa* (16%) and *Vibrio* spp. (10%) as the least, (Figure 2-9). The TSI agar slants inoculated with typical colonies formed yellow butts, red slants with some blackening and some with or without formation of gas (Figure 5-6). Bacterial growth and color change from green to blue are evident on the Simmons citrate agar slant. The formation of blue-purple color indicated a positive result for cytochrome oxidase test. Further confirmation was also performed using a series of biochemical tests (data not shown).



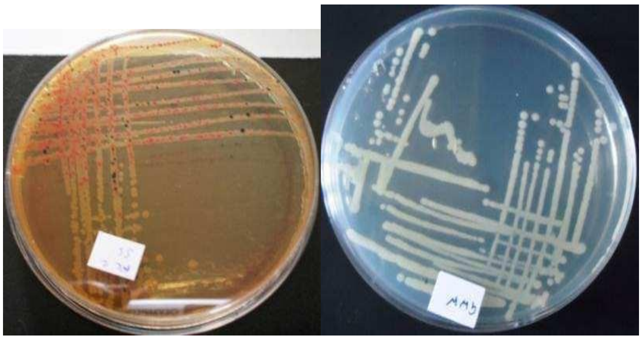
A B

Figure 2.A. Typical colonies of FS on KF agar, B. Typical colonies of FS on nutrient agar.



A B

Figure 3.A. Streptococcal strains showing the characteristic of short to long chains following gram stain reaction, B. Typical colonies of fecal coliforms on EMB media.



A B

Figure 4.A. Black *Salmonella* colonies on SS agar, B. Cream *Salmonella* colonies on nutrient agar plate.

Vibrio species

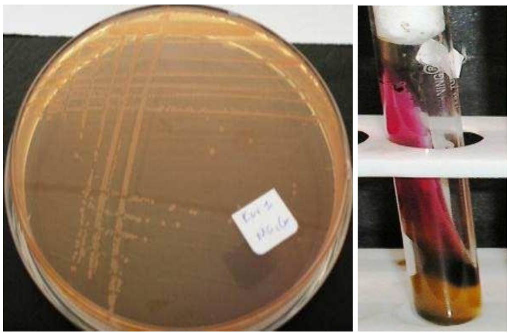
Vibrio spp. exhibited characteristic golden yellow colonies on TCBS (Figure 7.A) and constituted 10% of the pathogens detected. The identity of the isolates as *Vibrio* spp. was also confirmed positive test for Oxidase test.

Escherichia coli

Among total pathogens detected, 34% were confirmed Enteropathogenic *Escherichia coli* (Figure 8).

Pseudomonas species

Of the total pathogenic bacteria identified, 16% of them were *Pseudomonas aeruginosa* (Figure 9).

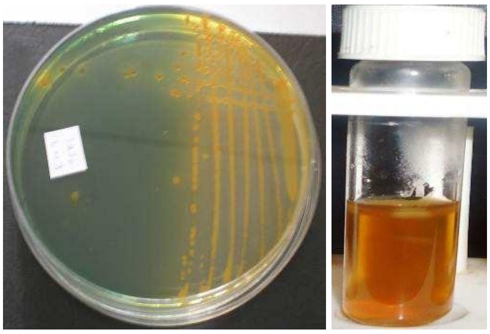


A B

Figure 5.A. *Salmonella* colonies on MacConkey agar plate, B. Yellow butt and red slant and some blackening on TSI slant showing the presence of *Salmonella* spp.

A B

Figure 6.A. Gas production in peptone water as indicated by bubble formation in Durham inserted in tube, B. Reaction in urea agar slants. Color change shows absence of *Salmonella* spp.



A B

Figure 7.A. *Vibrio* spp. on TCBS, B. *Vibrio* colonies showing motility on SIM media.

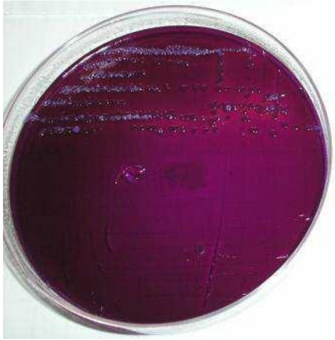


Figure 8. Colorless colonies of *E. coli* on MacConkey.

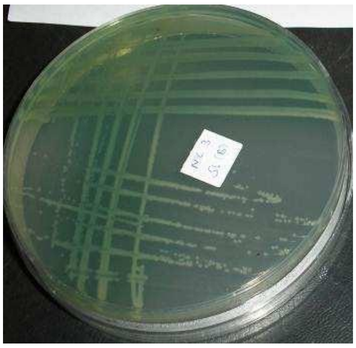


Figure 9. Green colonies of *Pseudomonas aeruginosa* on Nutrient agar.

Physico-chemical parameters

The season and site had significant interactions in determining the pond water temperature (Table 3). The temperature ranged between 18-29 ºC. The water temperature differed significantly between dry and wet season and varied greatly in all sites (Table 3). There was no significant interaction between location and season in determining the pond water pH. The water pH differed significantly (p=0.0058) between the dry and wet season in comparison with the sites. There was a significant difference in water pH in all the sites sampled (Table 3).

Water conductivity varied significantly between sampling sites. However, there was no significant interaction between season and location determining the water conductivity (Table 3). There was a considerable difference in water conductivity between the dry and wet season (Table 3). The site and season did not have a strong interaction in determining the water turbidity (Table 3). Pond water turbidity varied significantly between the dry and wet season. There was a significant difference in water turbidity in the various sites (Table 3).

Site and season did not interact significantly in determining the phosphates concentration in the pond water. Phosphate concentrations in the water fell in the narrow range between two seasons (Table 3). The phosphate concentrations did not differ significantly. Sulfate levels in the pond water did not change dramatically in the wet and dry season. However, the sulfate concentrations ranged considerably among the studied sites. No significant interaction was observed between location and season in determining the sulfate concentrations in pond water (Table 3).

The nitrate concentrations were significantly different among the sites. However, there was no significant interaction between location and season in determining the nitrate concentrations in pond water (Table 3). Nitrate concentrations differed significantly between the wet and dry season (Table 3). There was no significant interaction between site and season in determining ammonia concentration in fishpond water (Table 3). Ammonia concentration differed significantly between the wet and dry season. Ammonia concentration was significantly different depending on the location. Based on the two-way ANOVA, there was no significant interaction between location and season in determining the amount of DO in pond water. The amount of DO did not differ significantly based on the season. High DO was recorded during the dry season significantly (Table 3).

The pond water temperature was significantly different in all the sites during the dry and wet season. Higher temperatures were recorded during the dry seasons, with the highest being in Mbwinjeru and the lowest in Muri, both dry and wet seasons. There was no significant difference in pH across all sites during the dry and wet seasons. The pH of the water in all the ponds was more acidic during the dry season. The highest pH value recorded was in Kithirune, while the lowest value recorded was in Muri during the dry season. Meanwhile, Kianthumbi had the highest value with Muri having the lowest during the wet season. There was a significant difference in conductivity in all sites during the dry and wet seasons. The high conductivity of the pond water was observed during the dry season in all ponds. The highest electrical conductivity was observed in Mbwinjeru, while Muri had the lowest in both dry and wet seasons (Table 4).

The turbidity of the pond water was significantly different across the sites during both the dry and wet season (Table 4). The turbidity of the pond water was substantially higher during the wet season (Table 4). The pond water was significantly least turbid during the dry and wet season in Thu (Table 4). The turbidity of the sampling sites varied between 16 and 118.0 NTU during the study period. The highest value of turbidity examined was in Mbwinjeru, while the lowest value recorded was in Kianthumbi in both seasons.

There was no significant difference in phosphate concentrations across the sites between two seasons. Mbwinjeru recorded the highest levels of phosphate, while Kianthumbi showed the lowest in both seasons. Sulfates concentrations were significantly different in all the sites with the highest levels being recorded during the wet seasons. The highest level was recorded in Mbwinjeru and the lowest in Kianthumbi in both seasons (Table 4).

There was a significant difference in nitrate concentrations in all the sites during the dry and wet season. Higher concentrations of nitrates were observed during the wet season. Mbwinjeru had the highest levels, whereas Kianthumbi recorded the lowest nitrate concentrations in both seasons. Ammonia concentrations were not significantly different across the sites during the dry and wet seasons. The highest levels were recorded in Muri, while the lowest in Kianthumbi. Dissolved oxygen

(DO) concentration in the pond water remained the same in all the sites, during both the dry and wet season. The highest DO value was obtained in Muri, while the lowest in Mbwinjeru in both seasons (Table 4).

Heavy metals

The order of occurrence of heavy metals in water was Fe > Mn > Zn > Cu > Pb. Data on heavy metals were analyzed using two-way ANOVA. There was no significant difference in the concentration of lead in the ponds between the wet and dry seasons. There was no significant difference between the lead concentrations across all sites. No significant interaction was observed between the season and location in influencing the lead concentration in the ponds. Githongo recorded the highest level of lead, while Kianthumbi recorded the lowest. Data on copper concentrations showed a significant difference in copper concentration between the dry and wet season (Table 5). No significant difference in copper concentration was observed among the sites. Two-way ANOVA showed no significant interaction between location and season in influencing copper concentration in the ponds. Mbwinjeru had the highest level of copper, while Kianthumbi had the lowest.

Table 3. Interaction of Physico-chemical parameters between sites and seasons.

Treatment	Temp °C	pH	E. C (µs/cm)	Turbidity	PO_4_MgL$^-$	SO_4_MgL$^-$	NO_3_MgL$^-$	NH_3_MgL$^-$	DO_MgL$^-$
Season									
Wet season	21.13±0.355b	5.953±0.1303b	95.96± 11.2b	94.72±10.16a	0.324±0.09a	16.57±2.65a	21.57± 3.06a	0.26± 0.049a	3.997±0 .08a
Dry season	24.25±0.4003a	6.343± 0.101a	128.11±13.88a	71.0± 9.184b	0.28±0.104a	12.38±2.53a	14.68±2.90b	0.148± 0.028b	4.23± 0.092a
Sites									
Githongo	23.58± 0.542b	6.19± 0.11ab	146.58±13.47b	95.93±11.2ab	0.445±0.15a	19.08±2.58b	26.7±3.81b	0.293± 0.07ab	4.075± 0.096a
Kithirune	21.79± 0.66c	6.408±0.15ab	114.3± 13.47b	118.58±12.6a	0.128±0.03a	8.93±1.003c	17.18±2.61b	0.20± 0.078ab	4.192± 0.11a
Mbwinjeru	25.8± 0.534a	5.942±0.156bc	203.23±12.55a	111.4±11.94ab	0.605±0.25a	35.1± 4.51a	39.22±3.55a	0.13± 0.032ab	3.858±0.155a
Muri	20.33± 0.482d	5.53± 0.210c	35.30± 3.56c	72.35± 15.63b	0.28±0.108a	5.66± 1.05c	6.64 ±1.47c	0.33± 0.075a	4.18± 0.123a
Kianthumbi	21.92± 0.378c	6.67± 0.157a	60.78± 3.68c	16.09± 2.977c	0.046±0.01a	3.63± 0.51c	0.93± 0.187c	0.059± 0.008b	4.25± 0.177a
P value									
Season	0.0001	0.0058	0.0004	0.0247	0.7278	0.0565	0.0031	0.0464	0.0665
Site	0.0001	0.0001	0.0001	0.0001	0.0582	0.0001	0.0001	0.0137	0.2903
Season×Site	0.1322	0.6043	0.4628	0.8138	0.9995	0.6537	0.3048	0.9356	0.9772

Values (Means ±SE) followed by dissimilar letters along the columns are significantly different at P≤0.05 (Tukey's HSD test)

Note: E.C-Electrical conductivity; PO_4-phosphates; SO_4-sulfates; NO_3-Nitrates; NH_3-Ammonia; DO-Dissolved oxygen.

Table 4: Comparison of Physicochemical parameters between the wet and dry season.

SITE	Temp °C	PH	E. C (µs/cm)	TURBIDITY	PO_4_Mg_L^{-1}	SO_4_Mg_L^{-1}	NO_3_Mg_L^{-1}	NH_3_Mg_L^{-1}	DO_Mg_L^{-1}
Git D	25.17± 0.48b	6.33± 0.163a	165.20± 19.55b	84.47±18.00abcd	0.398± 0.21a	16.17± 4.36bcd	20.73 ± 6.34bc	0.225± 0.07a	4.167 ± 0.17a
Git W	22.83± 0.31b	6.16± 0.166a	127.95±16.65bcd	107.39±13.17ab	0.4917±0.24a	22.00± 2.62bc	32.62± 3.09ab	0.362± 0.13a	3.98± 0.098a
Kit D	24.58±0.201b	6.60 ±0.126a	135.7±18.39bc	100.50±21.71ab	0.115± 0.039a	7.37± 0.63cd	12.07 ± 4.42cd	0.12± 0.028a	4.317± 0.191a
Kit W	22.67± 0.21cd	6.22 ±0.259a	92.9±16.65cde	136.67± 9.795a	0.142± 0.06a	10.50± 1.75cd	22.28± 0.24bc	0.277± 0.155 a	4.067± 0.112a
Mbu D	27.33± 0.42a	6.34± 0.307a	230.0±16.03a	105.50±1.432ab	0.568± 0.455a	30.13± 8.162ab	33.93± 6.47ab	0.087± 0.044a	3.917± 0.229a
Mbu W	24.00± 0.00bc	6.10± 0.25a	176.46±12.22ab	117.21±24.73ab	0.642± 0.248a	40.00 ±3.64a	44.50±1.53a	0.173± 0.043a	3.80± 0.228a
Mur D	21.83± 0.17ed	6.33± 0.29a	41.67± 3.073ef	52.32±20.94bcd	0.270± 0.163a	4.82± 0.764d	5.93± 2.19dc	0.257±0.093a	4.37±0.2155a
Mur W	19.17± 0.31f	5.932±0.36a	28.93±5.482f	92.38± 21.80abc	0.297± 0.16a	6.50±1.996cd	7.35± 2.13dc	0.403± 0.119a	4.00± 0.082a
Thu D	23.00± 0.37cd	6.617± 0.24a	68.00± 4.00def	12.22± 0.95d	0.042± 0.01a	3.42± 0.712d	0.75± 0.28d	0.047± 0.01a	4.367± 0.23a
Thu W	20.58± 0.20e	6.610± 0.267a	53.55±4.721ef	19.97±5.66cd	0.050± 0.010a	3.83± 0.79d	1.10±0.256d	0.072±0.012a	4.13±0.279a
P value	<.0001	0.5742	<.0001	<.0001	0.3740	<.0001	<.0001	0.0466	0.4445

Values (Means ±SE) followed by dissimilar letters along the columns are significantly different at P≤0.05. (W): wet season, (D): dry season.

Note: E.C-Electrical conductivity; PO_4-phosphates; SO_4-sulfates; NO_3-Nitrates; NH_3-Ammonia; DO-Dissolved oxygen. Localities: Mbu-Mbwinjeru location, Git-Githongo location, Kit-Kithirune location, Mur-Muri location, Thu-Kiathumbi location

Table 5. Comparison of heavy metals between seasons and the sites.

TREATMENT	Lead_MgL^{-1}	Copper_MgL^{-1}	Zinc_MgL^{-1}	Iron_MgL^{-1}	Manganese_MgL^{-1}
Season					
Dry	0.035 ±0.02007a	0.0691± 0.01661b	0.0768±0.01478b	0.3174±0.09277a	0.1900±0.02851b
Wet	0.068± 0.031a	0.1272± 0.02101a	0.1397±0.02378a	0.3537±0.08965a	0.29667±0.04054a
Site					
Githongo	0.145± 0.0609a	0.10917± 0.0293a	0.1167±0.02401a	0.33417±0.04226a	0.19167±0.062107a
Kithirune	0.0993± 0.0614a	0.0967± 0.03192a	0.1025±0.01871a	0.3150±0.04384a	0.2083±0.064501a
Mbwinjeru	0.0101± 0.0032a	0.155± 0.0438a	0.1658±0.04498a	0.397±0.07995a	0.300±0.06963a
Muri	0.002± 0.00039a	0.0517± 0.017a	0.0533±0.00873a	0.1354±0.03053a	0.1917±0.03786a
Kianthumbi	0.0018± 0.0004a	0.07817± 0.022a	0.1029±0.04476a	0.4965±0.30404a	0.3250±0.04106a
P values					
Season	0.3619	0.0341	0.0290	0.7892	0.0384
Site	0.1929	0.1738	0.1752	0.5488	0.2800
Season×Site	0.9035	0.7814	0.8056	0.9998	0.8435

Values (Means ±SE) followed by dissimilar letters along the columns are significantly different at $P \leq 0.05$ (Tukey's HSD test).

The study showed a significant difference in zinc concentration in the water between the dry and wet season (Table 5). There was no notable difference in zinc concentrations among the sites. Two-way ANOVA showed no significant interaction between location and season in determining zinc concentration in the pond water. The highest value of zinc was seen in Mbwinjeru, while the lowest value was in Muri.

Iron concentrations were similar between the wet and dry seasons. Also, the iron concentrations in the various sites did not change dramatically. There was no significant interaction between season and location in determining the iron concentration in the pond water. Kianthumbi had the highest level of iron, while Muri had the lowest.

Manganese concentration in the water varied significantly between the dry and wet season; however, comparison of the sites showed no significant difference in Manganese concentration in the ponds (Table 5). There was no significant interaction between location and season in determining Manganese concentration in the pond water. Kianthumbi had the highest level of manganese while Muri had the lowest.

Discussion

Microbial quality of fishpond water

Fecal bacteria (fecal streptococcal and fecal coliform) were found in all the samples; thus, confirming the previous research (Ashbolt et al. 2001; Hunter et al. 2002; Emikpe et al. 2011). High fecal contamination detected in the present study is consistent to the findings reported in Pakistan (Nahiduzzaman et al. 2000) and in Italy (Maugeri et al. 2000). On the population of the fecal bacteria, there was significant variation in their populations across all the sites. The levels of fecal bacteria in all the locations exceeded 1.0×10^1 CFU/100 (FAO 1979); hence, all the water samples collected showed high levels of contamination. On the population of the two fecal bacteria, Pearson correlation analysis indicated that prevalence of FS from all sites showed a considerable positive correlation with that of FC. These results are consistent with the findings in Nigeria (Egbere et al. 2008) and Ghana (Fafioye 2011).

Lack of animal waste management, as well as wastewater, could directly affect water quality because of surface runoff (Ampofo and Clerk 2010). Surface runoff could be a plausible source of pond water contamination particularly during the wet season (Ampofo and Clerk 2010) in Ghana. Free roaming animals and pets, especially dogs, might also contribute to fecal contamination of surface water. Also, there are cattle reared by farmers that walk the region for green pastures. Along the rivers in this location, there is possible run-off from roads, parking lots and yards that could also be carrying animal wastes into natural watercourse and ponds (Emikpe et al. 2011). In the densely populated region of Meru, a factor that could contribute to pond contamination is the closeness of latrines to water points, washing and bathing in rivers that serve as the source of fishpond water (Doyle 2007; Adebayo-Tayo et al. (2012a).

Mbwinjeru had the highest population of fecal streptococci and fecal coliform during both the dry and the wet season, which could be attributed agricultural activities that take place around the site. Fertilizer and manure used by the farmers may have found their way into the ponds. The high temperature in this area is a favorable environment for the growth of the bacteria. The source of water used in this area is primarily from a river and therefore, it might be the source of contamination. The farmers mostly use streams of their fishpond water, which may harbor some bacteria. This data is consistent with those of Ghana as described by Emikpe et al. 2011.

Kianthumbi showed the lowest population of fecal streptococci and fecal coliform in both the dry and wet seasons, which could be associated with its source of water that is mainly from a swamp and the zone is also relatively cold. This data is consistent with those reported in Nigeria

(Egbere et al. 2008). Fecal coliform in fish demonstrates the level of pollution of their environment because coliforms are not the normal flora of bacteria in fish. Birds can be a significant source of fecal coliform bacteria. Swans, geese, and other waterfowl can elevate bacterial counts in ponds (Doyle and Ericson 2006). The presence of fecal coliforms in all the sampling sites was an indication that water sources in the four locations were contaminated.

Pathogenic organisms

From water analyzed in these fishponds, pathogenic bacteria were also detected. *Salmonella* spp. were the most prevalent, followed by *E. coli*, *Pseudomonas aeruginosa,* and *Vibrio* spp. These findings are consistent with previous studies in Kisumu performed in County, Kenya (Onyango et al. 2009); in Nigeria (Egbere et al. 2008); in India (Nabonita et al. 2011) and Cameroon (Kuitcha et al. 2010). These bacterial isolates are common intestinal bacteria of both animals and humans gut; however, this contamination may also have come from untreated public water or water taken by animals or cycling between the livestock and their environment or even contamination in feeds (Doyle 2007; Adebayo-Tayo et al. (2012a).

Potential sources of these pathogens in water include: wastewater effluents, run-off from urban land, combined sewer overflows, animal waste, and municipal waste sludges disposed of on ground or in water. Because different kinds of livestock manure are contaminated with pathogenic bacteria, such as *Streptococcus, Salmonella, Shigella, Pseudomonas, Vibrio,* and *E. coli* species (Abdelhamid et al. 2006), it shows that sources of contamination in the sites could have been because of human and livestock activities (Emikpe et al. 2011). The bacteria from fish will develop into pathogens when fish are physiologically unbalanced, nutritionally deficient, or there are other stressors namely: poor water quality, overstocking, which allow opportunistic bacterial infections to prevail (Austin 2011). Besides, physico-chemical characteristics influence the growth and diversity of microbial populations. The study has indicated that the fishponds were significantly contaminated with both fecal and pathogenic bacteria.

Physico-chemical parameters

The variations observed in the physicochemical properties of the fishponds in the present study are like earlier findings in Nigeria by Kumar (2004) and in Samburu County, Kenya by Mwaura (2005). The variations could be attributed to the influences of the micro-climatic, topographic, and edaphic conditions of fishponds in the area. Also, human and animal or livestock activities could another be a factor. Both the highest & lowest recorded temperatures (Table 4.2) were within the recommended limits of 20ºC to 30ºC Kumar (2004). The World Health Organization has recommended 27ºC as the acceptable limit for fish growth and productivity (WHO 2006), whereas the Environmental Management Authority of Zimbabwe has set a limit of 35ºC. In a region of high altitude bordering a forest such as in Muri and Kithirune, we measured the lower temperature. During the wet season, the temperatures were lower than to the dry season as observed some years ago in Samburu County, Kenya (Mwaura 2005).

We observed that water from Mbwinjeru and Githongo was found to be slightly warmer due to their lower altitude, in congruence with a previous study conducted in Kenya (Mwaura 2005). This provides better growth and food conversion than a low temperature (Chaudhari 2003; Mwaura 2005). The lowest value observed fell below the acceptable range between 24°C and 34°C for the tropics, according to a previous study in Nigeria by Udo (2007). Increased temperature causes an increase in the metabolic activity of the fish, while reducing the DO content in the system (Afzal et al. 2007). WHO recommends a temperature range of between 25°C and 32°C for the proper performance of fish (WHO 2006). High water temperature enhances the growth of the thermo-tolerant microorganism. At temperatures below 15°C, the growth of fish ceases and they may end up dying. While at very high temperatures, there is less solubility of oxygen, stress, and fish may eventually die. Aquatic organisms can tolerate a broader range of temperatures, if fluctuations are not so dramatic, sudden and of long duration (Priyadarshini et al. 2011).

The pH values measured are consistent with the findings by Mwaura (2005) in Samburu County, Kenya; in Nigeria (Ayanwale et al. 2012) in the USA (Sipaúba-Tavares et al. 2007) and in Brazil (Silapajarn et al. 2004). According to WHO, the desirable range for pond pH is 6.5-9.0, and range which is in between 5.5 to 10.0 (Stone and Thomforde 2003). The average value of the pH falls below the recommended pH range of 6.5-9.0 (WHO 2006) for better fish performance, which shows that all the fishponds were acidic. Only Kithirune (dry season) and Kianthumbi (wet season) had a pH above 6.5. During the wet season, the pH was alkaline. The low pH might be caused by acid sulfate runoff (Silapajarn et al. 2004). Low pH might also be caused by high rates of carbon dioxide release by zooplankton during respiration. This is a common phenomenon in aquaculture ponds (Silapajarn et al. 2004).

Higher acidic levels in the present study could be due to the chemical additives applied to the aquaculture pond for better production, high stocking density or lack of buffering activities in the farms as indicated by pH values. This observation was consistent with the findings in Nigeria by Thilza and Muhammad (2010). The values of electrical conductivity were within the permissible limit set by the WHO of 1000 µS/cm (WHO 2006). The data was also consistent with the findings in Brazil (Sipaúba-Tavares et al. 2007). The higher values of electrical conductivity in Mbwinjeru may be due to high temperatures. Likewise, those in Muri may be due to low temperatures associated with the altitude. Electrical conductivity is measurement of the ability of water to conduct an electric current. Since the electric current is done through the movement of ions in solution, it also indicates the number of ions or total dissolved salts in the water. The low levels during the wet season might be caused by the rainy season in which the samples were collected. Dilution of water during the rainy season lowers the levels of electrical conductivity

(Sipaúba-Tavares et al. 2007). During the dry season, the electrical conductivity corresponded to a study in Samburu County, Kenya (Mwaura 2005).

The values of turbidity varied significantly. The values read were within the permissible limit set by the WHO of 1000 mg/l (WHO 2006). The results in the present study are consistent with the findings in Nigeria (Ehiagbonare and Ogunrinde 2010) and Ghana (Takyi et al. 2012). Turbidity affects the appearance of water. Water with high turbidity usually is associated with high microbiological contamination, which is in congruence with the bacterial population in Mbwinjeru which had high values of turbidity. High turbidity may be because of rains, which cause flooding that may cause soil erosion and surface runoff. Hence, this may be responsible for depositing nutrients, silt, and domestic wastes into the water. Kianthumbi showed the lowest values of turbidity, due to the source of water which was a swamp.

The values of phosphates differed significantly, consistent with those obtained by in Nigeria (Ehiagbonare and Ogunrinde 2010). The values obtained from Mbwinjeru exceeded the permissible limit set by the WHO of 0.5 mg/l (WHO 2006). The high values are suggestive of possible pollution of the fishponds, which is supported by previous research in the USA (Wudtisn and Boyd 2005). The source of water for Mbwinjeru was a river, and it was in an agricultural zone. The higher phosphorus concentration may be associated with an increase in phosphorus produced during the decomposition of organic fertilizer and the feedthrough fish excreta. Each soluble organic phosphorus is released during the process of organic fertilizer decomposition under aerobic conditions (Wudtisn and Boyd 2005). Kianthumbi had low levels of phosphates in both seasons, which may be associated more with water source and less agricultural activities.

The values of sulfates hugely varied, consistent with the findings in the USA (Silapajarn et al. 2004). The World Health Organization has recommended 100 mg/l (WHO 2006) and therefore, they still fall within limits. The values obtained here are higher than those reported in Nigeria (0.66 to 1.09 mg/l) (Ehiagbonare and Ogunrinde 2010). The high amounts found in Mbwinjeru could be associated with the frequent use of detergent and soaps by people in the neighborhood or frequent usage of fertilizers in farming (Ehiagbonare and Ogunrinde 2010). The values of nitrates obtained from this study differed significantly (Table 4.3) yet fell within the recommendations (50.0 mg/L) (WHO 2006). These values are consistent with the findings reported in Nigeria by Ehiagbonare and Ogunrinde (2010). However, the values were lower than those reported in Brazil by Sipaúba-Tavares *et al.* (2007). The high value of nitrates in Mbwinjeru in both seasons suggested the presence of pollutants like bacteria and pesticides (Nzunga 2011) and can be remedied by water change and plant density. The higher levels can be attributed to agricultural and domestic activities, and surface runoffs during the rainy season (Nzunga 2011).

The values of ammonia were within the recommended amounts of less than 1 mg/l (Kumar 2004). The mean value was within the permissible limit set by the WHO of 0.5 mg/l (WHO 2006). The high values obtained in Muri in both seasons could be due to low temperature and the high rate of feeding and densities; hence the excess feed decomposes and pollutes the pond water (Thilza and Muhammad 2010). Ammonia is introduced into through dead phytoplankton, uneaten feeds, dead and decaying organic matter. It can be attributed to the addition of manure to fertilize the pond or through the process of nitrogen fixation by algae and water plants (Edwards 2008).

Ammonia at concentration >0.1 mg L^{-1} tends to cause gill damage, destroy mucous producing membranes, poor feed conversion, effects like reduced growth and reduced disease resistance. Ammonia affects fish in different ways; for example, when they are poisoned by ammonia, fish congregates close to the water surface, gasp for air and are restless (Edwards 2008). Their skin becomes light colored and is covered with a thick layer of mucus. In some cases, hemorrhages occur mainly at the base of the pectoral fins (Thilza and Muhammad 2010).

The DO values differ significantly across the sites. However, these levels were below the WHO recommended values of 5.0 mg/l (WHO 2006). The low DO may be attributed to the small size of the ponds and eutrophication because of over-fertilization with manure or fertilizer (Thilza and Muhammad 2010). This may be due to the presence of microbes and plants, as fish are not the only oxygen consumer in an aquaculture system. Low concentration of DO in water causes suffocation in fish, while its supersaturation may result in the gas bubble disease leading to the mass mortality of fish in both cases. Low DO increases the toxicity of ammonia to culture organisms. Oxygen depletion in water can cause poor feeding of fish, reduced growth, starvation, and higher fish mortality, either directly or indirectly. Bacteria, phytoplankton and zooplankton consume high quantities of oxygen (Thilza and Muhammad 2010). The solubility of oxygen in water decreases when the water temperature increases. The study has shown that the physicochemical parameters varied significantly in all the study sites.

Heavy metals

Heavy metals are chemical elements with a specific gravity, which is at least four to five times the specific gravity of water, at the same temperature and pressure (Duruibe et al. 2007). Fish and other aquatic organisms are always immersed in water containing pollutants. They absorb the contaminants through the skin, gut (from food) and respiratory surfaces (Wegu and Akanimor 2006). Except for Fe, the heavy metal concentrations in the tested water here did not exceed WHO (World Health Organization 1993), EC (European Community 1998), EPA (Environment Protection Agency 2002), CIW (Criterions of the Irrigation Water 1997) and TSE-266 (Turkish Standards 2005) guidelines. A high number of metals in the fishponds may be attributed to the increased coverage of the aquatic and higher plants which absorb minerals from water and sediments. Areas with high metal content had high bacteria counts which are consistent with a previous study in Kenya (Mutuku 2010) and Italy (Ozurtuk et al. 2010).

The data has indicated that the order of heavy metals amount in the fishpond water is iron > manganese > zinc > copper > lead, which is consistent with those reported in Egypt (Al-Afify et al. 2010). The WHO has recommended a permissible limit of 0.01 mg/l (WHO 1993). A high level of lead in some pond water in England has been attributed to industrial and agricultural discharge (Mason 2002). The pollution of the aquatic environment by heavy metals affects marine organisms and poses considerable environmental risks and concerns (Amisah et al. 2009). Heavy metal pollutants accumulate in tissues and organs of marine organisms. The high levels of lead in Githongo and Kithirune in the present study could also be attributed to a spill of leaded petrol from the combustion of gasoline in automobile cars and the closeness of these ponds to the two markets.

Lead is a cumulative toxin, sources include automobile exhaust fumes, from sewage effluent, runoff wastes, used dry-cell batteries, and atmospheric deposition (Mason 2002). Acute lead intoxication in fish can be recognized by damaged gills epithelium, erythrocytes, leucocytes, and nervous system. In human beings, lead binds with SH group of proteins, apart from that, it damages blood circulation, liver, central nervous system, and kidneys (Mason 2002). Also, lead can delay embryonic development, suppress reproduction, inhibit growth, increase mucus formation, neurological problem, enzyme inhalation and kidney dysfunction (Kori-Siakpere and Ubogu 2008).

The amount of copper in the present study showed interaction between the sites and the seasons, which is consistent with the findings reported in the USA (Silapajarn et al. 2004), Turkey (Ozuturk et al. 2010), and in Egypt (Samir and Ibrahim 2008). The data was within the permissible limits set by WHO of between 1 and 2 mg/l (WHO 1993). Copper toxicity in natural water arising from pollutants may lead to severe damage in gills and necrotic changes in the kidney and liver. Long term high exposure to copper can cause nausea, vomiting, stomach cramps, or diarrhea when ingested by humans (Javed and Usmani 2013).

The amount of zinc measured in this study varied significantly between the sites and the seasons yet falls the set values by WHO of 3.0 mg/l (WHO 1993). These amounts are consistent with the findings in the USA by Silapajarn *et al.* (2004) and in Egypt (Samir and Ibrahim 2008). The primary source of zinc entering water is dissolved zinc from zinc-related appliances such as galvanized pipes. Zinc accumulation results in several disturbances in fish. It exerts negative effects by accruing structural damage which affects the growth, development, and survival. Sublethal levels adversely affect hatchability, survival, and hematological parameters of fish (Kori-Siakpere and Ubogu 2008). Low levels can be attributed to less zinc load from industrial, agricultural, domestic, and urban wastewaters (Ozortuk *et al.* 2009).

The amount of iron detected was similar across the sites, consistent with previous studies in Egypt (Samir and Ibrahim 2008) and Turkey (Ozurtuk et al. 2010). The high amount of iron may be attributed to the high density of people settled in buildings having iron sheets roofs. Due to corrosion, the iron ions find their way into the fishponds. The amount of manganese recorded in the present study revealed the interaction between the sites and the seasons. The WHO has recommended 0.1 to 0.5 mg/l of manganese for optimal productivity of fish (WHO 1993). These values are consistent with those reported in the USA (Silapajarn et al. 2004) and those by Samir and Ibrahim (2008) in Egypt.

The primary sources for manganese in the air and water are iron and steel manufacturing and the burning of diesel fuel in the motor cars (Samir and Ibrahim 2008). High levels of manganese can disturb lung, liver and vascular, lowering blood pressure, causing brain damage in fish and failure in the development of animal fetuses (Javed and Usmani 2013). Dissociation of heavy metals into ions in water is a prolonged process, and metabolic processes cannot detoxicate them. These metals are generally very toxic to fish. The heavy metals in general in the study have indicated a low level of contamination except for iron; hence the fishponds are not significantly contaminated with heavy metals.

Conclusion

The fishponds were found to be significantly contaminated with fecal streptococci and fecal coliforms. The pathogenic bacteria detected in this study, *Salmonella, Vibrio* spp. *E. coli* and *Pseudomonas aeruginosa* indicate high contamination and a high risk of infection. Heavy metals were found to be within the WHO standards, except for iron. All the physicochemical parameters in the tested sites varied and the temperature, ammonia, sulfates, nitrates, phosphates, electrical conductivity, and turbidity were found to be within the values of WHO (2006). The pH and the dissolved oxygen fell below the limits provided by WHO (2006). The populations of fecal and pathogenic bacteria, the variability of physicochemical parameters, and concentration of heavy metal contamination were affected by the source of water, geographical patterns of the site, human and livestock activities.

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BIOTEKNOLOGI
Volume 15, Number 2, November 2018
Pages: 84-91

ISSN: 0216-6887
E-ISSN: 2301-8658
DOI: 10.13057/biofar/c150204

The effect of adding coconut shell liquid smoke by distillation and redistillation on the chemical, microbiological, and sensory properties of *pindang* layang fish (*Decapterus* spp.) during storage

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Manuscript received: 7 September 2018. Revision accepted: 8 November 2018.

Abstract. *Himawati E, Riyadi NH, Manuhara GJ. 2018. The effect of adding coconut shell liquid smoke by distillation and redistillation on the chemical, microbiological, and sensory properties of pindang layang fish (*Decapterus spp.*) during storage. Bioteknologi 15: 84-91. Pemindangan* (salted-boiled fish) is one way of processing fish with a combination of treatments between salting and boiling. But *pindang* layang fish (*Decapterus* spp.) only has a shelf life of about 2-4 days which quickly undergoes a process of decay. So we need a preservation technique that can minimize damage to *pindang Decapterus* spp.. Liquid smoke is a chemical that results from the distillation of smoke from burning biomass and acts as a disinfectant. Phenol, carbonyl, and organic acids in liquid smoke play an important role in fish preservation. Distillation liquid smoke is obtained from the first pyrolysis and has a fairly high content of tar compounds, while redistillation liquid smoke is the second product from the first distillation. The purpose of this study was to determine the effect of adding coconut shell liquid smoke by distillation and redistillation on the chemical properties (moisture content, pH, phenol), microbiological properties (TPC), and sensory properties (color, odor, texture, and overall) of *pindang Decapterus* spp. during storage. As well as knowing the best concentration of distillation liquid smoke (1%, 2%, 3%) and redistillation (25%, 30%, 35%) in maintaining the quality of *pindang Decapterus* spp. during storage. The analysis was carried out for 4 periods on 0,2,4,6 days. The experimental design used was a completely randomized design (CRD). The data obtained were analyzed by ANOVA at =5% and continued with the DMRT test if there was a significant difference. The results showed that the chemical characteristics were water content (61-67%), pH (5-7), phenol (0.226-0.566%), while microbiological characteristics (TPC) (1.85.104-3.15.105 Cfu/g). The sensory characteristics (color, odor, texture, and overall) of *pindang Decapterus* spp. with redistillation liquid smoke treatment were preferable to distillation liquid smoke treatment. In terms of chemical, microbiological, and sensory properties, the treatment of redistillation of liquid smoke was significantly different from that of the distillation of liquid smoke. A review of the chemical and microbiological properties of the 35% redistillation liquid smoke treatment was the best treatment, while from the sensory properties, the 30% redistillation liquid smoke treatment was the most preferred by the panelists compared to other treatments.

Keywords: *Decapterus,* distillation liquid smoke, *pindang,* redistillation liquid smoke

INTRODUCTION

The potential of Indonesian marine products, especially fisheries, is quite large, estimated at 6.7 million tons per year, consisting of 4.4 million tons in the waters of the archipelago and 2.3 million tons in the Indonesian Exclusive Economic Zone (ZEEI). Capture fisheries production from fishing at sea and in public waters in 2006 was around 4,468,010 tons (Direktorat Jenderal Perikanan Tangkap 2007). Meanwhile, aquaculture production in 2006 reached 2,625,800 tons (Direktorat Jenderal Perikanan Budidaya 2007).

Layang fish (*Decapterus* spp.) has the highest production compared to other types of fish, reaching 52% of the total catch, which is around 2,323,365 tons per year (Direktorat Jenderal Perikanan Tangkap 2007). In addition to high production, this fish has relatively low prices compared to other types. To add value to fishery products, *Decapterus* spp. has a high protein content of about 22%, 1% fat, and 109 calories of energy and is perishable; alternative processing or preservation needs to be made. Preservation aims to maintain the quality and stabilize the selling price of fish (Ridwansyah 2002). Traditional fish preservation in Indonesia includes salting, pemindangan, peda making, shrimp paste, petis, and others (Hadiwiyoto 1993).

Pemindangan (salted-boiled fish) is a method of processing, as well as a method of preservation, which is favored by the public because the final product has a distinctive taste and is not too salty (Winarno 2002). The material that is often used in pemindangan is *Decapterus* spp. (Purnomo et al. 2002), which has a fairly high nutritional content, namely the amount of protein 27%, fat 3%, energy 176 calories, water 60%, minerals 0.26%, and vitamin B 0.07 mg (Heruwati 1986). In the leaching process, fish are preserved by steaming or boiling in a tool containing salt at a rather high concentration (10-25%) and under normal pressure to inhibit or kill spoilage bacteria and enzyme activity (Afrianto and Liviawaty 1989). In addition, the fish collectors also preserve fish using chemicals that are harmful to health, such as formalin and H_2O_2; indeed, alternative natural preservatives must be sought, one of which is the addition of liquid smoke.

Liquid smoke is a chemical product of the distillation of smoke resulting from combustion. Liquid smoke can be a disinfectant, so food ingredients can last longer without harming consumers (Amritama 2007). According to Darmadji (1996), the pyrolysis of coconut shells that have become liquid smoke will have phenol compounds of 4.13%, carbonyl of 11.3%, and acid of 10.2%. These compounds can preserve food for a long time because they mainly inhibit bacterial development. Preservation with liquid smoke has several advantages, including being more environmentally friendly because it does not cause air pollution, can be applied quickly and easily, does not require fogging installations, the equipment used is simpler and easier to clean, and the concentration of liquid smoke used can be adjusted to the desired level. Important volatile compounds are easily controlled if desired. The resulting product has a uniform appearance, plays a role in forming sensory compounds, and provides food safety assurance (Swastawati 2008).

The acid content in liquid smoke can affect the taste, pH, and shelf life of smoked products; carbonyl reacts with protein and forms brown coloration and phenol, which is the main constituent of odor and shows antioxidant activity (Prananta 2005). Fatimah (1998) states the groups of compounds that make up liquid smoke are water (11-92%), phenol (0.2-2.9%), acid (2.8-9.5%), carbonyl (2.6-4.0%), and tar (1-7%). The compound that makes up liquid smoke greatly determines the organoleptic properties of liquid smoke and the quality of smoking products.

Pemindangan is one of the preservation methods used by the community, but *pindang Decapterus* spp., the resulting product, only has a short shelf life of about 2-4 days (Moedjiharto 2002). It is necessary to add a safe preservative, namely by adding liquid smoke into *pindang Decapterus* spp.. Adding liquid smoke to *pindang Decapterus* spp. can increase shelf life to make it more durable, produce a distinctive odor, smell, color, a more compact texture, and loss of flavor on easier control (Maga 1987).

The purpose of this study was to determine the effect of adding coconut shell liquid smoke by distillation and redistillation to the chemical properties (moisture content, pH, phenol), microbiological properties (TPC), and sensory properties (texture, color, odor, overall) of *pindang* layang fish (*Decapterus* spp.) during storage and to determine the best concentration of distillation and redistillation liquid smoke in maintaining the quality of *pindang Decapterus* spp. during storage.

MATERIALS AND METHODS

Materials and tools

The main ingredients used to preserve *pindang* are *Decapterus* spp. from Pasar Gedhe Market, Surakarta, Central Java, Indonesia, and Nutrient Agar (NA).

Procedures

Preparation of distillation and redistillation of liquid smoke solutions

Before the preservation process, each type of preservative was diluted using distilled water. The concentrations of redistillation liquid smoke preservatives used were 25%, 30%, 35% and 1%, 2%, and 3% of the distillation used.

Making of pindang layang

The process of preserving the *pindang Decapterus* spp. carried out in this study is as follows: weeded and washed the *pindang Decapterus* spp. until clean, then arranged in the besek. Then, the besek was soaked (15-30 minutes) in a 25% salt solution. Next, the fish are removed and drained. The result is *pindang Decapterus* spp..

Preservation of pindang Decapterus spp.

The preservation of *pindang Decapterus* spp. is carried out with two types of liquid smoke, namely: (i) immersion in 25%, 20%, 35% redistilled liquid smoke for 15 minutes and (ii) immersion in 1%, 2%, 3 % for 15 minutes. Followed by draining, and placing into a naya/besek, then stored at room temperature. Furthermore, on days 0, 2, 4, 6, tests were carried out, including: (i) Chemical properties (moisture content, pH, phenol), (ii) Microbiological properties (TPC), and (iii) Sensory properties (color, aroma, texture, and overall). Each analysis was carried out on days 0, 2, 4, and 6. The method of each analysis can be seen in Table 1.

Data analysis

The experimental design used in this study was a completely randomized design (CRD) with two factors: a combination of concentration and preservative types; each analysis was repeated twice. The data obtained were analyzed by ANOVA to determine whether there were differences in treatment. If there were differences between treatments, proceed with the DMRT (Duncan Multiple Range Test) tests with a significance level of 0.05, using SPSS 13.0.

RESULTS AND DISCUSSION

Pemindangan fish (salted-boiled fish) is one way of processing fish with a combination of treatments between salting and boiling. The fish type commonly used as raw materials are seawater fish such as tuna (*Euthynnus* spp.), tengiri/mackerel (*Scomberomorus* spp.), kembung/ mackerel (*Scomber* spp.), and layang (*Decapterus* spp.). The raw material used in this research was *Decapterus* spp. because it has a high protein content of ± 22% and has a high catch yearly, reaching 2,323,365 tons or ± 52%.

Table 1. Analysis method

Test	Method
Water content	Thermogravimetry (Sudarmadji et al. 1997)
pH	pH meter (Widowati 1986)
TPC	Total plate count (Fardiaz 1993)
Phenol test	Senter et al. (1989)
Organoleptic	Scoring (Kartika et al. 1988)

Pindang Decapterus spp. has a shelf life of about 2-4 days which quickly undergoes a perishable food; this is due to the high protein content and environmental conditions that are very suitable for the growth of spoilage microbes. A preservation technique is needed to minimize damage to *pindang Decapterus* spp. during storage. In this study, the preservative used was liquid smoke with a concentration of redistillation of 25%, 30%, 35%, and distillation of 1%, 2%, and 3%. The parameters observed were chemical properties, microbiological properties, and sensory.

Chemical properties of *pindang Decapterus* spp.

Water content

Moisture content is the amount of water contained in the material expressed in percent. Water content is also one of the most important characteristics of foodstuffs because water can affect appearance, texture, and taste. The water content in foodstuffs determines the freshness and durability. The high-water content makes it easy for bacteria, molds, and yeasts to breed, changing food ingredients (Afrianto and Liviawaty 1989).

The water content of *pindang Decapterus* spp. can be seen in Table 2. This table shows that all control treatments and adding redistillation and distillation liquid smoke on day 0 to day 4 decreased water content due to some of the water being evaporated because it was only stored at room temperature. Then, on the 6th day of control treatment and adding 1%, 2%, and 3% distillation liquid smoke, the water content increased because the fish had decayed. While in the treatment with the addition of redistillation liquid smoke, 25%, 30%, and 35% water content still decreased.

The statistical analysis shows that on day 0, all distillation and redistillation liquid smoke treatments did not have a significant difference. On day 2, the addition of 25% redistillation liquid smoke was not significantly different from the 30% redistillation liquid smoke and 3% distillation liquid smoke. Still, it differed significantly from the 35% redistillation liquid smoke, 1% distillation liquid smoke, and 2% treatment. Then for the 4th day, all redistillation and distillation liquid smoke treatments were not significantly different, except for the 25% redistillation liquid smoke treatment. On the 6th day, all treatments of distillation liquid smoke were significantly different from all treatments of redistillation liquid smoke.

The water content on day 6 for the control treatment and adding 1%, 2%, and 3% distillation liquid smoke increased due to the product's decay process. The process of spoilage is caused by the oxidation of fish fat which contains various unsaturated fatty acids, as well as the mineral content in salts such as iron and magnesium, these substances also play a role in accelerating fat oxidation. In addition to the activity of extremely halophilic bacteria (*Micrococcus*, *Serratia*, and *Sarcina*), which can grow at a salt content of 20-30%, producing water and mucus. Thus, the increase in water content on day 6 was caused by the result of the decomposition process by microbial activity (Supardi and Sukamto 1999).

Table 2. The results of the analysis of the water content (%) in the treated *pindang Decapterus* spp.

Treatment	Observation on day			
	0	2	4	6
Control	65.61 ^{a}	64.16 ^{ab}	62.68 ^{a}	64.87 ^{b}
Redistillation 25%	65.46 ^{a}	64.46 ^{ab}	64.37 ^{b}	62.28 ^{a}
Redistillation 30%	65.96 ^{a}	63.41 ^{a}	63.13 ^{a}	61.80 ^{a}
Redistillation 35%	66.46 ^{a}	65.81 ^{cd}	64.78 ^{b}	62.68 ^{a}
Distillation 1%	66.57 ^{a}	65.81 ^{cd}	65,16 ^{b}	66.98 ^{c}
Distillation 2%	66.86 ^{a}	66.50 ^{d}	65.46 ^{b}	66.93 ^{c}
Distillation 3%	66.46 ^{a}	65.02 ^{bc}	64.73 ^{b}	66.22 ^{bc}

Note: Numbers followed by the same letter show no significant difference at the 5% level (applies to the same column)

The spoilage process in the control was due to the very low content of antibacterial preservative contained in the material, while the spoilage in the addition of 1%, 2%, and 3% distillation liquid smoke was due to the addition of a very low concentration of liquid distillation smoke so that spoilage microbes were still present and can grow. In the additional treatment of 25%, 30%, and 35% redistillation liquid smoke, the water content continued to decrease until the 6th day; this was because the microbial activity in *pindang Decapterus* spp. was hampered by the addition of a fairly high concentration of liquid smoke.

According to the Indonesian National Standard (SNI-01-2717-1992), the value of water content that meets the quality requirements of *pindang Decapterus* spp. is 60%-70%. The water content of *pindang Decapterus* spp. in this study still meets the quality requirements according to SNI (1992) because the water content value ranges from 61.80% - 66.98%.

pH

Measurement of pH was carried out to determine the tendency of increasing/decreasing pH during storage. The amount of pH is related to the formation of alkaline compounds during storage and will affect microbial growth. In general, fish that is not fresh, the meat has a more basic (higher) pH than fish that are still fresh. This is due to the emergence of basic compounds such as ammonia, trimethylamine, and other volatile compounds (Hadiwiyoto 1993). The pH analysis of *pindang Decapterus* spp. can be seen in Table 3.

From Table 3, it is known that from day 0 to day 6, all treatments increased. Then from Table 3, the average fish pH can be seen on day 0; generally, the average pH of fish with the control treatment was significantly different from the liquid smoke redistillation treatment but not significantly different from distillation. Still, on the 2nd day, the control significantly differed with all treatments (redistillation and distillation of liquid smoke). The use of redistillation liquid smoke has a significantly different effect from that of distillation liquid smoke. *pindang Decapterus* spp. preserved with liquid smoke redistillation has a lower pH (more acidic) than fish preserved with distillation liquid smoke. This is due to the relatively high concentration of redistillation liquid smoke added to the product, so the acid content also increases. The acidic compounds in liquid smoke are mostly carboxylic acid derivatives such as furfural, furan, and glacial acetic acid, inhibiting microbial growth (Darmadji 1996). The higher the concentration of liquid smoke added to the product, the lower the pH value.

Table 3 show the average pH pattern of fish with redistillation liquid smoke preservative is more significant, which indicates a more acidic pH (pH 5-6), while the control pH pattern is almost the same as the addition of distillation liquid smoke (pH 6-7), which indicates tend to grow. This indicates that the control treatment and distillation liquid smoke conditions are getting worse because the concentration of liquid smoke added to the product is very low. According to Winarno (2002), in storage conditions, there will be changes in humidity and temperature, which are the determining factors for the speed of reshuffling of enzymes and bacteria in food which can cause changes in pH during a certain period. The increase in fish pH is also caused by a decay process in which the protein content of amino acids is converted into alkaline ammonia compounds.

Atmadjaja (1994) stated that fish pH values ranging from 6.0 to 7.0 and temperatures around 25-30ºC were ideal for the growth of spoilage bacteria. While a low pH can inhibit contamination of spoilage microorganisms, pathogenic microorganisms and poison-producing microorganisms will die (Sperling 1968).

Phenol levels

Phenol compounds are very important in smoked products because phenol plays a role in contributing to the specific odor and taste of smoked products (Girard 1992). Maga (1987) stated that phenols with higher boiling points would show better antioxidant properties than phenolic compounds with lower boiling points. The purpose of this phenol analysis was to determine the number of phenolic compounds attached to *pindang Decapterus* spp.. The phenol content of *pindang Decapterus* spp. can be seen in Table 4.

Table 4 shows that on all days of observation, the samples with the addition of redistillation and distillation liquid smoke (except the 1% distillation liquid smoke treatment on the 2nd and 6th days) had phenol levels that were significantly different from the control. The higher the concentration of liquid smoke added, both distillation and redistillation type liquid smoke resulted in increased phenol levels in *pindang Decapterus* spp. From days 0 to 6, the phenol content in *pindang Decapterus* spp. decreased; this was due to the phenol content in the material experiencing evaporation due to the storage process at room temperature (Sundari 2008). Even in the control treatment, there was phenol content due to the presence of phenolic compounds in water and salt from seawater and industrial and agricultural wastes (Romimohtarto 2009).

According to Girard (1992), the amount of safe limits in smoking products ranges from 0.06mg/kg to 5000 mg/kg or 0.0006 to 0.5%. Thus, the phenol content in *pindang Decapterus* spp. with the addition of this liquid smoke for the treatment of adding redistillation liquid smoke (0.471 to 0.566%) exceeds the safe limit. The same thing also happened in Sundari's (2008) study, which produced phenol levels of 0.4502 to 0.5269 % with concentrations of redistillation liquid smoke of 25%, 30%, and 35%. These levels also exceed the safe limit of phenol in foodstuffs (0.0006 to 0.5%). Phenol has acidic properties, is easily oxidized, volatile, sensitive to light and oxygen, and is antiseptic. The phenol levels will decrease, among others, by washing, boiling, and processing to make ready-for-consumption products (Sundari 2008).

Microbiological properties of *pindang Decapterus* spp.

The number of microbes in foodstuffs affects the speed at which food spoils. According to Hadiwiyoto (1993), the rate of microbiological deterioration of fishery products depends on the growth rate of existing microbes, especially spoilage bacteria. Bacterial growth is generally defined as an increase in the number of constituents in a cell or its mass, followed by cell multiplication so that the number of cells increases. The TPC of *pindang Decapterus* spp. can be seen in Figure 1.

Figure 1 shows that from day 0 to day 6, the TPC of all treatments increased. The addition of redistillation liquid smoke tends to produce significantly lower TPC than distillation and the control during storage. In addition, the higher the concentration of liquid smoke added to the product, the lower the value of TPC levels. This shows that 25-35% of redistillation liquid smoke significantly inhibits microbial growth, with better inhibitory ability than adding 1-3% distillation liquid smoke during storage.

Table 3. Results of pH analysis on *pindang Decapterus* spp.

Treatment	Observation on day			
	0	2	4	6
Control	6.07 cd	6.82 e	7.40 g	7.63 f
Redistillation 25%	5.61 b	5.86 b	6.30 c	6.40 b
Redistillation 30%	5.53 b	5.60 a	6.20 b	6.31 a
Redistillation 35%	5.33 a	5.61 a	6.08 a	6.29 a
Distillation 1%	6.13 d	6.48 d	7.13 f	7.49 e
Distillation 2%	6.06 cd	6.32 c	7.02 e	7.35 d
Distillation 3%	6.02 c	6.27 c	6.95 d	7.27 c

Note: Numbers followed by the same letter show no significant difference at the 5% level (applies to the same column)

Table 4. The results of the analysis of phenol levels (%) in *pindang Decapterus* spp.

Treatment	Observations on day			
	0	2	4	6
Control	0.264 a	0.264 a	0.241 a	0.226 a
Redistillation 25%	0.471 e	0.460 d	0.416 d	0.399 c
Redistillation 30%	0.531 f	0.517 e	0.432 e	0.413 d
Redistillation 35%	0.566 g	0.547 f	0.494 f	0.449 e
Distillation 1%	0.283 b	0.271 a	0.241 a	0.227 a
Distillation 2%	0.312 c	0.292 b	0.252 b	0.242 b
Distillation 3%	0.340 d	0.311 c	0.265 c	0.249 b

Note: Numbers followed by the same letter show no significant difference at the 5% level (applies to the same column)

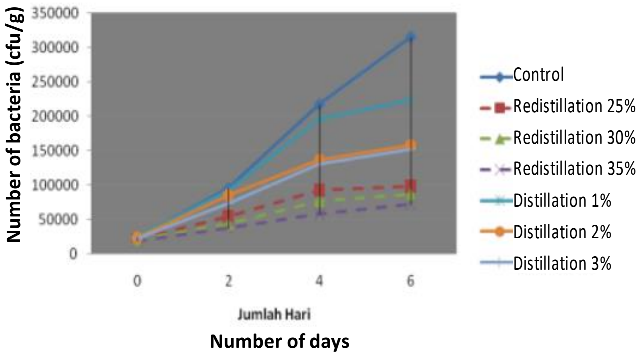

Figure 1. The average number of bacteria on *pindang Decapterus* spp.

The value of the total plate count of *pindang Decapterus* spp. from day 0 to day 6 has increased. On the 4th day of control treatment and adding 1%, 2%, and 3% distillation liquid smoke, it did not meet the threshold value following the SNI (1992) requirements for *pindang* microbiological quality with a maximum TPC per gram of 1.105 Cfu/g. This is because the value of the TPC content in the product (1.3.105-2.2. 105 Cfu/g) has exceeded the existing requirements limit.

While in the addition of 25%, 30%, and 35% redistillation liquid smoke, the value of TPC levels on day 4 to day 6 was about 5.8. 104-9.9. 104 Cfu/g still meets the requirements listed in SNI (1992) for *pindang* laying fish microbiological quality with a maximum TPC per gram of 1.105 Cfu/g. Thus, *pindang Decapterus* spp. in the treatment with the addition of redistillation liquid smoke (25%, 30%, 35%) stored until the 6th day was still suitable for consumption. This is because the high phenol content in the added liquid smoke can inhibit microbial growth and low pH, even though the water content in *pindang Decapterus* spp. is quite high.

Sensory properties of *pindang Decapterus* spp.

Sensory testing is one way of assessing a food product by humans as a measure using their five senses. The senses that play a role in this sensory test are sight (color), smell (smell), touch (texture), and overall level.

In this study, the sensory properties tested on 20 panelists were color, odor, texture, and overall. In general, the storage of *pindang* layang from day 0 to day 6 experienced a decrease in sensory value. This shows that the longer the *pindang* layang product is stored at room temperature, the more panelists dislike it because the product begins to experience changes that lead to a decline in color, odor, texture, and overall specifications.

Color

Color plays an important role in food acceptance, and color can give clues about chemical changes in food. According to Fennema (1985), color is the most important quality attribute; even though a product has high nutritional value, good taste, and texture, if the color is not attractive, it will cause the product to be less attractive. Color is the first parameter that determines the consumer acceptance level of a product. Subjective research with vision is still very decisive in color organoleptic testing; if the color seen by consumers is not attractive, it will result in low consumer ratings of the food product. The color of *pindang Decapterus* spp. can be seen in Table 5.

Table 5 shows that all treatments decreased from day 0 to day 6 of color analysis. The decrease in color parameters in the redistillation liquid smoke treatment was not as significant as in the distillation and the control. Then from the results of statistical analysis, on the color parameter on day 0, all treatments of redistillation liquid smoke (25%, 30%, 35%) were not significantly different from the distillation liquid smoke treatment and the control. At the same time, the control treatment had a significant difference from distillation liquid smoke treatments of 1%, 2%, and 3%. Of all the treatments, the one with the highest value was the control treatment. On the day of manufacture (day 0), all treatments of *pindang Decapterus* spp. followed the quality standards of high-quality *pindang Decapterus* spp. according to Wibowo (2000), namely having a specific color of grayish white, whole fish, not broken, clean, and not moldy.

On the 2nd day, the redistillation liquid smoke treatment was not significantly different from the 1% and 2% distillation liquid smoke treatment. Then for the 4th and 6th days, all treatments with the addition of redistillation liquid smoke were significantly different from those of the control and distillation liquid smoke. The fastest decrease in color parameters was in the control treatment, while the slightest decrease was in the treatment with the addition of redistillation liquid smoke. For all treatments, the addition of redistillation liquid smoke has a score above 5 which means that consumers are in a neutral condition and rather like it, while for the control treatment and the addition of 1%, 2%, and 3% distillation liquid smoke, the score is below 3 which means that consumers are at the very dissatisfied rating, so the product has not been received. This is because the control product does not meet the quality standards of *pindang Decapterus* spp., where the color of the fish is yellow, not intact, and moldy. It can be concluded that the panelists preferred fish given the addition of redistillation liquid smoke compared to distillation and the control liquid smoke.

Odor

Kartika et al. (1988) state that odor can be defined as the result of the sense of smell response caused by the evaporation of slightly fat-soluble substances in a food product into the air so that it can be responded to by the sense of smell, namely the nose, and then recognized by the body-system as a certain scent. In the food industry, testing for odor (*aroma*) is important because it can quickly assess whether the product is acceptable. In addition, the odor can also be used as an indicator of damage to the product. The odor of *pindang Decapterus* spp. can be seen in Table 6.

Table 6 shows that from day 0 to day 6, the odor parameters in all treatments decreased. The decrease in odor parameters in the redistillation liquid smoke treatment was not as significant as in the distillation and the control liquid smoke treatment.

Table 5. Results of the color analysis on *pindang Decapterus* spp.

Treatment	Observations on day			
	0	2	4	6
Control	7.30 [b]	5.70 [c]	1.75 [a]	1.25 [a]
Redistillation 25%	6.75 [ab]	6.40 [c]	6.05 [d]	5.10 [c]
Redistillation 30%	6.90 [ab]	6.00 [c]	5.85 [d]	4.85 [c]
Redistillation 35%	6.90 [ab]	5.95 [c]	5.50 [d]	5.10 [c]
Distillation 1%	6.20 [a]	3.30 [a]	2.15 [ab]	1.40 [a]
Distillation 2%	6.20 [a]	4.90 [b]	2.45 [b]	1.60 [ab]
Distillation 3%	6.45 [a]	5.85 [c]	3.20 [c]	1.90 [b]

Note: Numbers followed by the same letter show no significant difference at the 5% level (applies to the same column). Description of quality attributes: 1. very much dislike (unacceptable), 2. strongly dislike, 3. dislike, 4. slightly dislike, 5. neutral, 6. somewhat like, 7. like, 8. very much like , 9. very much like it

Table 6. The results of the odor analysis on *pindang Decapterus* spp.

Treatment	Observation on day			
	0	2	4	6
Control	7.05 [c]	5.70 [d]	2.00 [ab]	1.10 [a]
Redistillation 25%	6.50 [bc]	5.95 [d]	6.20 [d]	6.05 [e]
Redistillation 30%	5.80 [ab]	4.65 [c]	5.10 [c]	5.35 [d]
Redistillation 35%	5.25 [a]	3.75 [b]	5.00 [c]	4.50 [c]
Distillation 1%	6.10 [b]	3.10 [a]	1.75 [a]	1.40 [ab]
Distillation 2%	6.10 [b]	4.45 [c]	1.95 [ab]	1.60 [b]
Distillation 3%	5.85 [ab]	4.70 [c]	2.45 [b]	1.80 [b]

Note: Numbers followed by the same letter show no significant difference at the 5% level (applies to the same column). Description of the quality attribute: 1. very strongly dislike (unacceptable), 2. very much dislike, 3. dislike, 4. somewhat dislike, 5. neutral, 6. somewhat like, 7. like, 8. very much like, 9. very much like it

The fastest decrease in odor parameters was in the control treatment, while the presence of phenolic compounds caused a slight decrease in the addition of redistillation liquid smoke. From the statistical analysis results, the odor parameters on day 0 and day 2 of the control treatment were not significantly different from the 25% redistillation liquid smoke treatment and significantly different from all treatments.

On day 0, the control treatment had the highest score of the other treatments, then for the second day, the control treatment and 25% redistillation liquid smoke had the highest score from the panelists, which was around 6-7, which means the panelists were in a condition of somewhat like to like. For day 4, the control treatment was not significantly different from all distillation treatments but significantly different from all redistillation liquid smoke treatments. The highest value was from adding 25% redistillation liquid smoke, namely > 6, then for the control treatment and distillation liquid smoke < 2, which means the panelists did not like it very much. Then for the 6th day, the control treatment was not significantly different from the 1% distillation liquid smoke treatment and significantly different from all treatments. The lowest value of this treatment was in the control treatment, a score of 1.1 which means that the panelists strongly disliked it. The highest score was in the 25% redistillation liquid smoke treatment; the score was ± 6, which means the panelists somewhat liked the product.

From the data above, it can be concluded that on the 0th and 2nd days, the control treatment was the most preferred, while the liquid smoke treatment that used high concentrations was somewhat like; this was due to the addition of a high concentration of liquid smoke into the product which would produce a pungent smoke odor. But for the 4th and 6th day, the most preferred treatment was the addition of redistillation liquid smoke. This is because the longer the storage of the smoke odor will evaporate, while in the control treatment, the odor is rotten. According to Wibowo (2000), the high-quality standard of *pindang Decapterus* spp. is the specific odor of *pindang Decapterus* spp., without rancid, sour, and rotten odors. So that the control treatment and the addition of distillation liquid smoke on the 4th and 6th days were not suitable for consumption because they were not following the quality standards of *pindang Decapterus* spp..

The presence of phenolic compounds causes the odor of the liquid smoked fish. According to Girard (1992), phenol compounds play a significant role in giving smoke odor. Daun (1979) stated that the flavor characteristics of smoked products were caused by the presence of phenolic components absorbed on the product's surface.

Texture

The texture is a sensation of pressure that can be observed using the mouth (when bitten, chewed, and swallowed) or by palpation with the fingers (Kartika et al. 1988). To feel the texture of a product, the sense of touch is used. The sense of touch used for food is usually in the mouth using the tongue and the parts in the mouth; it can also be using the hands to feel the texture of a food product. The texture is also one of the determinants of quality that needs to be considered. The texture of the *pindang Decapterus* spp. can be seen in Table 7.

Table 7 shows that from day 0 to day 6, the texture parameters in all treatments decreased. The decrease in texture parameters in the redistillation liquid smoke treatment was not as significant as in the distillation and the control. The higher the concentration of liquid smoke added to *pindang Decapterus* spp., the better the texture. The fastest decrease in texture parameters was in the control treatment, while the slightest decrease was in the treatment with the addition of redistillation liquid smoke.

From the texture analysis results, it can be seen that, on day 0 and day 2, all redistillation liquid smoke treatments were not significantly different from the 3% distillation liquid smoke treatment. For day 0, the control treatment had a higher value than the other treatments. On the 4th and 6th days, all treatments of redistillation liquid smoke were significantly different from all treatments of distillation and the control.

On days 0 and 2, the control treatment and redistillation of liquid smoke were the most preferred. All treatments on days 0 to 2 still met the quality standards of *pindang Decapterus* spp.. According to Wibowo (2000), the high-quality standard of *pindang Decapterus* spp. is compact, dense, dry enough, not runny/muddy, and with no visible bacteria or mold. From the 4th day onwards, the control treatment and all the distillation treatments did not meet the quality standards of *pindang Decapterus* spp. because the product had molds visible, and the texture was not compact, so the panelists gave a score of 2-3, which meant the panelists were in a state of dislike and very strong dislike. However, in contrast to the redistillation liquid smoke treatment, the product was still following the quality standards of *pindang Decapterus* spp., so the panelists still gave a score of 5-6 which means the panelists were in a neutral condition and somewhat liked it. From the data above, it can be concluded that the addition of redistillation liquid smoke is preferred by panelists and can maintain quality longer than other treatments.

Overall analysis

The overall preference test assesses all quality factors, including color, odor, and texture, which is intended to determine the level of panelists' acceptance of the product.

Table 8 shows that from day 0 to day 6, the overall parameters in all treatments decreased. The decrease in overall parameters in the redistillation liquid smoke treatment was not as significant as in the distillation and the control. The higher the concentration of liquid smoke added to the *pindang Decapterus* spp., the more preferred by the panelists. The fastest decrease in overall parameters was in the control treatment, while the slightest decrease was in the treatment with the addition of redistillation liquid smoke.

From Table 8, the results of the overall analysis can be seen that on day 0, the redistillation liquid smoke treatment was not significantly different from the 2%, 3% distillation liquid smoke, and the control treatments. For the 2nd day, all the redistillation liquid smoke treatments were not significantly different from the control treatment. For days 0 and 2, the control treatment had a higher value than the other treatments. On the 4th and 6th days, all treatments of redistillation liquid smoke were significantly different from all treatments of distillation and control liquid smoke. For the 4th and 6th days, the most preferred treatment was the addition of redistillation liquid smoke; the panelists gave a score >5, which means the panelists were in a neutral condition. From the data above, it can be concluded that the redistillation liquid smoke treatment is the most preferred by the panelists and the treatment that has the longest shelf life compared to the control and the addition of distillation liquid smoke.

According to Girard (1992), the effects of acids are less specific but generally affect the overall organoleptic quality. Daun (1979) added that the main effects of liquid smoke are changes in color, odor, bactericidal properties, and antioxidant properties. According to Hadiwiyoto (1993), phenol is a compound that can provide odor, taste, and color, as well as antioxidant and preservative effects.

The organoleptic value of *pindang Decapterus* spp. decreased with increasing storage time. This is due to the decomposition of compounds in fish such as protein, amino acids, lactic acid, and reducing sugars by decomposing bacteria, resulting in a foul odor. In addition, there are spoilage bacteria found in fish meat and bacteria from the outside environment.

Table 7. The texture analysis results on *pindang Decapterus* spp.

Treatment	Observation days on			
	0	2	4	6
Control	7.30 ^{c}	6.20 ^{c}	2.35 ^{a}	1.20 ^{a}
Redistillation 25%	6.85 ^{abc}	5.60 ^{bc}	6.05 ^{c}	5.30 ^{c}
Redistillation 30%	6.95 ^{bc}	6.25 ^{c}	6.10 ^{c}	5.80 ^{d}
Redistillation 35%	6.95 ^{bc}	5.60 ^{bc}	6.15 ^{c}	5.65 ^{cd}
Distillation 1%	6.35 ^{ab}	4.35 ^{a}	2.50 ^{ab}	1.30 ^{a}
Distillation 2%	6.20 ^{a}	5.15 ^{b}	2.70 ^{ab}	1.40 ^{a}
Distillation 3%	6.55 ^{ab}	5.25 ^{c}	3.05 ^{b}	1.90 ^{b}

Note: Numbers followed by the same letter show no significant difference at the 5% level (applies to the same column). Description of quality attributes: 1. very much dislike (unacceptable), 2. strongly dislike, 3. dislike, 4. slightly dislike, 5. neutral, 6. somewhat like, 7. like, 8. very much like , 9. very much like it

Table 8. The overall analysis on *pindang Decapterus* spp.

Treatment	Observation days on			
	0	2	4	6
Control	7.35 ^{c}	6.05 ^{d}	2.20 ^{a}	1.10 ^{a}
Redistillation 25%	7.00 ^{bc}	5.75 ^{cd}	5.75 ^{c}	5.00 ^{c}
Redistillation 30%	6.75 ^{abc}	6.05 ^{d}	5.70 ^{c}	5.00 ^{c}
Redistillation 35%	6.70 ^{abc}	5.45 ^{cd}	5.55 ^{c}	4.65 ^{c}
Distillation 1%	6.05 ^{a}	3.90 ^{a}	2.20 ^{a}	1.65 ^{b}
Distillation 2%	6.20 ^{b}	4.80 ^{b}	2.55 ^{ab}	1.00 ^{a}
Distillation 3%	6.25 ^{ab}	5.20 ^{bc}	2.90 ^{b}	1.95 ^{b}

Note: Numbers followed by the same letter show no significant difference at the 5% level (applies to the same column). Description of quality attributes: 1. very much dislike (unacceptable), 2. strongly dislike, 3. dislike, 4. slightly dislike, 5. neutral, 6. somewhat like, 7. like, 8. very much like , 9. very much like it

Based on the research that has been carried out, it can be seen that: (i) In the control treatment and the 6th-day observation of redistillation liquid smoke, the water content value increased, while in the distillation liquid smoke treatment, it still decreased. (ii) On the 6th day of observation, the water content of the redistillation liquid smoke treatment significantly affected the distillation liquid smoke treatment. (iii) During the storage of the redistillation liquid smoke treatment, the increase in pH (0.8) was not as significant as the distillation liquid smoke treatment (1.4), which was stated at the level of 0.5%. (iv) Total Plate Count (TPC) in the redistillation liquid smoke treatment was significantly different from the distillation liquid smoke treatment; the longer the storage time, the higher the number of bacteria. (v) In the control treatment and distillation, liquid smoke can only maintain quality until the 4th day in terms of the Total Plate Count (TPC) 1.3. 105-3.2. 105 Cfu/g exceeds the standard of SNI (1992), while the redistillation liquid smoke treatment can maintain quality until the 6th day. (vi) Based on the sensory properties (color, odor, texture, and overall), the panelists preferred the redistillation liquid smoke treatment to the distillation liquid smoke treatment. (vii) In terms of chemical, microbiological, and sensory properties, the redistillation treatment can maintain the quality longer (6th day) and is preferred by the panelists compared to the distillation liquid smoke treatment. (viii) From the chemical and microbiological properties, the 35% redistillation liquid smoke treatment can maintain its quality longer than the other treatments. (ix) In terms of sensory properties, the 30% redistillation liquid smoke treatment is preferred by panelists over other treatments. (x) The higher the phenol content contained in the liquid smoke, the better the quality of the chemical, microbiological, and sensory properties.

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BIOTEKNOLOGI
Volume 15, Number 2, November 2018
Pages: 92-98

ISSN: 0216-6887
E-ISSN: 2301-8658
DOI: 10.13057/biofar/c150205

Biological characterization of *Fusarium* sp. isolates on red chili plants (*Capsicum annuum*) from Boyolali, Central Java, Indonesia

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Manuscript received: 7 September 2018. Revision accepted: 8 November 2018.

Abstract. *Nugraheni ES, Widadi S, Supyani. 2018. Biological characterization of* Fusarium *sp. isolates on red chili plants (*Capsicum annuum*) from Boyolali, Central Java, Indonesia. Bioteknologi 15: 92-98.* This study examines the biological character, and virulence diversity of *Fusarium* sp. isolates in red chili plants (*Capsicum annuum* L.) from Boyolali, Central Java, Indonesia. This study was conducted from October 2009 to May 2010 at the Laboratory of Plant Pests and Diseases, Faculty of Agriculture, Universitas Sebelas Maret, Surakarta, Central Java, Indonesia. This study was designed as exploratory research in the field and an experiment in the laboratory. Observation variables included colony growth rate, colony phenotype, sporulation, and virulence test. Data from growth rate observations, colony phenotype, and sporulation were presented in descriptive form. Meanwhile, data from the virulence test were analyzed for variance with the F test at a 5% level. If the treatment has a significant effect, it is continued with Duncan's Multiple Distance Test (DMRT) at a 5% level. The results showed that the fungus of *Fusarium* sp. on red chili plants from Boyolali had distinguished macroscopic and microscopic diversity, with different levels of virulence. The colony group of *Fusarium* sp. isolates that had a low virulence level was the isolate of group D. In contrast, the biological characters of isolate D showed white mycelium like cotton, long macroconidia shape with a tapered tip, macroconidia with 3-6 septa, microconidia with 0-1 septa, size [(±5.35x3.15)-(±4.75x2.5)] µm x [(±27.36x4.10)] µm, spore density between 25.64×10^6-27.76×10^6 spores/ml. This isolate has the potential as a hypovirulent isolate that can be used as an environmentally friendly biological control agent, particularly by applying the hypovirulent isolate in a biological control system with induced resistance and virocontrol with mycovirus methods.

Keywords: Biological characterization, *Fusarium* sp., red chili

INTRODUCTION

Horticultural products such as fruits and vegetables are always in demand by the community every day to meet the needs of vitamins for the body. One of the horticultural products people need daily is red chili, both used for vegetables and as a mixture in cooking spices. Chili plants are one of the horticultural crop commodities whose fruit has a fairly high nutritional value, especially the content of vitamins A and C. The nutritional content of chili peppers in 100 grams of the edible part contains 71.00 mg of vitamin A and 18.00 mg of vitamin C (Wirakusumah 1995 cit. Harpenas and Dermawan 2010). The demand for red chili has been increasing over the years in line with the increase in population. Yet, chili production is still insufficient. In 2006, the harvested area of red chili in Central Java, Indonesia, reached about 20,415 hectares with a production level of 1,248,392 quintals and productivity of 61.15% per year. However, in the 2003-2006 period, the harvested area of red chili in Central Java seems to have a downward trend of 6.10% per year. Those are due to the emergence of pests and diseases (Agriculture Office of Central Java 2006 cit. Supriyati and Roosganda 2009).

Efforts to increase the production of chili plants still experience obstacles. Insects, mites, nematodes, and pathogens are threats that are always present in every planting. Plant pathogens can be caused by fungi, bacteria, mycoplasma-like organisms, and viruses (Gunawan et al. 2004 cit. Samoosir 2007). One of the pathogens caused by fungi is fusarium wilt caused by *Fusarium* sp. This fungus attack is one of the limiting factors that cause a decrease in red chili production. The spread of *Fusarium* is highly fast and can spread to other plants by infecting plant roots using sprout tubes or mycelium. Plant roots can be infected directly through the root tissue or lateral roots and wounds, which settle and develop in the vascular bundles. Once entering the plant roots, the mycelium will develop until it reaches the root cortex tissue. When the fungal mycelium reaches the xylem, this mycelium will develop to infect the xylem vessels. Mycelium that has infected the xylem vessels will be carried to other parts of the plant so that it interferes with the circulation of nutrients and water in plants which causes plants to wither (Semangun 2005). *Fusarium* forms a polypeptide called lycomarasmin, which can interfere with the permeability of the plasma membrane of plants (Walker 1952 cit. Susetyo 2010).

When the vascular tissue dies, and the air is moist, the fungus forms purplish-white spores on the infected roots. Their spread can occur through wind, irrigation water, and agricultural tools. Complete wilting may occur between 2-3 weeks after infection. Generally, when plants wilt, it is started from the lower leaves, and the leaves turn yellow. When an infection develops, plants wilt within 2-3 days after infection. Diseased plants cut near the understock showed symptoms of a brown ring from the vascular bundle. The color of the root and stem tissue becomes brown. The infected site is covered with white hyphae like cotton (Directorate of Horticultural Protection 2003 cit. Huda 2010).

The decline in red chili production in the Boyolali area resulted from *Fusarium* sp. attacks which caused huge losses to farmers. Efforts to control fusarium wilt have been carried out, but farmers have not found an effective and environmentally friendly control method. The rapid spread of fusarium wilt has exacerbated the situation, so the use of synthetic pesticides (fungicides) is the last alternative to control *Fusarium* sp. pathogens in red chili plants in the Boyolali area. Various things have been considered before using these fungicides because, in addition to their high price, they also cause pathogens to become resistant to fungicides, create new *Fusarium* familia, and may cause environmental pollution. However, the control of *Fusarium* sp. pathogens can be done naturally without having to cause negative residues for the environment, particularly by applying the induced resistance method and virocontrol with mycoviruses. According to Istikorini (2002), biological control mechanisms can occur through some mechanisms, two of which are virocontrol with mycoviruses and induced resistance. Virocontrol with mycoviruses is controlled by viruses that can infect fungi. Meanwhile, induced resistance develops after plants are inoculated early with biotic elicitors (avirulent, non-pathogenic, saprophytic microorganisms) and abiotic elicitors (salicylic acid, 2-chloroethyl phosphonic acid).

The objective of this study is to examine the biological character, and virulence diversity of *Fusarium* sp. isolates on red chili (*Capsicum annuum* L.) from Boyolali, Central Java, Indonesia.

MATERIALS AND METHODS

Materials

The main materials used in this study were chili plant parts infected with *Fusarium* sp. (understock, interstock, scion, root) and PDA (Potato Dextrose Agar) media.

Research design

This study was designed as exploratory research in the field and an experiment in the laboratory.

Research implementation

Collection of isolates of Fusarium *sp.*

Fusarium sp. isolates were collected from the Boyolali area. The collection was done by visiting the area. Red chilies that showed a general symptom of wilting were dug up with a shovel. The entire plant (including all the roots) was removed, then the attached soil grains were released by immersing them in water. Parts of the plant, especially the understock and roots, were examined for additional symptoms of pathogens in the form of spots, wounds, rot, or signs of pathogens in the form of sclerotia.

The plant parts that show symptoms or signs of the pathogen were then put in a plastic bag and labeled. They were then put in a cooler right away. Once arriving at the laboratory, the plant samples were immediately transferred to a refrigerator at 4°C to be further cultured in PDA media.

Cultures of Fusarium *sp. isolates in PDA media*

Cultures of *Fusarium* sp. isolates were carried out in LAF (Laminar Air Flow). Tissue surfaces containing spots, wounds, or rot were sterilized with 90% alcohol. A small section of the border area between diseased and healthy plant tissue was cut, removed, and placed in the center of an 8 cm diameter sterile Petri dish containing 20 mL of PDA (Potato Dextrose Agar). The preparations were incubated under standard conditions at 22-26°C for 7-10 days. All isolates were labeled with an identification number corresponding to the label's identity when they were isolated from the field. When the culture was 1 week old, photo shoots were taken for documentation.

While taking the photo shoot, each isolate was stocked, particularly by culturing it on a regeneration medium in a 4 cm diameter Petri dish. The making of stock began with inoculating 3x3x3 mm cubic inoculum taken from the edge of the culture, then placed in the middle of the medium that had been provided. The preparations were incubated under standard conditions at 22-26°C for 1 week. After that, the preparation was stored in a refrigerator at 4°C as stock for the next tests. Each of these stocks was labeled with an identification number corresponding to the identification number of the isolate on the PDA media.

Characterization of Fusarium *sp. isolates*

Morphological characterization was carried out by inoculating 3x3x3 mm^3 inoculum, taken from the edge of 1-week-old stock culture, in the center of an 8 cm diameter Petri dish containing 20 mL of PDA. The Petri dish was then incubated under standard conditions of 22-26°C. At the same time, virus-free *Fusarium* sp. preparations were also cultured for comparison. The culture was observed on days 3, 5, and 7. The characteristics observed and recorded were colony growth rate, colony phenotype, and sporulation. If the observed isolates showed different characteristics, such as smaller colony diameter of the colony growth rate, darker/lighter color of the colony phenotype, and rough colony surface, the isolates were marked/selected and documented. These isolates had a high chance of low virulence (hypovirulence). The selected isolates were then tested by virulence testing using apples and chili plants.

Virulence test

The virulence test was carried out on apples using selected hypovirulent isolates based on the characterization of the isolates. Selected isolates were tested for virulence. First, ripe apples were disinfected with 90% alcohol. Four points around the fruit with a balanced spread position were determined. Each point was then inoculated with *Fusarium* sp. isolates on the part that had been wounded. The inoculum was inserted into each wound in an inward-facing position. It was pressed with a sterile spatula so that there was complete contact with the apple tissue. The inoculated parts were then wrapped with parafilm to prevent drying. Next, the apples were incubated in a plastic tray measuring 35x25x7 cm at room temperature. The diameter of the lesions was measured on days 5, 7, 9, and 12. Based on the results of the virulence test, it was possible to determine which *Fusarium* sp. isolates had lower virulence (hypovirulent) than the control (virulent).

Data analysis

Data on the growth rate, colony phenotype, and sporulation were presented in descriptive form. The virulence test data were then analyzed for variance with the F test at a 5% level. If the treatment has a significant effect, it is continued with Duncan's Multiple Distance Test (DMRT) at a level of 5%.

RESULTS AND DISCUSSION

The general condition of the location

The sampling location is in Boyolali. Boyolali, particularly the sampling location, has an altitude of ±400 m above sea level with an average rainfall of 3000-4000 mm/year, 70% air humidity, and an average temperature of 21-30°C/month (www.scbdp.net). The red chili plantation area at the sampling site looks quite dry (especially the soil). It lacks water because the sampling was carried out during the dry season, and it was difficult to find water for irrigation. Around the location, cassava and peanuts are planted, cultivated food crops. During the sampling, the condition of red chili plantations showed withered leaves and brown stems, and the plants seemed to no longer have the potential to produce red chilies (Appendix 8). Those are presumably caused by the attack of the pathogenic fungus, *Fusarium* sp., which is one of the limiting factors for red chili production in the Boyolali area. Various efforts have been carried out to control the pathogen that causes this wilt, such as using healthy seeds or fungicides. Yet, they have not yielded satisfactory results.

The symptoms caused by the *Fusarium* sp. pathogen to red chili plants are that the affected plants wilt and die. Those are because the fungus attacks the vascular tissue and causes wilting of the host plant by inhibiting the flow of water in the xylem tissue (De Cal et al. 2000). The most typical symptoms are found on the inside of the plant stem. If the understock is split longitudinally, dark brown streaks are seen running in all directions, from the stem upwards through the vascular tissue to the base of the leaves and stems. The root vascular bundles are usually not discolored, but the roots of diseased plants are often black and rot.

Isolation and pure culture

Pathogen isolates were taken from the scion, interstock, understock, and roots of red chili plants affected by fusarium wilt. The parts were then aseptically sliced, grown on PDA media, and isolated (Appendix 9). The results of the isolation of *Fusarium* sp. obtained from red chili plantations from Boyolali were 100 isolates with morphological diversity. The 100 isolates obtained were divided into five groups based on the color of the mycelium colonies formed on PDA media, including cream, purple, pink, white, and creamy white colonies. The grouping of the isolate colonies was also based on the macroscopical variation of other morphological characteristics. According to Machmud et al. (2003), one way to determine the type of pathogen is to know its biological character. Therefore, studying various biological and ecological characteristics of pathogenic microbes is also related to the epiphytology and control of agricultural plant diseases.

Macroscopic observation of *Fusarium* sp. isolates

Macroscopic observations of *Fusarium* sp. isolates were carried out from the 3rd to the 7th day after isolation. Macroscopic observations were carried out directly by evaluating the development of each colony, starting from the diameter of the colony, colony color, air mycelium, and colony profile. Based on these morphological differences, *Fusarium* sp. isolates were grouped into five colony groups: A, B, C, D, and E (Appendix 10). In each group of isolates that had been isolated, there were 5 selected isolates consisting of 1 isolate with the fastest colony growth (control isolate: K) and 4 isolates with relatively slow colony growth (treatment isolate). The control isolates were used as a comparison with treatment isolates. The results of macroscopic observations of *Fusarium* sp. isolates are presented in Table 1.

Table 1. Macroscopic observation of control and non-control of *Fusarium* sp. isolates

Isolate	Control isolate diameter (cm)	The mean diameter of the treatment isolates (cm)	Colony color	Air mycelium	Colony profile
A	7.8	5.6	The base of the colony is cream; the top is white with a smooth surface	little	soft
B	6.5	4.2	The base of the colony is purple; the top is white	little	soft
C	6.8	4.5	The base of the colony is pink; the top is white	little	wavy
D	7.3	4.8	White like cotton.	little	like cotton, uneven edges
E	7.5	5.3	The base of the colony is creamy white	little	rough, forming a ring

Notes: A: Group, AB: Group, BC: Group, CD: Group, DE: Group E

The observation results showed that the isolated isolates could be identified based on the macroscopic characteristics that appeared, including colony diameter, colony color, air mycelium, and colony profile (Table 1). Each of the selected isolates shows different morphological characters. The red chili plant parts taken as isolation material for the fusarium wilt pathogen may affect the characteristics of the *Fusarium* sp. isolates. The isolate colony of group A is from the scion of red chili plants, the isolate colony of group B is from the interstock of red chili plants, and the isolate colony of group C is from the understock of red chili plants. Meanwhile, the isolate colony of groups D and E are from the root of the red chili plants.

Observation of the growth rate of the isolate colony was carried out by evaluating the size of the colony diameter in each group, which showed different sizes of the isolate diameter (Figure 1). The control isolates in each colony group had a higher growth rate than the treatment isolates. The control isolate and the treatment isolate colonies showed that the colony groups with the highest and lowest diameter values were in the colony of group A and colony of group B. In the control isolate colonies, the highest colony diameter values were found in isolates group A of 7.8 cm. In comparison, the lowest diameter value found in isolates group B was 6.5 cm. In the treatment isolate colonies, the highest colony diameter value was found in the isolate of group A at 5.6 cm.

In comparison, the lowest average diameter value was found in the isolate of group B at 4.2 cm. Gandjar (1999) stated that *Fusarium* sp. on PDA media had colonies reaching a diameter of 3.5-5.0 cm. Those indicate that the isolates obtained from the isolation of red chili plants have a high growth rate. The isolate colony's diameter affects the conidia formation process, which in turn will also affect the level of development of *Fusarium* sp. The growth of the next fungal isolate colony will still occur even though it is slow. That is because the conidia are developmental tools in the Deuteromycetes class, which are produced asexually so that their number determines the development of the next generation. Under favorable conditions, the number of conidia tends to be directly proportional to the rate of development of the *Fusarium* (Burnett and Hunter 1988). In addition to observing the colony growth rate, observations were also made on the color of the colonies in each group. The appearance of the color of the colonies of *Fusarium* sp. isolates in each group of isolate colonies was different. The color difference of this isolate colony was based on the color that appeared at the bottom of the colony and the top surface.

The color of the colonies appeared to be smooth cream, purple, pink, white like cotton, and creamy white (Figure 2). The appearance of the isolate colony of group A had a cream color, smooth colony surface, little air mycelium formation, and hyaline mycelium appearance. The appearance of the isolate colony of group B had purple color, smooth colony surface, and medium air mycelium. The appearance of the isolate colony of group C had pink color, wavy edges, and medium air mycelium. The appearance of the isolate colony of group D had white colors like cotton, uneven edges, and little air mycelium. Finally, the appearance of the isolate colony of group E had a creamy white color, a rough and ring-forming colony profile, and a little air mycelium formation.

Based on these macroscopic observations, it can be considered that, in fungal cultures in solid media such as Potato Dextrose Agar (PDA), *Fusarium* sp. can have different appearances even though they come from the same host plant. And in general, the air mycelium first appears white and then can change to various colors according to the strain (or special shape) of *Fusarium* sp. Some *Fusarium* sp. isolates will also form red pigment in the medium (Agrios 1996; Susetyo 2010). According to Semangun (1996), the hyphae pigments of *Fusarium* sp. are generally varied, hyaline pigmented (colorless), if colored means the fungus is pigmented, generally it is melanin pigment bound to hyphae cell walls.

In Sastrahidayat (1986), the fungus growing on a PDA medium was initially white, then the color became cream or pale yellow. Under certain circumstances, it was pinkish-purple with the mycelium insulated and forming branches. The effect of light on the growth of fungal vegetative hyphae is usually in the form of inhibiting or triggering their growth so that light may affect the concentration of pigment production and hyphae growth. Generally, fungi grown in bright conditions will have more air mycelium than in other conditions. That is due to the nature of fungi that grow in the direction of light (phototropy).

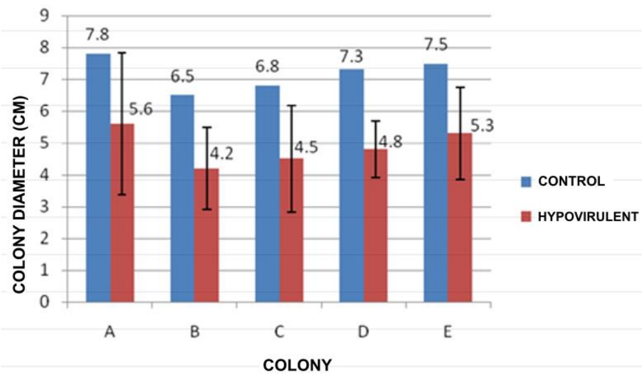

Figure 1. Colony diameter of *Fusarium* sp. isolates at the age of 7 days. Notes: A: Isolate colony, AB: Isolate colony, BC: Isolate colony, CD: Isolate colony, DE: Isolate colony E

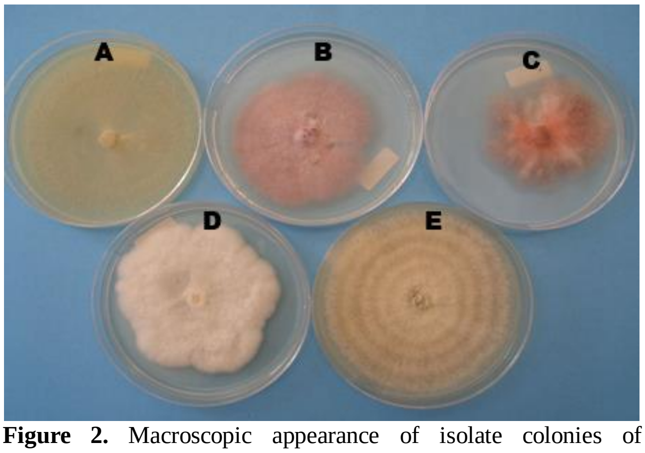

Figure 2. Macroscopic appearance of isolate colonies of *Fusarium* sp. Notes: A: Isolate colony, AB: Isolate colony, BC: Isolate colony, CD: Isolate colony, DE: Isolate colony E

Microscopic observation of *Fusarium* sp.

Observations of *Fusarium* sp. microscopically were done by observing the size and shape of the parts of *Fusarium* sp. and measuring the spore density of each isolate from each colony group (Figures 3 and 4).

Tables 2 and 3 show the differences in shape, size, conidia septa, and spore density in *Fusarium* sp. control and treatment isolates. In general, *Fusarium* sp. isolates microscopically have an ovoid microspore shape which generally has 0-1 septa, while macrospores generally have a long tapered tip and 2-6 septa.

Observation of the fungal isolates of *Fusarium* sp. is done by observing the size and shape of the conidia/spores of the isolates and the density of the spores of *Fusarium* sp. The colony measurements on control isolates showed that the longest macroconidia were found in the isolate colony of group C of 31.56x4.10 µm; 3-5 septa with microconidia size of 4.38x1.5 µm; 0-1 septa. Meanwhile, the smallest macroconidia size was found in the isolate of group B of 27.36x2.20 µm; 2-5 septa with microconidia size of 3.25x2.15 µm; 0-1 septa. On the other side, in the treatment isolates, the longest macroconidia size was found in the isolate of group D at 31.16x3.2 µm; 3-6 septa with microconidia size of 4.75x2.5 µm. Meanwhile, the smallest macroconidia were found in the colony of group B of 24.16x1.6 µm; 2-5 septa with microconidia size of 3.25x2.15 µm; 0-1 septa (Appendix 12). The size of microconidia and macroconidia in each isolate differed in control and treatment isolate colonies. Still, the general shape of macroconidia and microconidia was the same, particularly ovoid (microconidia) and elongated with tapered tips (macroconidia). In Domsch et al. (1993), macroconidium is spindle-shaped, oval, the sharp tip has 3-5 septa, and the size of the spores is [(20-27) - (46-60) x (3.5-4.5)] µm. Microconidia are arranged in 1 cell, transparent, in long basipetal chains. According to Agrios (1996), microconidium has one or two cells, is present in large numbers, and is often produced in all conditions. This type of spore is commonly found in infected plant tissues. Meanwhile, macroconidium has two to five cells and is curved. This type of spore is commonly found on the surface of plants that die due to this fungal infection.

Observation of spore density of *Fusarium* sp. isolates in the five groups of colonies was seen from the number of spores of each isolate. The spore density was observed by making a suspension for each isolate, particularly by diluting the mushroom culture with 10 mL of distilled water into a Petri dish with a diameter of 8 cm. First, the surface of the mushroom culture was scratched with an L glass for 5 minutes.

Table 2. Microscopic observation of *Fusarium* sp. control isolates

Isolate	Conidia/spora		Conidia septa	Sporulation
	Shape	Size		
A0	Ovoid (microconidia), macroconidia with a tapered tip	Microconidia of 3.75x2.5µm, Macroconidia of 29.76x3.12 µm	Macroconidia consists of 2-6 cells	$17.86x10^6$
B0	Ovoid (microconidia), macroconidia with a tapered tip	Microconidia of 5.25x1.87µm, Macroconidia of 27.36x2.20 µm	Macroconidia consists of 2-5 cells	$44.20x10^6$
C0	Ovoid (microconidia), macroconidia with a tapered tip	Microconidia of 4.38x1.5µm, Macroconidia of 31.56x4.10 µm	Macroconidia consists of 3-5 cells	$15.34x10^6$
D0	Ovoid (microconidia), macroconidia with a tapered tip	Microconidia of 5.35x3.15µm, Macroconidia of 27.36x4.10 µm	Macroconidia consists of 5-6 cells	$27.76x10^6$
E0	Ovoid (microconidia), macroconidia with a tapered tip	Microconidia of 3.05x1.75µm, Macroconidia of 30.16x3.26 µm	Macroconidia consists of 2-4 cells	$37.18x10^6$

Notes: A0: Control isolate colony A, B0: Control isolate colony B, C0: Control isolate colony C, D0: Control isolate colony D, E0: Control isolate colony E

Table 3. Microscopic observation of *Fusarium* sp. treatment isolates

Isolate	Conidia/spora		Conidia septa	Average sporulation
	Shape	Average size		
A	Ovoid (mikrokonidia), makrokonidia with a tapered tip	Microconidia of 3.15x1.5µm, Macroconidia of 27.26x2.72 µm	Macroconidia consists of 2-5 cells	$15.94x10^6$
B	Ovoid (mikrokonidia), makrokonidia with a tapered tip	Microconidia of 3.25x2.15µm, Macroconidia of 24.16x1.6 µm	Macroconidia consists of 2-5 cells	$42.76x10^6$
C	Ovoid (mikrokonidia), makrokonidia with a tapered tip	Microconidia of 2.52x2.5µm, Macroconidia of 29.36x3.2 µm	Macroconidia consists of 2-4 cells	$13.82x10^6$
D	Ovoid (mikrokonidia), makrokonidia with a tapered tip	Microconidia of 4.75x2.5µm, Macroconidia of 31.16x3.2 µm	Macroconidia consists of 3-6 cells	$25.64x10^6$
E	Ovoid (mikrokonidia), makrokonidia with a tapered tip	Microconidia of 2.64x1.45µm, Macroconidia of 28.76x2.16 µm	Macroconidia consists of 2-6 cells	$36.25x10^6$

Notes: A: Isolate colony A, B: Isolate colony B, C: Isolate colony C, D: Isolate colony D, E: Isolate colony E

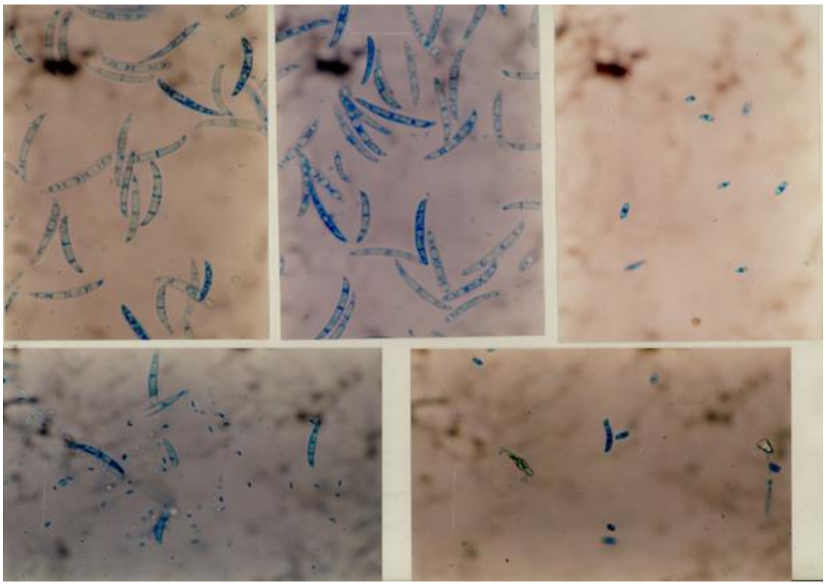

Figure 3. Microscopic appearance of *Fusarium* sp. isolates

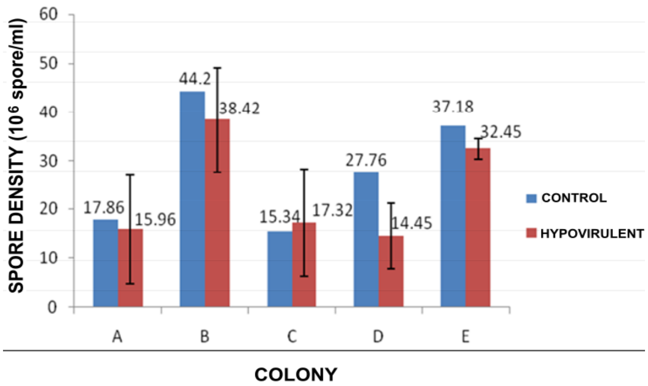

Figure 4. Spore density of *Fusarium* sp. isolates. Notes: A: Isolate colony A, B: Isolate colony B, C: Isolate colony C, D: Isolate colony D, E: Isolate colony E

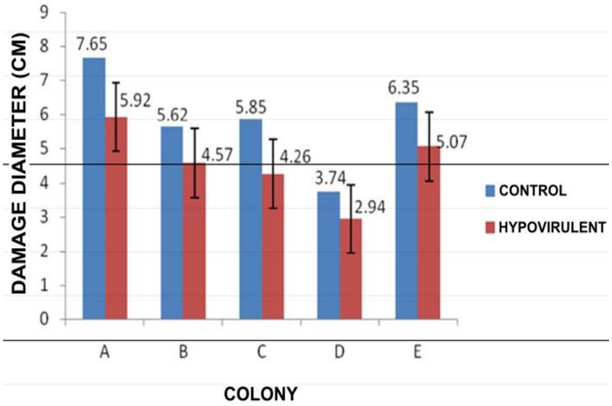

Figure 5. Diameter of apple damage on virulence test of *Fusarium* sp. Notes: A: Isolate colony A, B: Isolate colony B, C: Isolate colony C, D: Isolate colony D, E: Isolate colony E

After that, it was filtered and put into a prepared falcon. The suspension dilution was carried out three times. The results of this dilution were used to calculate the spore density of *Fusarium* sp. isolates. The highest spore density of control isolates was found in the isolate colony of group B at 72.38x10^6 spores/ml, while the lowest spore density was found in the isolate colony of group C, which was 15.34x10^6 spores/mL. On the other hand, in the treatment isolates, the highest spore density was found in the isolate colony of group B at 38.42x10^6 spores/mL. In contrast, the lowest density was found in the isolate colony of group D of 14.45x10^6 spores/mL (Tables 2 and 3).

Based on the results of observations of the spore density of *Fusarium* sp. isolates that have been obtained and identified, it can be seen that these isolates have spore density values that are almost the same. Specifically, the spore density in both control and treatment isolates has a spore density value ranging from 10^6 spores/mL.

Virulence test of *Fusarium* sp. isolates

The isolates that had been identified macroscopically and microscopically were then tested for virulence to determine the ability of *Fusarium* sp. isolates to cause both symptoms and damage to apples. The parameter observed was the diameter of apple damage caused by *Fusarium* sp. isolates which had been successfully characterized macroscopically and microscopically (Appendix 13). The highest damage diameter in the control isolate's virulence test was found in the isolate colony of group A at 7.65 cm. The lowest was found in the isolate colony of group D at 3.74 cm. Likewise, for the diameter of damage to the treatment isolates, the highest diameter of damage in the isolate colony of group A was 5.92 cm. The lowest damage diameter was found in isolate D at 2.94 cm (Figure 5).

The results of measuring the diameter of the damage caused by the *Fusarium* sp. isolate on the virulence test showed that the isolate colony of group A had the appearance of cream colony color, smooth colony surface, a little air mycelium formation, and hyaline mycelium appearance as well as fast colony growth rate with high colony diameter values, gave the most serious damage when tested for virulence on apples. In contrast, the isolate colony of group D had a slow colony growth rate with low diameter values. It appeared with pigmented colony colors (purple, pink), smooth colony surface, and medium air mycelium, which gave low damage during virulence tests on apples.

The virulence test of *Fusarium* sp. isolates was carried out to determine the ability of *Fusarium* sp. isolates isolated in artificial culture (PDA) to infect apples. Based on the observations, it was known that *Fusarium* sp. isolates in each colony had different levels of infecting ability. Boisson and Lahlou (1984) and Hadisutrisno (1987) stated that fungi treated with culture will lose their pathogenicity after being transferred several times in the medium or after the isolates have been stored for a long time. The exception is the hyaline variant, from which variant fungi can be obtained stable variants with weak or strong pathogenicity.

Based on the macroscopic, microscopic, and virulence testing of each colony group, it can be seen which isolates are virulent and which are hypovirulent. Isolates considered virulent were control isolates with high and fast growth rates and gave serious damage when virulence tests were carried out on apples. Meanwhile, isolates considered hypovirulent had a slow colony growth rate and gave low damage during the virulence test on apples. Hypovirulent isolates inoculated on apples showed minor disease symptoms; in contrast, control apples that had been inoculated with virulent isolates showed serious damage. According to Latterot (1982) cit. Hadisutrisno (2004), variants characterized by clear or hyaline mycelium with air mycelium, such as cotton, are called *une souche faible* or weak isolates, which are identified with avirulent isolates, while variants that have thin air mycelium are identified with strong isolates or virulent isolate. Avirulent isolates differed from virulent isolates in terms of morphology, the growth rate of macro-microconidium production, and protein content (Susanto, 2000). In addition, the mechanism that causes pathogens to turn non-pathogenic is caused by biochemical changes in these non-pathogenic strains, particularly reduced production of extracellular pectic lyase enzymes, decreased polygalacturonase activity, and deficient secretion of extracellular enzymes (Yamaguchi et al. 1992).

Related to biological control mechanisms based on antagonistic microbes, it can occur directly (competition and antibiosis) or indirectly with host-induced resistance. Induced resistance is resistance that develops after plants are inoculated early with biotic elicitors (avirulent, non-pathogenic, saprophytic microorganisms) and abiotic elicitors (salicylic acid, 2-chloroethyl phosphonic acid) (Agrios 1996). For example, hypovirulent fungi can be used in the biological control of virocontrol mechanisms with mycoviruses, which is by utilizing the viruses present in fungi that play a role in weakening the growth of the fungus so that the growth of the fungus is inhibited. Meanwhile, the control mechanism of induced resistance is resistance that develops after plants are inoculated early with biotic elicitors (avirulent, non-pathogenic, saprophytic microorganisms) and abiotic elicitors (salicylic acid, 2-chloroethyl phosphonic acid) (Agrios 1996). For example, suppose a certain part of the plant or fruit is inoculated with a pathogen or a weak pathogen. In that case, a reaction will occur in the plant body that produces a defense system so that the plant will be resistant to attacks by the same pathogen that is more virulent or even resistant to other pathogens. The occurrence of resistance induction due to local infection is considered to be caused by the presence of infected plant cell fluids that do not have sufficient or suitable food supplies or because the infected plants form toxic compounds that inhibit the development of pathogens (Caruso and Kuc 1979 cit. Hadisutrisno 2004).

Based on the research that has been done, it can be seen: (i) *Fusarium* sp. isolates on red chili plants from Boyolali that have been successfully isolated, obtained five groups of isolate colonies. (ii) The colony group of *Fusarium* sp. isolates that had a high virulence level was the isolate colony of group A, while the biological character of isolate A was cream-colored mycelium, long macroconidia shape; tapered tip, macroconidia with 2-6 septa, microconidia with 0-1 septa, conidia size of [(3.15 x 1.5) - (3.75 x 2.5)] µm x [(27.26 x 2.72) - (29.76 x 3.12)] µm, spore density between (15.94×10^6-17.86×10^6) spores/ml. (iii) The colony group of *Fusarium* sp. isolates which had low virulence was the isolate colony of Group D, while the biological character of isolate D was white mycelium like cotton, long macroconidia shape; tapered tip, macroconidia with 3-6 septa, microconidia with 0-1 septa, conidia size of [(4.75 x 2.5) - (5.35 x 3.15)] µm x [(31.16 x 3.2) - (27.36 x 4.10)] µm, the spore density was between (25.64×10^6-27.76×10^6) spores/ml. (iv) The isolate colony of group D had the potential as hypovirulent isolates that can be developed as environmentally friendly biological control agents, particularly by the induced resistance method.

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