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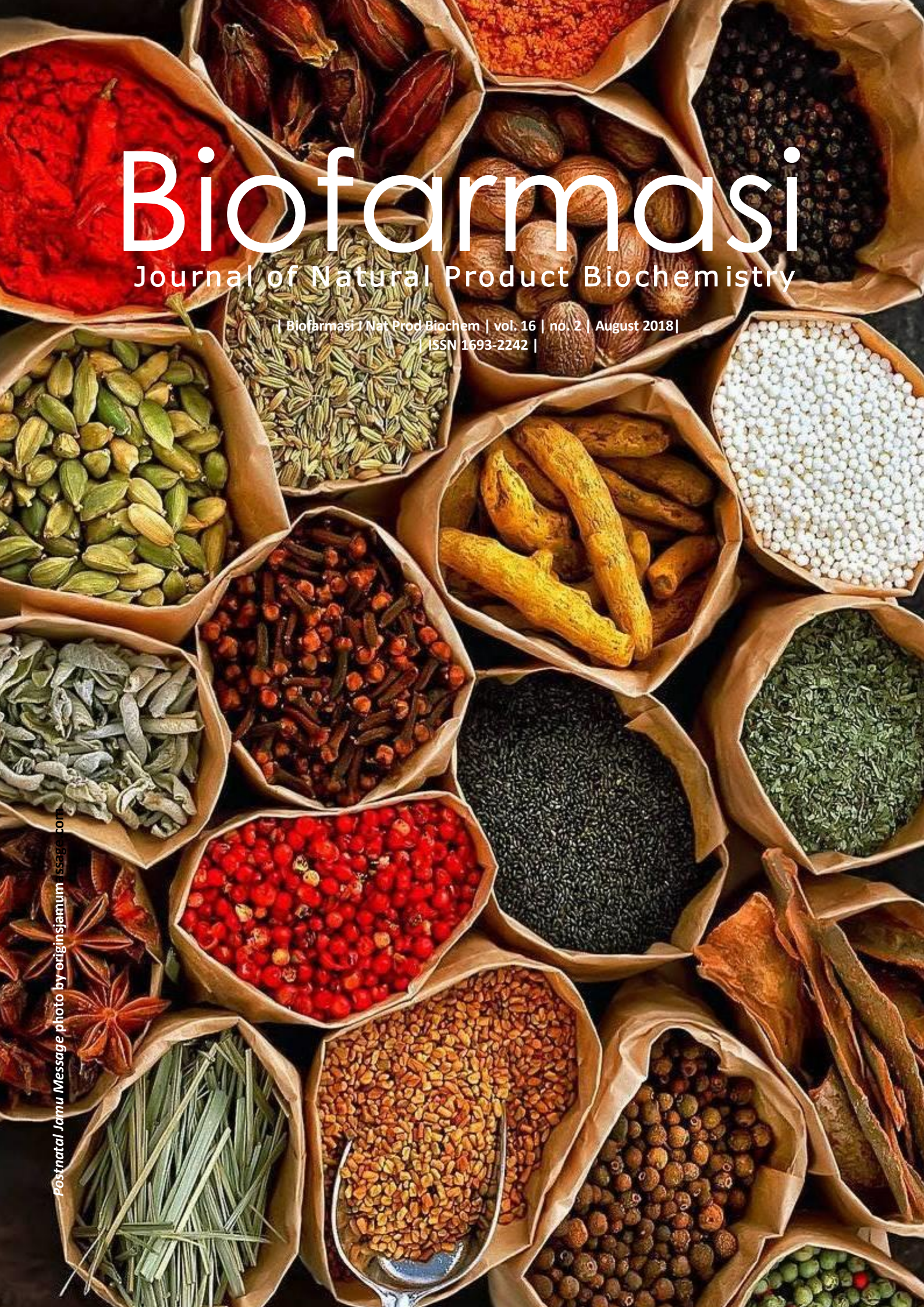

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Chapter in book:

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:

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

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The effect of jengkol (*Archidendron pauciflorum*) fruit peel ethanolic extract to heart histologic of rat induced by streptozotocin

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Department of Biology, Faculty of Mathemathics and Natural Sciences, Universitas Padjadjaran. Jl. Raya Bandung Sumedang km.21, Jatinangor, Sumedang 45363, West Java, Indonesia. Tel.: +62-228-4288828, ♥email: alamandaselma@gmail.com, ♥♥ desak_malini@yahoo.com

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Abstract. *Abadi SA, Illiyyin Z, Rachmadina JR, Malini DM. 2018. The effect of jengkol (*Archidendron pauciflorum*) fruit peel ethanolic extract to heart histologic of rat induced by streptozotocin. Biofarmasi J Nat Prod Biochem 16: 59-63.* Indonesia is ranked 7th out of 10 countries with the highest number of diabetic patients globally. Diabetes mellitus is a disease that can cause heart disorders, and adults who suffer from DM are four times more likely to develop heart disease. Jengkol fruit peel has been used traditionally as a drug for diabetes mellitus. The aim of this research was to know the effect of fruit peel ethanolic extract of jengkol (*Archidendron pauciflorum*) (JFPEE) on rat's (*Rattus norvegicus*) heart histological structure and to obtain an effective dose from JFPEE. This research used an experimental method in the laboratory with Completely Random Design (CRD) using 6 treatments and 4 replications. Treatment was given for 14 consecutive days consisting of negative control, positive control, comparison (glibenclamide dose 10 mg/kg BW), P1, P2, and P3 (JFPEE dose 385, 770, and 1.540 mg/kg BW). Diabetic induction was performed with a 65 mg/kg BW streptozotocin dose in female Wistar rats except for the negative control group. The observed parameters were several necroses and cell damage scores, including fat degeneration, hydropic degeneration, and inflammatory cell. The obtained data were analyzed by ANOVA-Tukey's test with a 95% confidence level using SPSS version 21 for Windows. The result of the histological structure showed that several necroses and cell damage scores in a group of rats treated with a JFPEE dose of 385 mg/kg BW (174.25±6.34; 1.25±0.50) were not significantly different from the negative control rats (172.00±7.62; 1.00±0.00). The effective dose of JFPEE that can repair the damage to heart cell's rat induced by streptozotocin was 385 mg/kg BW.

Keywords: *Archidendron pauciflorum*, heart, jengkol fruit peel, rat, streptozotocin

INTRODUCTION

The total population of Diabetes Mellitus (DM) patients in Indonesia is estimated to reach 8.2 million patients over the age of 20 by 2020. Indonesia is ranked 7th out of 10 countries with the highest number of people with diabetes globally (IDF 2015). Diabetes Mellitus is a chronic metabolic disease disorder because the pancreas cannot produce enough insulin or the body cannot effectively use insulin production, resulting in an increase in glucose concentration in the blood or hyperglycemia (Kemenkes 2014).

DM can affect all organs of the body and cause various complaints, one of which is heart trouble, such as coronary heart disease (CHD), congestive heart failure, and stroke (Fatimah 2015). According to the American Heart Association, in May 2012, less than 65% of people with diabetes died of heart disease or stroke. In addition, adults who suffer from DM are two to four times more likely to develop heart disease than people without diabetes.

One of the organs that has the most important function is the heart. The heart serves as a pumping device to circulate blood, either to the lungs or all other organs of the human body, because of the importance of this heart function, so if there is a disturbance or damage to this organ will result in disruption of all system performance in the body of mammals (Anggraeni et al. 2017).

Streptozotocin (STZ) is a chemical compound in the form of broad-spectrum antibiotics. It is a toxic compound for pancreatic beta cells that produce the hormone insulin because it can destroy its cells. The induction of STZ as a diabetic agent is very convenient and easy to use (Abeeleh et al., 2009). STZ is an N-nitrosourea and D-glucosamine derivative structurally isolated from *Streptomyces achromogenes* (Raza and John 2013).

A chemical drug that people with diabetes mellitus often use is type glibenclamide. According to Mulyanti (2010), diabetes Mellitus requires serious handling. The adverse effects of synthetic drugs used to treat DM are the main reason for searching for natural antihyperglycemic drugs. Currently, traditional medicine is often used by the community for self-medication. Before modern medicine was discovered and marketed, traditional medicine in Indonesia had been going on for thousands of years. Traditional medicine is widely used to treat chronic diseases such as diabetes mellitus (Pramono 2002). One of the plants that have been used traditionally in some areas in Indonesia as a drug for diabetes mellitus is jengkol peel (Syafnir et al. 2014).

Based on the results of research Rahayu and Pukan (2008) disclosed that the content of chemical compounds in jengkol peel are alkaloid, steroid/triterpenoid, saponin, flavonoid, and tannin, and the jengkol fruit peel also contains protein, vitamin A, vitamin B, phosphorus, and

calcium. Jengkol fruit peel is a good source of protein because it plays a role in the body's development and can repair damaged cells. Therefore, the peel of jengkol fruit (*A. pauciflorum*) is thought to have the potential to keep the heart organ healthy in people with diabetes, observed through damage to cells in the heart. In this research, the effect of ethanol extract of jengkol peel on histology of heart organ in rat/mouse (*Rattus norvegicus*) induced by streptozotocin was observed.

MATERIALS AND METHODS

Material preparation and diabetes induction

Production of ethanol extract of jengkol fruit peel was done by maceration method using ethanol 70%. The obtained maceration was then filtered and then concentrated with a rotary evaporator at a temperature of 40 ºC (Khan et al. 2012) to obtain an extract in the form of a paste.

The test animal was acclimatized in the Biology Department animal cage for seven days with a temperature of 22-30ºC. Test animals were given feed and drink with tap water by ad-libitum (IACUC 2015). Replacement of the chamber cage is done twice a week.

The test animal was checked for its glucose level and fasted for 12 hours. The test animals were then induced with streptozotocin dissolved in 0.1 M citrate buffer (pH 4.5) with a single dose of 65 mg/kg BW intravenously. After 72 hours of STZ induction, the blood glucose levels of test animals were examined. Rats used as test animals were rats possessing blood glucose >250 mg/dl on day 3 of the test (72 hours after STZ induction) (Furman 2015).

Ethanol extract of jengkol fruit peel is given orally every day for 14 days in a row according to the dose of each treatment. The extract was administered on day 4 after STZ injection and was considered to be the 1st day and lasted up to 14 days (Sajedianfard 2014).

Histological incision preparation

After administration the extract for 14 days, the rat dislocated the neck, dissected, and isolated the heart organ. The organ was washed with 0.9% NaCl to remove blood residue and dried with filter paper. The histological incision of the cardiac organ is made by isolating the cardiac organ fixed in the Bouin solution for 24 hours. Then the heart organ was cut transversely and washed in alcohol 70% for 24 hours. Afterward, the organ was dehydrated in a series of alcohol and 100% alcohol-based clarification: xylol. Then, the heart organ was infiltrated in xylol: paraffin and embedding in paraffin in the oven at 60-70ºC. Then the organ was cut using a microtome with a thickness of 5 microns at a temperature <24ºC.

Staining was done using Hematoxylin-Eosin (HE). The stages performed in this staining began with deparaffinization. Then rehydration process in a series of alcohol, which was then put into Hematoxylin solution for 25 minutes, and the histological incision was washed with tap water flowing. The histological incision was then introduced into a solution of Eosin for 10 seconds and dehydrated in a series of alcohols. It was then purified in a solution of xylol series and dried at room temperature, and covered with a sliding glass cover.

Histological sample observation

Histological incision of heart dyed in Hematoxylin-Eosin was then observed histological structure using a light microscope. The observation of necrosis was performed by looking at 1000 myocardial cells, which were then counted. Other changes were observed, such as infiltration of inflammatory cells, fat degeneration, and hydropic degeneration, were scored degrees of its severity using the method Karthikeyan et al. (2007), as follows: (0) no change; (1) mild (focal damage to myocytes or small multifocal degeneration with slight inflammatory processes); (2) moderate (broad myofibrillar degeneration and/or inflammatory of the difusa); (3) severe (widespread degeneration with inflammation of the difusa).

Data analysis

The histologic observation of rat heart (*Rattus novergicus*) Wistar was analyzed by One-Way ANOVA parametric statistic and continued with the Tukey test using SPSS for Windows version 21 (Sudjana 2012). In One-Way ANOVA, H_0 is accepted when $F_{count} < F_{table}$, and H_1 is accepted when $F_{count} > F_{table}$ at the real level ($\alpha = 0.05$). If H_1 is accepted, the statistical test continues with the Tukey test.

RESULTS AND DISCUSSION

Observation of cardiac histological structure

Observations of cardiac histological structures include the total necrotic cell, fat degeneration illustrations, hydropic degeneration, and inflammatory cell infiltration. The histological cross-section of the heart organ of the post-treatment 14-day is presented in Figure 1. The positive control induced by streptozotocin showed necrotic cells, hydropic degeneration, and inflammatory cell infiltration. The negative control did not occur without streptozotocin induction, with only necrotic cells and hydropic cell degeneration. Fat degeneration was observed in the treatment of P1 and P3. The average number of normal cells and necrosis cells is presented in Table 1, whereas the illustrations of fat degeneration, hydropic degeneration, and inflammatory cell infiltration are presented in Table 2.

Necrosis cell

The result of the ANOVA test for necrotic cell count shows that F_{count} (61,040)> F_{table} (2,77), which means that there is a significant difference in at least one treatment group showing that jengkol fruit peel extracts affected the histological structure of female Wistar rat (*R. novergicus*) heart organ. This also means that the administration of jengkol fruit skin extract significantly affected the number of female Wistar rat (*R. novergicus*) heart necrosis cells. The data analysis continued with the Tukey test to determine the most significant difference between all treatments.

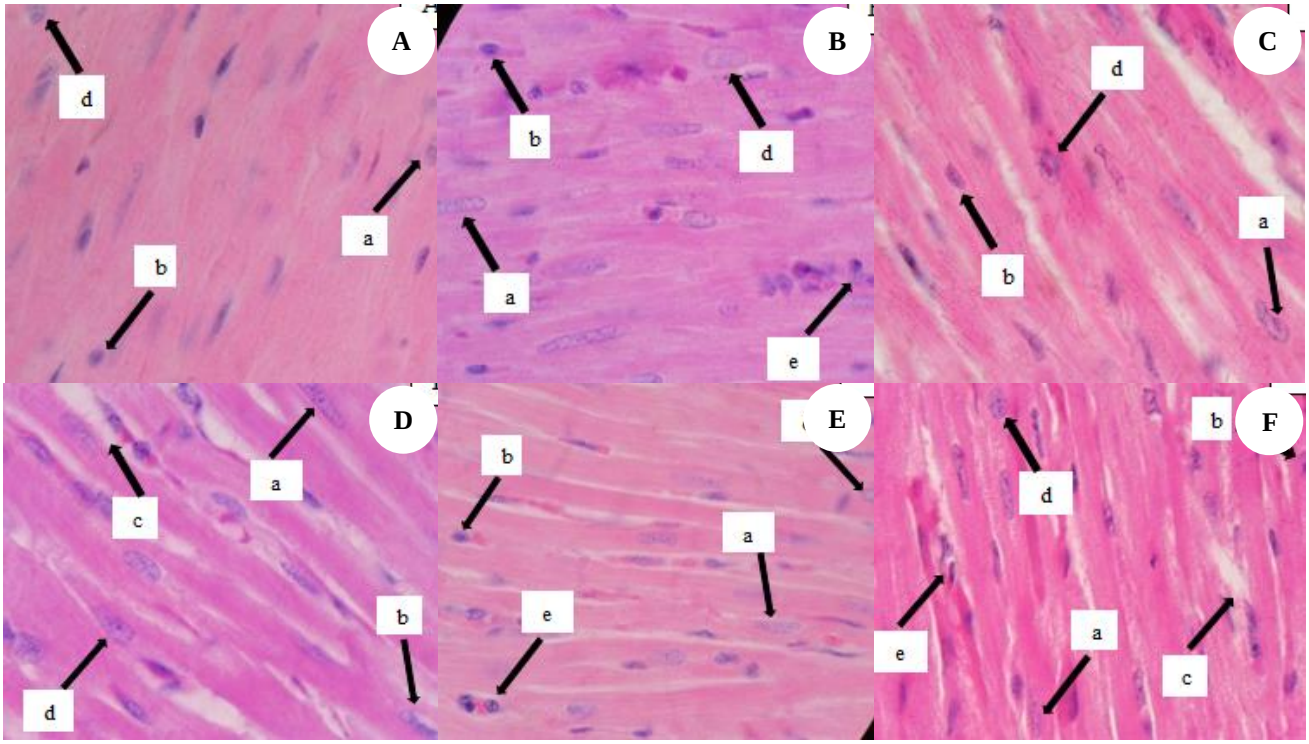

Figure 1. Histological cross section incision of rat heart. Note**:** (A) NC (CMC 0,5%); (B) PC (STZ 65 mg/kg BW + CMC 0,5%); (C) Pb (STZ 65 mg/kg BW + CMC 0,5% + Glibenklamide 10 mg/kg BW); (D) P1 (STZ 65 mg/kg BW + CMC 0,5% + Jengkol Fruit Peel Extract 385 mg/kg BW); (E) P2 (STZ 65 mg/kg BW + CMC 0,5% + Jengkol Fruit Peel Extract 770 mg/kg BW); (F) P3 (STZ 65 mg/kg BW + CMC 0,5% + Jengkol Fruit Peel Extract 1.540 mg/kg BW). [a] normal cell; [b] necrosis cell; [c] fat degradation; [d] hydrophic degradation; dan [e] inflammatory cell inflammation

Table 1. The average number of necrosis cells in rat heart myocardium post-treatment

Treatment group	Average of necrosis cell
Negative control	172.00±7.62^{a}
Positive control (STZ)	234.50±6.56^{b}
Pb (Glibenklamide)	185.50±6.76^{c}
P1 (385 mg/kg BW)	174.25±6.34^{ad}
P2 (770 mg/kg BW)	184.25±6.18^{cd}
P3 (1540 mg/kg BW)	222.75±7.04^{e}

Note: Data were analyzed using ANOVA and Tukey test with a 95% confidence level. Different letters in one column show a real difference ($p<0.05$).

Table 2. Average of scoring damage to mouse heart post-treatment myocardium

Treatment group	Average of scoring
Negative control	1.00±0.00^{a}
Positive control (STZ)	2.75±0.50^{c}
Pb (Glibenklamide)	1.25±0.50^{ab}
P1 (385 mg/kg BW)	1.25±0.50^{ab}
P2 (770 mg/kg BW)	1.00±0.00^{a}
P3 (1540 mg/kg BW)	2.00±0.00^{bc}

Note: Data were analyzed using ANOVA and Tukey test with a 95% confidence level. Different letters in one column show a real difference ($p<0.05$).

Results of cardiac histologic observations showed that streptozotocin-induced rats had higher numbers of necrotic cells (234,50 ± 6.56) than a streptozotocin-non-induced rats (172.00 ± 7.62). Normal cells in people with diabetes will experience a decrease in the amount caused by damage to cells. This is in accordance with the results of research conducted by Sari (2015) that the induction of streptozotocin causes heart organ permanent cell damage in the form of myocardium cell cytoplasm vacuolization.

Necrosis is the death of tissue cells due to injury when an individual is alive. Microscopically, the core changes are the loss of chromatin image, the core wrinkle, not vesicular anymore, the core appears denser, the color is dark black (pyknosis), the core is divided into fragments, torn (karyokinesis), and no longer to take much color because it is pale, not real (karyolysis) (Suhita et al. 2013).

Tukey test results showed that rats treated with jengkol fruit peel extract at each dose were significantly different from those given streptozotocin. This means that the administration of jengkol fruit peel extract can repair cell damage in the heart of diabetics by reducing the number of necrosis cells. In rats treated with jengkol fruit peel extract at a dose of 385 mg/kg BW, it does not differ significantly from the control group rats without streptozotocin-induced induction. This means that the extract of jengkol fruit peel at a dose of 385 mg/kg BW had a better effect on reducing the number of necrosis cells than the comparison

(glibenclamide drug) effect was close to the normal condition of the streptozotocin-non induced rat. The rat treated with jengkol fruit peel extract at a dose of 385 mg/kg BW had fewer necrotic cells (174.25 ± 6.34) than the glibenclamide-treated rat (185.50±6.76).

The peel of jengkol fruit contains tannin and flavonoids, which apparently prevent and repair cell damage by increasing the number of normal cells. Karodi et al. (2009) state that tannin performs wound healing activity by increasing the regeneration and organization of new tissues. In contrast, according to Harisaranraj et al. (2009), flavonoids are water-soluble antioxidants that clean up free radicals and prevent oxidative cell damage. They also have strong anti-cancer and anti-inflammatory activity. The results of microscopic histological observation show that the changes in rat heart happen due to the administration of streptozotocin, glibenclamide drug, or treatment given in three doses (385 mg/kg BW, 770 mg/kg BW, and 1540 mg/kg BW), which could be observed from the degeneration of fat, degeneration of hydropic, and inflammatory cell infiltration.

Scoring Damaged CellThe result of the ANOVA test for cell damage score showed that F_{count} (15,533)> F_{table} (2,77), which means there is a significant difference in at least one group of treatments that showed that fruit jengkol skin extract affected the histological structure of female rat (*R. novergicus*) Wistar heart. It also means that the administration of jengkol fruit peel extracts significantly affected the degree of mast cell damage in female rats (*R. novergicus*) Wistar heart. The data analysis was continued with the Tukey test to find out the most significant different treatments.

Tukey test results showed that mice treated with jengkol fruit peel extract at a dose of 385 mg/kg BW and 770 mg/kg BW did not differ significantly from the negative control group that was not induced by streptozotocin (Table 2). This means that the jengkol fruit peel extract of doses of 385 mg/kg BW and 770 mg/kg BW had a better effect on the reduction of the cell damage caused by fat degeneration, hydropic degeneration, and inflammatory cell infiltration when they were compared with comparison (glibenclamide drug) so that their effects were near the normal condition of rats with no streptozotocin induction. In rats treated with jengkol fruit peel extract at a dose of 1540 mg/kg BW was not significantly different from that of the positive control group induced by streptozotocin. This shows the extract of ethanol skin jengkol dose 1540 mg/kg bb had not been able to repair the damage to heart cells in people with diabetes mellitus.

Based on the observation, jengkol fruit peel extract is known to reduce necrosis in the cell. Fat degeneration is a metabolic disorder of cells that will cause cell damage and initiate the occurrence of necrosis. According to Aisyah et al. (2014), the damage to heart cells is characterized by the presence of vacuoles that accumulate on the walls. The vacuoles are fatty deposits, which are known as foam cells. If sedentary, the foam cell hardens and may clog the blood vessels, known as atherosclerosis. Fat degeneration is abnormal fat deposits in cells between connective tissue or degenerative changes leading to cellular necrosis. This occurs in diabetes mellitus, malnutrition, ischemic, and severe anemia conditions.

Discussion

Fat degeneration is an abnormal fat accumulation in the cytoplasm, vacuoles, and urgent nuclei to the edges. Fat degeneration describes the abnormal accumulation of triglycerides in parenchymal cells. The etiology of fatty degeneration is a toxin, protein malnutrition, diabetes mellitus, obesity, and anoxia. The consequences of changes in fat depending on the amount of fat deposits. If there are not too many fat deposits, the cell function is not disrupted, but if there are excess fat deposits, it will cause changes in fatty cells and cause necrosis (Suhita et al. 2013).

Cell cytoplasm vacuolization is a feature of hydropic degeneration, which is the accumulation of further water in the cells due to mitochondrial damage, the cessation of ATP production, and the sodium pump's failure, causing an increase in osmotic pressure in the cell. Severe hydropic degeneration results in necrosis of the cells (Salim and Balqis 2017). Hydropic degeneration occurs when a vacuole containing water in a cytoplasm containing no fat or glycogen is present; the cytoplasm becomes pale and swollen with fluid retention. This change is generally a result of metabolic disorders such as hypoxia or chemical toxicity. This change is reversible, although it can also be irreversible if the cause of the injury persists. If a rupture of the plasma membrane occurs and changes in the nucleus, the cell becomes irreversible and dies (Kasno 2005).

Hydrophic degeneration is characterized by cellular swelling, the presence of empty spaces (vacuoles), enlarged and docked cells. Hydropic degeneration is a reversible cell lesion with more severe intracellular accumulation when it is accompanied by albumin. Its etiology is similar to cell swelling, only the intensity of pathological stimuli is more severe, and the duration of exposure to pathologic stimulation is longer. Hydropic degeneration is common in epithelial cells (Suhita et al., 2013).

According to Braun and Anderson (2010), dead cells are chemically altered, and adjacent living tissues respond to the change and cause an inflammatory reaction. Cell inflammation is a vascular reaction that results in the delivery of fluids, dissolved substances, and cells from the blood circulation to the interstitial tissues of the necrosis region.

Inflammation or an inflammatory reaction is an important mechanism that the body needs to defend itself from various dangers that disrupt the balance and improve the structure and disruption of tissue function caused by the hazard. Inflammation is characterized by plasma protein fluid transfer and leukocytes from the blood circulation into the tissues in response to hazards. Inflammation can be characterized by redness, heat, swelling, pain, and disruption of body functions. Histopathologically, inflammation is characterized by the infiltration of inflammatory cells (Baratawidjaja 2002).

The results showed that the treatment using jengkol fruit peel extract (*A. pauciflorum*) could affect normal cell count, necrosis cell count, fat degeneration, hydropic

degeneration, and inflammation of inflammatory cells almost the same with a negative control without streptozotocin induction. Fruit ethanol extract of jengkol fruit has a good effect on each parameter at a dose of 770 mg/kg BW, increasing normal cell count, decreasing cell necrosis, repairing cell damage based on fat degeneration, hydropic degeneration, and inflammatory cell infiltration. This suggests that jengkol fruit peel extract (*A. pauciflorum*) has the potential to improve the histologic damage of rat (*R. novergicus*) heart induced by streptozotocin.

Biochemical compounds in the peel extract of jengkol fruit (*A. pauciflorum*) include tannins, flavonoids, alkaloids, quinones, steroids/triterpenoids, saponins, and polyphenols can reduce cellular necrosis and increase regeneration of new tissues in cardiac myocardium. These compounds also act as antioxidants that help repair tissue damage to the heart. Jengkol fruit peel also contains protein, vitamin A, B vitamins, phosphorus, and calcium that play a role in the body's development and can repair back damaged cells.

Based on the results of this study, it can be concluded that the provision of jengkol fruit peel extract (*A. pauciflorum*) can improve the damage to the histological structure of the heart, which could be observed from the number of necrosis cells and score damage picture caused by fat degeneration, hydropic degeneration, and inflammatory cell inflammation on Wistar rat (*R. norvegicus*) induced by streptozotocin. The dose of ethanol extract from the jengkol fruit peel (*A. pauciflorum*) effectively repairs cell damage in female rats (*R. norvegicus*). Wistar heart induced by streptozotocin was 385 mg/kg BW. However, a toxicity test of ethanol extract of jengkol fruit peel for 28 days is required to know the toxic level of jengkol fruit peel to other body organs to prevent other negative effects inflicted on people with diabetes mellitus. In addition, further research on the effect of the duration of jengkol fruit peel extract on the histological and morphological structure of Wistar female rats is required. The optimum duration of time in the extract of jengkol fruit required peel can be obtained.

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The hydroxyproline content of fish bone gelatin from Indonesian *Pangasius catfish* by enzymatic hydrolysis for producing the bioactive peptide

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Abstract. *Atma Y, Lioe HN, Prangdimurti E, Seftiono H, Tahufik M, Fitriani D, Mustopa AZ. 2018. The hydroxyproline content on fish bone gelatin from Indonesian Pangasius catfish by enzymatic hydrolysis for producing of bioactive peptide. Biofarmasi J Nat Prod Biochem 16: 64-68.* Gelatins have been widely used in the food, medicines, cosmetics, and photography industries. In the food industry, gelatin is used as a food additive and in functional foods. The applications of gelatin as a functional food are due to its bioactivity in the form of peptides. Bioactive peptides from gelatin are mostly obtained through enzymatic hydrolysis processes. This study was conducted to measure the hydroxyproline content of the gelatin bone of Indonesian Pangasius catfish (*Pangasius sutchi*) before and after enzymatic hydrolysis. Hydroxyproline is one of the dominant amino acids in gelatin. Gelatin hydrolysis was carried out using a flavourzyme at a concentration of 6% and the incubation series of 0, 4, 6, and 8 hours. The standard hydroxyproline concentration was used in the range of 0-1 µg. The results showed that the linear curve of the standard hydroxyproline solution was y=0.0554x+0.0406, with the coefficient of determination (R^2) = 0.9435. The incubation time of enzymatic hydrolysis (6% enzyme concentration) affected the hydroxyproline content. The hydroxyproline from fishbone gelatin was 18.91±2.87 mg/mL, 63.81±1.28 mg/mL, 46.21±1.28 mg/mL and 37.64 ± 0.64 mg/mL respectively during 0, 4, 6 dan 8 h incubation time. This hydroxyproline content was significantly different at a 95% confidence level from each treatment time.

Keywords: Bioactive peptide, enzymatic hydrolysis, fishbone gelatin, hydroxyproline

INTRODUCTION

Gelatin is a collagen hydrolysate with molecular weight ranging from 97 kDa to>250 kDa, produced in acid or alkaline conditions. It has been used in various industries, including food, medicines, cosmetics, and photographs (Mariod and Fadul 2013). In food industries, gelatin was used as food additives and functional food. As a food additive, gelatin was used as an emulsifier, stabilizer, gelling former, thickener, adhesive agent, and biofilm. While as functional food, gelatin has been developed in peptides for antidiabetic, antimicrobial, antioxidant, and antihypertensive (Gómez-Guillén et al. 2011).

The research regarding gelatin as a functional food has grown rapidly, especially about fish-based gelatin utilization (Aleman et al., 2011; Koli et al., 2014; Nikoo et al., 2014). Fish-based gelatin could potentially replace mammalian gelatin, which is the most source of gelatin; however, it is unacceptable due to religious, socio-cultural, and health considerations (Nurul and Sarbon 2015). Many previous studies have reported that the fish gelatins have their bioactivities, for example, the gelatin from salmon, hake, halibut, nila tilapia, pangasius catfish, etc. (Li-Chan et al. 2012; Mahmoodani et al. 2014a; Wang et al. 2015). Fortunately, most fish-based gelatin comes from by-products of fish processing waste (Karayannakidis and Zotos 2016). Therefore, the utilization of this source could be promising in the future, especially in Indonesia.

Indonesia is a country with a wide water area, so it provides biodiversity in fisheries. There are two types of fish based on their habitat: warm-water fish and cold-water fish. In cases of gelatin sources, it has been known that the gelatin from warm-water fish has superior characteristics compared to the other one (Gómez-Guillén et al. 2009). One warm-water fish with a high production yield in Indonesia is the Pangasius catfish (*Pangasius sutchi*). In Indonesia, Pangasius catfish spread out in Sumatera and Kalimantan. In addition, the consumption and production rate of this fish has increased every year. The Ministry of Marine Affairs and Fisheries targeted the Pangasius catfish production in 2018, reaching 604.587 tons. It will inflict waste, especially fishbone, contributing about 12.44% of the total fish weight. Previous studies concluded that gelatin from the bone of Pangasius catfish was better than other fish-based on gelatin in physicochemical characteristics. The gelatin from this source was also comparable with commercial bovine gelatin (Mahmoodani et al. 2014b).

The aim of this research was to analyze the hydroxyproline of gelatin from Indonesian Pangasius catfish (*Pangasius sutchi)* before and after hydrolysis enzymatic. Most bioactive peptide extraction from gelatin resulted from gelatin hydrolyzation followed by hydroxyproline content measurement (Li-Chan et al. 2012; Mahmoodani et al. 2014a; Nikoo et al. 2014). The hydrolysis of gelatin is mostly and more efficient using protease. Determining this hydroxyproline content could become a basic knowledge to know and characterize the influence of hydrolysis toward hydroxyproline of fishbone gelatin from Pangasius catfish. According to the bioactive peptide production, it could also become a comparable study in the hydroxyproline content of fish-based gelatin.

MATERIALS AND METHODS

Study area

This experimental research was conducted in four stages, including the gelatin extraction of *Pangasius catfish* bone, fishbone gelatin hydrolysis, the measurement of the hydroxyproline content, and data analysis. The fish bones used for gelatin extraction were *Pangasius sutchi* from Indonesian rivers in Riau province.

Procedures

Gelatin extraction

The gelatin extraction was done in two steps, i.e., pre-treatment and main extraction. The pre-treatment of fish bones was done by soaking them with a mild acid (citric acid) for 48 h. After the pre-treatment steps, the leached bone (ossein) was separated with pre-treatment solvent using a centrifuge (Hettich, USA) at 4000 rpm for 15 minutes, followed by main extraction steps using hot water (75°C) for 5 h. Afterward, the main extraction solvent was separated with liquid extract using filter paper (Whatman Grade 4, USA). Finally, this liquid extract, namely liquid extract of fishbone gelatin, was collected in a tube and stored at 4°C until further hydrolysis and analysis.

Gelatin hydrolysis

Fishbone gelatin obtained from gelatin extraction stages was firstly incubated at 50°C for 10 minutes. Subsequently, the flavourzyme (Sigma, Germany) was added with enzyme/substrate (E/S) with a ratio of 6%. Afterward, this solution contained gelatin and flavourzyme (6%) and was incubated for 4, 6, and 8 h. The enzymatic hydrolysis process was stopped using hot water (100°C) for 10 minutes, then soaked in cool water for 20 minutes. In the last step, the hydrolysis gelatin was obtained by separation using a centrifuge (Hitachi, Japan) at 1000 rpm for 15 min at 4°C. The supernatant, called fishbone gelatin, was stored at-18°C for measurement analysis of their hydroxyproline content.

Hydroxyproline analysis

Hydroxyproline analysis of fishbone gelatin was carried out as follows: preparation sample, reagent addition, and absorbance measurement, as described by Koli et al. (2014). The preparation sample of gelatin was done by adding 12 N hydrogen chloride into the sample, followed by incubation at 100ºC for 3 h. Afterward, the gelatin was filtered through filter paper. Whatman no. 4. The filtrate gelatin was then added to a chloramine reagent containing 1.4% chloramine T (Biovision, USA) and oxidant buffer (Biovision, USA) ratio of 1: 10. After the incubation for 5 min at room temperature, the sample of gelatin was added *p-dimethylaminobenzaldehyde* (DMAB) reagent containing 10% of DMAB concentration (Biovision, USA) and 60% of perchloric acid (Biovision, USA) in ratio 1: 1 (v/v). This solution was then incubated at 60°C for 90 minutes and continued with cooling for 2-3 minutes. At last, the solution was measured for its absorbance using a spectrophotometer (Thermo Multiskan, USA) at a wavelength of 540 nm. Hydroxyproline e kit (Biovision, USA) was used to measure the hydroxyproline content in fishbone gelatin before and after enzymatic hydrolysis. The concentration of hydroxyproline used in this analysis was 0-1 µg/well. The hydroxyproline quantification was done after the linear curve of the standard hydroxyproline solution was obtained.

Data analysis

Data analysis was performed using one-way Analysis of Variant (ANOVA) at level 5% and continued with Tukey's HSD (Honestly Significant Different) test or Tukey's range test to determine the statistical analysis at the level of significant differences between data.

RESULTS AND DISCUSSION

Fishbone gelatin hydrolysis

There are some stages for the production of the bioactive peptide from the gelatin, including (i) gelatin extraction, (ii) gelatin hydrolysis, (iii) the hydrolysate filtration by ultrafiltration, (iv) gelatin purification, and (v) analysis of the sequence after purification (Zhang et al. 2012; Li-Chan et al. 2012). The bioactivities of gelatin in each stage must be measured. In this research, a part of gelatin extraction and gelatin hydrolysis using the bone of Pangasius catfish (*Pangasius sutchi*) as a source of gelatin (without bioactivities analysis) was obtained. The most alternative source of gelatin came from fish processing products, especially skin and bone. Previous studies showed that the gelatin from the bone of Pangasius catfish had a better gelatin yield and ash composition than the commercial gelatin (Mahmoodani et al. 2014b). In addition, this source was also abundant in Indonesia. Figure 1 presents three series of stages to obtain the fishbone gelatin after hydrolysis.

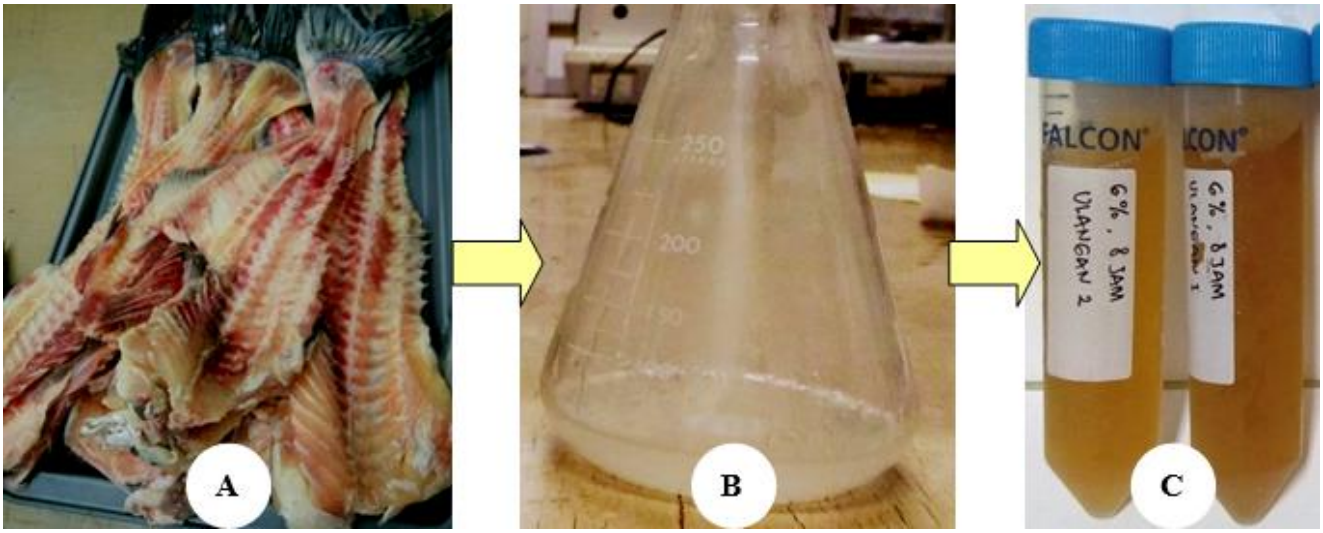

Figure 1. The fishbone (A), gelatin before (B) and after hydrolysis (C) derived from Pangasius catfish

Hydrolysis is one of the key parts of producing bioactive peptides. The successful process of the hydrolysis would determine the activities of peptides. Most of the gelatin from fish processing products was hydrolyzed by the enzymatic method. Some enzymes have been used for the hydrolysis of gelatin, such as alcalase, flavourzyme, pepsin, trypsin, chymotrypsin, bromelain, and papain (Choonpicharn et al., 2014; Himaya et al., 2012; Zhang et al. 2012; Li-Chan et al. 2012; Wang et al. 2015). The research of Li-Chan et al. (2012) concluded that the gelatin from fish processing products was the best in their bioactivities after hydrolyzed using flavourzyme. Some fish-based gelatin hydrolyzed using flavourzyme to obtain the bioactive peptides was gelatin from the skin of tuna, nila tilapia, and salmon (Atma 2016).

Hydroxyproline content

Hydroxyproline is one dominant amino acid in gelatin besides glycine, proline, alanine, and glutamic acid (Atma 2017). The hydroxyproline was analyzed to determine the extraction yield, representing the successful process of the extraction in gelatin production (Mahmoodani et al. 2014b; Sanei et al. 2013). Recently, most research regarding gelatin extraction from fish skin, bone, head, or other parts usually quantified their hydroxyproline content. This amino acid is unique and has become a differentiator between gelatin and other proteins (Taheri et al., 2009; Sanei et al., 2013). This study analyzed the hydroxyproline content of fishbone gelatin from Pangasius catfish before and after enzymatically hydrolysis. The quantification of hydroxyproline was done to determine the influence of enzymatic hydrolysis of fishbone gelatin toward hydroxyproline concentration.

In quantifying hydroxyproline, a series of standard hydroxyproline solutions were set up. It is to quantify the linear curve for determining the relation between hydroxyproline absorbance and hydroxyproline concentration. In this research, the standard hydroxyproline concentration was in the range of 0-1 µg, and the linear curve of the standard hydroxyproline solution was y=0.0554x+0.0406 with the coefficient of determination (R^2) = 0.9435. Figure 2 presents the standard curve of hydroxyproline in a concentration ranging from 0-1 µg.

Based on the linear equation of the hydroxyproline standard curve, the hydroxyproline content in sample gelatin was quantified. The y value is the absorbance at 560 nm wavelengths, while the x is the quantified hydroxyproline concentration. Table 1 presents the hydroxyproline content of fishbone gelatin from Pangasius catfish at different incubation times during enzymatic hydrolysis. The quantification was done on twice repetitions with the average of hydroxyproline of gelatin from the fishbone of Pangasius catfish was from 18.91±2.87 mg/mL to 63.81±1.28 mg/mL. The hydroxyproline content of gelatin before the hydrolysis process was lower than after the hydrolysis process. In this research, the fishbone gelatin was hydrolyzed using 6% flavourzyme and incubated for 4 h and had a higher hydroxyproline content than other fishbone gelatin.

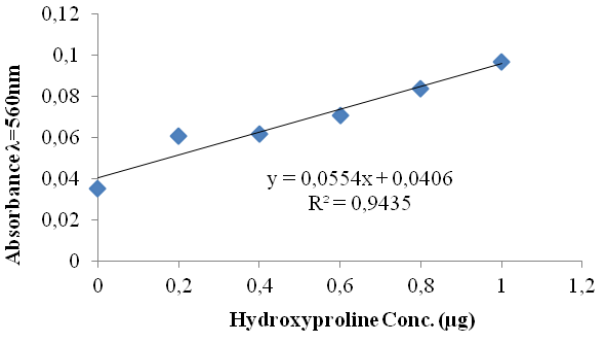

Figure 2. The standard curve for absorbance at wavelength 560 nm of hydroxyproline in the concentration range of 0-1 µg

Table 1. The hydroxyproline content on fishbone gelatin from Pangasius catfish (*Pangasius sutchi*) at different times of incubation during enzymatic hydrolysis

Hydrolysis incubation time	Hydroxyproline content (mg/mL)		
	1	2	Average
0 h (before hydrolysis)	16.88	20.94	18.91±2.87 a
4 h	62.91	64.71	63.81±1.28 b
6 h	45.31	47.11	46.21±1.28 c
8 h	37.18	38.09	37.64±0.64 d

Note: Average are mean ± standard deviation from twice analysis. Value of average in row bearing different letters are significantly different at $p < 0.05$

Discussion

Hydroxyproline is an amino acid contained in fishbone gelatin, around 5.3-9.6% (Atma 2017). The study conducted by Mahmoodani et al. (2014b) stated that fishbone gelatin from Pangasius catfish contained hydroxyproline 5.97 g/100 g. It means around 5970 mg/mL hydroxyproline was obtained from the fishbone gelatin of Pangasius catfish. In this study, Pangasius catfish's gelatin contained about 18.91 mg/mL of hydroxyproline. However, the extraction method in the research conducted by Mahmoodani et al. (2014b) used hydrochloric acid (strong acid) in the pre-treatment, while this study used citric acid in the pre-treatment. The extraction method of gelatin affected the yield of hydroxyproline. Therefore, the hydroxyproline content in the previous study was higher. Furthermore, Mahmoodani et al. (2014b) optimized the gelatin extraction by response surface methodology, whereas no optimization is done in this study. The optimized condition for gelatin extraction from Pangasius catfish bone is obtained by pre-treatment using hydrochloric acid. However, this chemical utilization for extraction has been limited because of safety and environmental issues consideration.

In this study, the hydroxyproline content increased after the hydrolysis process. The higher hydroxyproline concentration was on fishbone gelatin incubated for 4 h (63.81 mg/mL). The hydrolysis of a polypeptide chain probably caused the increase in hydroxyproline content in gelatin, so it increased the detectable amount of hydroxyproline. The hydroxyproline content decreased if the incubation time was longer (6 and 8 h). It might cause by the denaturation or another factor that influences the hydroxyproline in the gelatin solution for being detected. The hydroxyproline content of fishbone gelatin during various incubation times was significantly different ($p < 0.05$) in each treatment (Figure 3).

Overall, the hydroxyproline concentration in this research is different from other studies. Li-Chan et al. (2012) reported that the hydrolysis of gelatin derived from Atlantic Salmon skin using flavourzyme found that the gelatin contained hydroxyproline with a concentration around 88.24 mg/mL. Furthermore, Li Chan et al. (2012) also used an enzyme with the concentration of 6% enzyme-substrate [E/S] in order to obtain the gelatin hydrolysate with the inhibitory activity toward dipeptidyl peptidase IV (DPP-IV) as an antidiabetic treatment approach. Previously, Benjakul et al. (2009) also measured the hydroxyproline of gelatin from two species of bigeye snapper, i.e., *Priacanthus tayenus* and *Priacanthus macracanthus*, with the result of the hydroxyproline content in each species were 87.75 mg/mL and 90.86 mg/mL, respectively. This has been done to characterize the gelatin from the fish skin of the bigeye snapper. Another study conducted by Sun et al. (2012) was conducted to analyze the antiphotoaging, and antioxidant activity of gelatin from Tilapia (*Oreochromis niloticus*) using in vivo method,. The hydroxyproline was quantified after the gelatin was fed to mice. Furthermore, the hydroxyproline in mice after feeding was 50 mg/kg, 100 mg/kg and 200 mg/kg fish gelatin were 0.018 mg/mL, 0.020 mg/mL and 0.022 mg/mL respectively (Sun et al. 2012). The hydrolysis of fish skin gelatin in the animal's intestine occurs due to the protease that contains in the digestive tract (Tabata et al., 2017).

The hydroxyproline content of fish bone gelatin was affected by enzymatic hydrolysis. The enzymatic hydrolysis increased the hydroxyproline content. The hydrolysis with flavourzyme (6% enzyme/substrate concentration) was better during 4 h incubation than 6 and 8 h. Nevertheless, their bioactivities are the most important in hydrolysis gelatin for producing the bioactive peptide.

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Evaluation of antimicrobial and antioxidant attributes of Tanzanian honey from two agro-ecological areas

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Abstract. *Luvanda FT, Lyimo ME. 2018. Evaluation of antimicrobial and antioxidant attributes of Tanzanian honey from two agroecological areas. Biofarmasi J Nat Prod Biochem 16: 69-82.* In order to appraise the characteristic of Tanzanian honey based on antioxidant attributes and antimicrobial activity on bacteria and fungi about its physicochemical attributes, the study was conducted. The examined honey was sampled from two kinds of bees: stinging and stingless honey bees. It was gathered from the western area (Tabora and Shinyanga) and the central area (Singida and Dodoma). They were examined for their antimicrobial activity, anti-oxidant attributes (total phenol, vitamin C), and physicochemical attributes of pH, color, pH, sugar, and minerals. 54 % of tested honey samples inhibited microbial growth, but the remaining 46% did not inhibit the growth. Microbial impediment areas range from 8.5 to 14.16 mm (stinging honey bee) and 10.56 to 15.13 mm (stingless honey bee). Honey yielded by stinging honey bees Shinyanga, Bukombe, and Nzega and the cities of Singida and Issuna are more effective in impeding microbial growth than that other cities. *Candida albicans* is more sensitive (23.1%) to honey than stingless honey bees, followed by *Staphylococcus saprophyticus* (33%), *Salmonella typhi* (32%), *Escherichia coli* (19.2%), and *Aspergillus flavus* (16.0%). The substance of antioxidant, total phenol, and vitamin C in the honey of stingless honey bees were importantly ($P < 0.05$) higher than that of honey of stinging honey bees. Antioxidants (FRAP) ranged from 322.16-973.57 µMFe (II) /100g, total phenols were from 13.87-33.55 mg /100g, and vitamin C was from 2.54-10.99 mg /100. There was no substantial dissimilarity ($P > 0.05$) between the two honey samples at pH value. Potassium possesses the highest value, while Zn possesses the lowest value in honey samples of stinging and stingless honey bees. The gradation color of honey of stingless honey bees ranged from extra light amber-light amber, while that of honey of stinging bees ranged from white water-light amber. There was a positive and substantial correlation ($P < 0.001$) between the color of honey, antioxidants, total phenol, and mineral substance. Stingless honey bees' honey from the western region is superior to stinging honey bees' honey in terms of antioxidant and antimicrobial attributes. Therefore, it is prompted to be utilized as a valuable diet product, preventive and curative medicine.

Keywords: Agro-ecological areas, antimicrobial, antioxidant, honey, Tanzanian

INTRODUCTION

Honey is an organic natural sweet substance yielded from nectar and saccharine exudation of plants by honey bees recognized as *Apis mellifera* and *Trigona meliponini* (Codex Standard 2001). The nectar of flowers is stockpiled, modified, stashed in the comb, and altered by honey bees into honey by regurgitation and evaporation (Stefan 2009).

Honey is a natural product with several characteristics that are advantageous for human beings. It is believed to be significant in human nutrition and health (Alisi et al. 2012). For thousands of years (since 2100 BC), archaic Greeks utilized honey as a traditional diet and healing agent (Alisi et al. 2012). Its attributes cause it to be capable of acting as a natural diet with antioxidants and antimicrobial, which are conscientious of its medicinal attributes. It possesses robust immune procedure stimulus and carbohydrates that supply power and energy to the body. The availability of enzymes in honey succors increases the digestive procedure and reduces muscle lethargy in the body (Rodriguez 2004).

Dissimilar kinds of honey possess dissimilar mixtures depending on the kind of honey bees, geographical origin, and maturity attribute of honey before reaping (Alvarez et al. 2010). Honey is composed of carbohydrates, enzymes hydroxymethylfurfural (HMF), proteins, minerals, amino acids, vitamins, and antioxidants such as phenolics and flavonoids (Candiracci et al., 2012). Its health advantage is related to the availability of these precious nutrients and phytochemicals.

The most honey-yielding and consuming nations globally are China, Turkey, Argentina, and Mexico (FAOSTAT 2014). Tanzania possesses the capability of yielding 138,000 metric tons of honey every year. Almost all areas participate in beekeeping and honey production. The primary beekeeping and honey production areas are the Western area (Tabora, Shinyanga, Rukwa, and Kigoma), Southern Area (Lindi, Mbeya, Iringa, and Ruvuma), Central Area (Singida and Dodoma), Eastern Area (Morogoro, Dar es Salaam and Coast) and North Eastern Area (Manyara, Arusha, Tanga, and Kilimanjaro). In Tanzania, honey is yielded from dissimilar provenances involving plants (perennial plants, fruits, sunflower, palm oil, rice, maize, sorghum), soil, and dissimilar geographical origins such as arid, wet, or coast (URT 2003). Tanzania possesses a tropical climate with regional diversity because of the topography (nation's landscape). The temperature is high in the primary land and tropical in the coastal areas

and the islands. The excellent climate diversity in Tanzania results in diversity in Tanzania's flora and fauna.

Currently, the consumption of honey is rising due to its advantageous biological and physical-chemical attributes, involving antioxidant and antibacterial activities. More than half of the honey yielded in the nation is consumed locally for diet and medicine. At the same time, the rest is sold to other nations, particularly European Union member nations such as the UK, Netherlands, and Germany. Other nations are Oman, United Arab Emirates, Iran, Uganda, Kenya, and Rwanda (Mwakatobe and Mlingwa 2004).

The objectives of this research were: (i) To specify the antimicrobial activities of bacteria (*Staphylococcus saprophyticus*, *Escherichia coli*, and *Salmonella typhi*) and fungi (*Aspergillus flavus* and *Candida albicans*) the on the raw honey from stinging and stingless honey bees. (ii) To evaluate raw honey's physicochemical attributes (pH, decreasing sugar and minerals) from stinging and stingless honey bees. (iii) To investigate the raw honey's antioxidant activities (total phenol and vitamin C) in stinging and stingless honey bees. (iv) To correlate the physicochemical attributes of honey to the antioxidant and antimicrobial attributes of raw Tanzanian honey from stinging and stingless honey bees.

MATERIAL AND METHODS

Materials

Two kinds of honey, i.e., raw honey of stinging and stingless honey bees, were gathered from beekeepers in two selected agro-ecological areas of Tanzania; the Western area, including Tabora (Inyonga, Tabora town, and Nzega) and Shinyanga (Kahama, Bukombe, and Shinyanga town), Central area including Singida (Issuna, Manyoni and Singida town) and Dodoma (Kibaigwa, Bahi, and Dodoma town). The central area of Tanzania is mostly savanna, bushland, and thickets, whereas the western area is embodied mostly by miombo woodland trees.

The bacteria (*S. saprophyticus, E. coli, S. typhi*) and fungi (*A. flavus* and *C. albicans*) to participate in the test organism were achieved from Veterinary Microbiology Laboratory, Sokoine University of Agriculture (SUA), Morogoro.

Methods

Sampling procedure

A purposive sampling method was utilized to pick out areas, followed by a simple random sampling method to gather honey samples from beekeepers in two selected areas (Western and Central) of Tanzania. Two kinds of honey were gathered in triplicate from each sampling area (3 replications). Thus, 3 replicates per honey kind x 2 honey bee kinds x 3 sampling areas x 2 regions in each area = 36 honey samples from each area, making a total of 72 honey samples gathered from the two areas. The honey samples were packaged in sterilized plastic bottles (330 mL), transported at ambient temperature (25±2ºC), and stashed at the Laboratory of Food Technology, Nutrition, and Consumer Sciences, SUA, Morogoro.

Research design

The study utilized a completely randomized block design in a factorial experiment with 3 replications. The principal factors were located at two levels (two areas) and bee kind at two levels (honey of sting honeybees and stingless honey bees). The effect of these factors on pH, decreasing sugar, color, antioxidant activity, antimicrobial activity, vitamin C, minerals, and total phenol was considered. The following model was assumed.

$$Y_{ijkl} = \mu + Z_i + R_j + A_k + T_l + (Z*R*A*T)_{ijkl} + e_{ijkl,} \ldots\ldots (1)$$

Where Y_{ijkl} = Dependent variable, μ=General mean, Z_i= the effect of i^{th} agro-ecological area, R_j= Effect of j^{th} sampling region A_k= Effect of k^{th} sampling area T_l=The effect of an l^{th} honey kind, $(Z*R*A*T)_{ijkl}$= Interaction effect between i^{th} area j^{th} region k^{th} area and l^{th} honey kind e_{ijkl}= Error term or Random term.

Antimicrobial sensitivity test

SpecifiedAgar Wells Diffusion technique, as described by Clinical Laboratory Typical Institute (2009), was utilized to consider the antimicrobial sensitivity test of the honey samples. To test antimicrobial susceptibility, Mueller-Hinton Agar medium was utilized, and following the manufacturer's instructions, the medium was arranged. After autoclaving (121ºC for 15 min.), the medium was left to cool to 50ºC. Then 25 mL per plate (15x100mm) was quantified on a level pouring surface to a uniform depth of 4 mm and hatched in an incubator (35 ±2ºC) for 24 hours.

The test organisms comprised of bacteria (*Staphylococcus saprophyticus, E. coli, S. typhi*) and fungi (*A. flavus* and *C. albicans*), and they were streaked onto a non-inhibitory agar medium (broth agar) to gain isolated colonies. After hatching at 35ºC overnight, 4 to 5 colonies were selected, injected into the broth (Mueller-Hinton broth), and hatched at 35ºC for 24 hours. A sterile cotton swab was dipped into the suspension, pressed firmly against the inside wall of the tube just above the fluid level, then streaked over the entire surface of the medium, rotating the plate approximately 60 degrees after each administration to ensure an equal location of the inoculum, finally swabbed all around the edge of the agar surface. Small holes of 5 mm were created on the Petri dishes with agar using glass pastor and sterile loops, then 100 µL of the honey sample was situated in the agar holes using a sterile micropipette. The plates were inverted and hatched at 37 ±1ºC for 24 hours for tested bacteria and 48 hours for yeast. After the hatching period, the diameter of the areas of complete impediment (involving the disk's diameter) was quantified and recorded in millimeters. The quantifications were performed with a ruler on the undersurface of the plate without opening the lids.

Chemical analyses

Antioxidant activity

By using the Ferric reducing ability of plasma (FRAP) assessment following the method described by Benzie and Strain (1996), antioxidant activity in honey samples was specified. About 0.1 g of honey was liquefied with refined

water and made up to 10 mL. Then 200 µL of liquefied honey (0.1 g/mL) was blended with 1.5 mL of FRAP reagent. The reaction mixture was then hatched at 37°C for 4 minutes, and its absorbance was quantified spectrophotometrically at 593 nm against a blank arranged with refined water. Fresh FRAP reagent was arranged by mixing 10 volumes of 300 mM/l acetate buffer (pH 3.6) with 1 volume of 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM/l HCl, containing 1 volume of 20 mM ferric chloride ($FeCl_3.6H_2O$). Trolox (vitamin E analog) was used for the calibration curve with serial dilutions of 0, 25, 50, 100, 150, 200, 250, and 300 µM/mL.

The typical curve was plotted, and the unknowns were computed using the linear regression equation of the typical plot. The ferric reducing ability of the honey sample was described as FRAP value (µM of Fe^{II}) per 100g of honey as per the following formula:

$$\text{FRAP value}\left(\frac{\mu M\ Fe(II)}{100g}\right) = \frac{\text{Change in absorbance of sample}}{\text{Change in absorbance of standard}} \times \text{FRAP value of typical}$$

Vitamin C determination

According to the AOAC (2007) procedure using method number 967.21, vitamin C (Ascorbic acid) was specified based on the oxidation-reduction reaction principle. Using 10 mL of 10% Trichloroacetic acid (TCA), about 5g of the homogenized honey sample was weighed and extracted, and the extract was gathered into a volumetric flask. Then, using Whatman sieve paper No.1, the liquefied sample extract was sifted. Next,10 mL of the clear filtrate was pipetted into a 250 mL Erlenmeyer flask. A blank solution was arranged using 10 mL of 10% TCA solution. The burette was filled with a typical indophenol solution. Slowly, the flask contents were titrated with a typical indophenol solution until the faint pink color was achieved and existed for 10 seconds. The volume of indophenol solution utilized to oxidize the ascorbic acid present in the sample extract and the blank solution was recorded. Then vitamin C substance in the honey sample extract was computed using the following formula:

$$\text{Vitamin C substance of the sample (mg/100g)} = \frac{(A-B)\ x\ C\ x\ V\ x\ 100}{D\ x\ S}$$

Where;

A = Volume in mL of the indophenol solution utilized for the sample

B = Volume in mL of the indophenol solution utilized for blank

C = Mass in mg of ascorbic acid equivalent to 1.0 mL indophenols solution

S = Mass of sample in (g) taken for analysis

V = Total volume of extract in milliliters

D = Volume of sample filtrate in milliliters taken for analysis

Total phenolic compound

Using the Folin-Ciocalteu reagent (FCR) described by Singleton et al. (1999), the total phenol of the honey samples was specified. Each sample was analyzed using the spectrophotometer Wagtech CECIL 2021, and a 20µL sample was added to 100 µL FCR (liquefied 1: 10 with refined water), blended hatched at 37ºC for 60 seconds before the addition of 80µL 7.5% (w/v) sodium bicarbonate solution. The samples were blended again and hatched at 37ºC for 15 minutes prior to absorbance reading at 760 nm. Total phenol was computed against a calibrated typical curve of gallic acid, and the results were expressed as mg gallic acid equivalents (GAE) per 100 sample weight (SW).

$$\text{Total phenol}\left(\frac{mg}{100g}\right) = \frac{\text{Reading value in ppm x Dilution factor x 100}}{\text{sample weight}}$$

pH determination

Using a digital portable pH meter-JENWAY, UK 3305P, in accordance with Harmonised International Honey Commission (2009), the pH of honey samples was specified. In between the readings of dissimilar samples, the electrode was washed with refined water, dried with tissue paper, slipped into prepared honey samples, and recorded. The experiment was performed in triplicates.

Color determination

According to the USDA Honey Colour Grading Chart (USDA 1985), the classification of honey color was named and assigned a rank. Honey samples were situated in clean and clear McCartney bottles and determined against the color grading chart by Panoramic Hill Honey Collective (2013).

Determination of total decreasing sugar

Following the AOAC (2000) procedure using method number 920.183, the total decreasing sugar of honey was specified. About 1g of honey sample in conical flask was deliquesced using 100 mL hot water to deliquesce all sugars. The solution was purified by adding 5 mL Carrez 1 solution followed by Carrez 2 solution; then, the substance was sifted with Whatman sieve paper No. 1. Then, 10 mL of the filtrate was situated in duplicate in a conical flask blended with 10 mL of copper reagent (sodium carbonate solution, copper sulphate solution, and citric acid), then boiled for 30 minutes and left to cool; after that, 1 mL of saturated solution KI was added followed by 10 mL of 6N HCL. About three drops of 1%w/v starch were added as an indicator, and the substance was titrated against 0.1N (NaS_2O_3) up to the endpoint (blue-black to cream). Luff Table was utilized after titration volume to sugar substance, and the amount of decreasing sugar per gram of honey samples was computed as follows:

$$\text{Amount of total decreasing sugar in the original sample (mg/g)} = \frac{\text{Decreasing sugar (mg) from table}}{\text{Weight of sample (g)}} \text{ X } \frac{\text{Total volume of extract (ml)}}{\text{Volume of extract analysed}}$$

Mineral analysis

According to AOAC (2000) procedures using method number 999.11 by Atomic Absorption Spectrophotometer (AAS UNICAM 919) and Flame Analyzer (Model 2655-00), the mineral substance of honey samples (Fe, K, Zinc, Ca, Mg) were specified About 5 g of honey samples were burnt in the muffle furnace at 550°C for 4 hours and ash substance of each sample was deliquesced using 10 mL of 6N HCl. The achieved ash solution was sifted using Whatman sieve paper No.1. The serially liquefied reference typical solutions for Atomic Absorption Spectrophotometer (Element wavelength flame-gases) were; zinc 213.9 nm, iron 248.3 nm, potassium 766.5 nm, calcium 422.7 nm, and Magnesium 285.2 nm.

The Typical curve plot of absorbance against the recognized concentration of typical solutions (0.5, 1, 1.5, 2.0, 2.5, and 3.0 ppm) was utilized to specify the concentration of minerals in samples and expressed as in the following formula.

$$\text{Mineral content} = \left(\frac{\text{mg}}{\text{100g}}\right) \frac{\text{Reading Value in ppm x Dilution factor x 100}}{\text{Sample weight(g)}}$$

Statistical data analysis

Data achieved from antimicrobial analysis, antioxidant analysis, and physicochemical analysis were examined using SAS software (version 9.1) for Analysis. Analysis of Variance (ANOVA) was utilized to specify the effect of the primary factor on the dependent variables; the factor effect was believed to be substantial when $p<0.05$. Mean comparison was performed using the probability of difference (pdiff), and results were expressed as Least Squares Mean ± SE. Statistical Package for Social Scientists (SPSS, Version 17) software was utilized to appraise the percentage of various quantified parameters.

RESULTS AND DISCUSSION

Antimicrobial properties of raw honey from stinging and stingless bee honey

Tables 1.A, B, and C declare the capability of honey to inhibit microbial growth from selected areas, regions, and areas, respectively. Table 1.D shows two kinds of honey gathered from various honey-yielding areas, neighborhoods, and areas concerning its capability to inhibit micro-organism growth. Honey gathered from the western area had more capability of impeding microbial growth than that from the central area. However, the impediment area (diameter) between the western and central area was not important ($P>0.05$) dissimilar (Table 1.A).

Honey from Tabora and Shinyanga in the western area and Singida in the central area had a relatively higher capability to inhibit microbial growth than honey from Dodoma. Shinyanga honey showed a significantly larger area diameter of impediment, followed by Singida, Dodoma, and Tabora (Table 1.B).

The areas where honey samples were gathered are clearly displayed in Table 1.C shows honey samples from Nzega and Inyonga had the highest capability to resist microbial growth, followed by samples from Shinyanga town Bukombe and Kahama. In contrast, honey from Kibaigwa in the Dodoma region presented the lowest capability to resist microbial growth and, importantly, ($P<0.05$) lowest impediment area. The dissimilarity incapability to inhibit microbial growth could probably be because of dissimilar vegetation kinds between these areas (Kumar et al. 2010). Dissimilar vegetation contains dissimilar floral kinds that vary in pollen and nectar, influencing the honey mixture.

Table 1.A. Effect of honey gathered from central and western areas of Tanzania on microbial growth (N =360)

Area	Response%		Inhibition area
	+ Ve	-Ve	(mm)
Central	22.2	27.8	11.87 ± 0.43^{a}
Western	31.9	18.1	12.24 ± 0.30^{a}
Total/Significance level	54.2	45.8	NS

Note: NS = Not substantial at $P>0.05$; +ve response = Ability of honey to inhibit microbial growth; -ve response = Inability of honey to inhibit microbial growth

Table 1.B. Effect of honey gathered from various areas of Tanzania on the response of microbial growth (N =360)

Region	Response		Inhibition area
	+Ve	-Ve	(mm)
Dodoma	9.2	15.8	11.31 ± 0.57^{b}
Singida	13.1	11.9	12.13 ± 0.48^{ab}
Tabora	16.4	8.6	10.97 ± 0.42^{b}
Shinyanga	15.6	9.4	13.80 ± 0.43^{a}
Total/Significance level	54.2	45.8	*

Note: * = Substantial at $P<0.05$. +ve response = Ability of honey to inhibit microbial growth;-ve response = Inability of honey to inhibit microbial growth

Table 1.C. Effect of honey gathered from various areas of Tanzania on microbial growth (N =360)

Areas	Response		Inhibition area
	+Ve	-Ve	(mm)
Kibaigwa	2.5	5.8	9.78 ± 1.04^{c}
Dodoma town	3.1	5.3	13.80 ± 0.99^{a}
Bahi	3.6	4.7	10.46 ± 0.87^{bc}
Issuna	4.4	3.9	13.63 ± 0.78^{a}
Manyoni	4.2	4.2	10.60 ± 0.81^{bc}
Singida town	4.4	3.9	12.06 ± 0.81^{b}
Nzega	5.8	2.5	10.90 ± 0.68^{bc}
Inyonga	5.8	2.5	10.62 ± 0.68^{bc}
Tabora town	4.7	3.6	11.47 ± 0.76^{b}
Kahama	5.0	3.3	13.78 ± 0.74^{a}
Bukombe	5.0	3.3	15.11 ± 0.74^{a}
Shinyanga town	5.6	2.8	12.65 ± 0.70^{b}
Total/Significance level	54.2	45.8	*

Note: * = Substantial at $P<0.05$; +ve response = Capability of honey to inhibit microbial growth;-ve response = Incapability of honey to inhibit microbial growth

Table 1.D. The ability of stingless and stinging honey bees from central and western areas in Tanzania to inhibit microbial growth (N = 360)

Area	Region	Area	Honey type	Response%		Inhibition area (mm)
				+Ve	-Ve	
	Dodoma	Bahi	Stingless	3.24	0.8	10.79 ± 0.88^{b}
			Stinging	0.2	3.78	12.16 ± 3.06^{b}
		Town	Stingless	2.43	1.62	13.25 ± 2.16^{a}
			Stinging	0.54	3.5	10.16 ± 1.08^{bc}
		Kibaigwa	Stingless	2.43	1.62	10.56 ± 1.02^{b}
Central			Stinging	0	4.1	NA
	Singida	Issuna	Stingless	4.1	0	13.40 ± 0.79^{a}
			Stinging	0.27	3.78	14.16 ± 1.53^{a}
		Manyoni	Stingless	3.24	0.8	11.71 ± 0.88^{b}
			Stinging	3.5	3.24	9.51 ± 1.76^{c}
		Town	Stingless	4.1	0	11.82 ± 0.82^{b}
			Stinging	0.27	3.78	12.16 ± 3.06^{b}
	Tabora	Inyonga	Stingless	3.78	0.27	11.27 ± 0.92^{b}
			Stinging	1.89	2.16	10.21 ± 1.25^{bc}
		Nzega	Stingless	4.1	0	11.33 ± 0.79^{b}
			Stinging	1.62	2.43	8.67 ± 1.25^{d}
		Town	Stingless	2.97	1.08	12.13 ± 0.92^{b}
			Stinging	1.62	2.43	8.88 ± 1.25^{d}
	Shinyanga	Bukombe	Stingless	4.1	0	15.13 ± 0.79^{a}
Western			Stinging	0.8	3.24	12.16 ± 1.37^{b}
		Kahama	Stingless	3.78	0.27	14.63 ± 0.82^{a}
			Stinging	1.08	2.97	10.34 ± 1.53^{bc}
		Town	Stingless	4.1	0	13.53 ± 0.79^{a}
			Stinging	1.35	2.70	8.50 ± 1.37^{d}

Note: Means bearing the same superscript along the column are not statistically dissimilar according to pdiff at P>0.05; +ve response = Ability of honey to inhibit microbial growth;-ve response = Inability of honey to inhibit microbial growth

Table 2. The effect of honey kinds on the growth of pathogenic micro-organisms (N = 360)

Honey kind	Micro-organism	Response %		Inhibition area
		+Ve	-Ve	mm
Stingless	*Escherichia coli*	19.2	25.0	9.22 ± 0.54^{b}
	Salmonella typhi	20.5	16.7	10.47 ± 0.53^{ab}
	Staphylococcus saprophyticus	21.2	12.5	10.86 ± 1.22^{ab}
	Candida albicans	23.1	0.0	11.67 ± 0.50^{a}
	Aspergillus flavus	16.0	45.8	10.97 ± 0.60^{ab}
Sub-Total		100.0	100.0	
Stinging	*Escherichia coli*	0.0	25.5	0.0
	Salmonella typhi	7.7	23.4	0.83 ± 0.06^{f}
	Staphylococcus saprophyticus	15.4	21.3	1.78 ± 0.09^{e}
	Candida albicans	30.8	17.0	2.89 ± 0.10^{d}
	Aspergillus flavus	46.2	12.8	6.56 ± 0.48^{c}
Sub-Total		100.0	100.0	***

Note: Means bearing the same superscript along the column are not statistically different according to pdiff at P>0.05; +ve response = Ability of honey to inhibit microbial growth;-ve response = Inability of honey to inhibit microbial growth

This study presented that about 54 % of all tested honey samples, regardless of the kind of honey or the area gathered, or the tested microorganism, presented a positive response to impede microbial growth, whereas 46% presented a negative response (Table 1.C). Incapability to inhibit microbial growth could be attributed to the vegetation where these honey samples were gathered, with contents of nectar and pollen that do not possess medicinal attributes that can kill pathogenic microorganisms (Manning 2000). Alternatively, the tested honey could not influence the pathogenic microorganisms utilized to test honey impediment.

Results in Table 1.D further comply that stingless honey had more capability to impede microbial growth than a stinging honey bee, suggesting that a stingless honey bee possesses higher medicinal attributes than the stinging honey bee. The superiority of the stingless honey bees could be attributed to the flowers and trees visited. Also, stingless bees do not blend pollen with honey, whereas larger bees blend honey and pollen together to produce the so-called bee bread (Cortopassi et al. 2006). Another

reason could be the smaller size (5 mm) of the stingless bee, which enables the bees to penetrate more into the flower and extract medicinal ingredients than larger bees that can't penetrate deep into the flower (Roubik 2006).

The microbial impediment area ranged from 8.5 (stinging honey bee) to 15.13 mm (stingless honey). The observed impediment area in this study is more or less similar to that informed by Rahman et al. (2010) (13-15). They worked with honey and propolis samples that presented the capability to inhibit the growth of *E. coli* and *Staphylococcus aureus*, where propolis attained a higher impediment limit. Mohapatra et al. (2010) informed higher impediment area resulted from raw and processed honey from India on gram-positive bacteria (*S. aureus, B. subtilis, B. cereus, E. faecalis,* and *Micrococcus luteus*) and gram-negative bacteria (*E. coli, P. aeruginosa* and *S. typhi*) where impediment area ranged from 6.94 mm to 37.97 mm. The informed upper limit is higher than the observed upper limit in this study. The dissimilarity could be attributed to dissimilarities in vegetation kind, honey processing, and kind of tested micro-organisms.

The effectiveness of stingless and stinging honey bees in inhibiting the growth of microorganisms is shown in Table 2. The results presented that *C. albicans* was more influenced by stingless honey bees, followed by *S. saprophyticus, S. typhi, E. coli,* and *A. flavus*.

The growth of *E. coli* was not influenced by stinging honey at all; on the contrary, *A. flavus* and *C. albicans* were more influenced. Bacteria are more resistant than fungi because bacteria cells possess a high spontaneous mutation rate (about 10-7 per cell division. This means that they can change their characteristics rapidly, thus, providing a greater diversity on which natural selection can act, which helps them survive in an ever-changing environment (French et al. 2005). These results are dissimilar from that informed by Mohamad (2012), who tested antimicrobial attributes of stinging honey from Mauritius using bacteria (*E. coli* and *S. aureus)* and fungal (*Aspergillus niger* and *C. albicans)* and observed that fungi were more resistant than the bacteria, this could probably be because of the concentration of honey utilized where Mohammad et al. (2012) liquefied the honey utilized while in this study honey was not diluted.

The general characteristics of honey to inhibit bacterial or fungal growth possess been explained by various scientists (Garcia et al. 1986; Wahdan 1998; Molan 1999a and Khan et al. 2007) who informed impediment of pathogenic microbial growth from the presence of hydrogen peroxide resulting from the action of glucose oxidase enzyme yielded from hypopharyngeal glands of workers bees on glucose in the presence of oxygen that inhibits microbial and fungal growth. Presence of inherent physical-chemical attributes such as high sugar substance (about 80% w/w) that results in a high osmotic effect that dehydrates the micro-organisms has been informed to contribute to the impediment of microbial growth (Molan 1992; Bogdanov et al., 1997). White (1978); Aparna and Rajalashmj (1999) suggested inhibition of microbial growth is because of the presence of diverse organic acids, such as gluconic acids, that actually creates an acidic micro-environment (pH 3-4.5) that inhibits the growth of several micro-organisms. Apart from hydrogen peroxide as a factor that inhibits microbial growth, Cabrera et al. (2006) elucidated the impediment of microbial growth because of the presence of non-peroxidic substances such as polyphenols that possess anti-microbial activity.

Physico-chemical properties of honey of the stingless and stinging honey bee

pH and total decreasing sugars of honey

Results in Table 3.A declare that there was no substantial (P>0.05) dissimilarity in the pH of the honey. More importantly, total decreasing sugar was importantly (P<0.001) higher in honey from the western area than in the central area.

Looking at the effect of areas (Table 3.B), it is clear that honey gathered from Shinyanga had importantly (P<0.05) higher total decreasing sugars than that of Tabora, Singida, and Dodoma areas. The dissimilarity could be because of dissimilarity in vegetation growing in areas where the samples were gathered.

Results in Table 3.C state no substantial (P>0.05) variations in pH in honey between areas where honey samples were gathered. Honey from Shinyanga town had the highest total decreasing sugars substance (322.03 mg/g), and honey of Kibaigwa had the lowest total decreasing sugars substance (172.04 mg/g) (Table 3.C). These scrutinies suggest that honey from Shinyanga town could be a better provenance of energy to the body compared to the one gathered from Kibaigwa (Eva 1999).

Table 3.A. Effect of honey gathered from western and central zones on pH and total decreasing sugars substance.

Area	pH	Total decreasing sugars (mg/g)
Central	3.46^{a}	211.97^{b}
Western	3.45^{a}	267.58^{a}
Mean	**3.45**	**239.77**
SE	±0.02	±10.04
Significance	NS	***

Note**:** Means bearing the same superscript within the same column are not statistically dissimilar (p≥ 0.05) according to pdiff. NS =Not substantial (P>0.05)

Table 3.B. Effect of region on pH and total decreasing sugars substance of honey

Region	pH	Total decreasing sugars (mg/g)
Dodoma	3.43^{ab}	182.62^{c}
Shinyanga	3.41^{b}	304.88^{a}
Singida	3.49^{a}	241.31^{b}
Tabora	3.48^{a}	230.27^{b}
Significance	*	*
Overall mean	**3.45**	**239.77**
SE	±0.02	±13.13

Note**:** Means bearing the same superscript within the same column are not statistically dissimilar (p≥ 0.05) according to pdiff. ***** =Substantial at P<0.05

The present research presented that area where honey samples were gathered, honey kind, and interactions between the two factors importantly (P<0.01) influenced the pH and total decreasing sugars substance of the tested honey samples. Comparisons between sampling areas and honey kinds presented that there was no substantial (P>0.05) dissimilarity in pH between stinging and stingless honey bees (Table 3.D).

Various authors within the range inform the examined pH values in this study within and outside Tanzania. Muruke (2014), working with 26 honey samples from Tanzania, informed a pH range of 2.6 to 4.4. The present research results also meet with those informed by Aloisi (2010), who did the research on Apis honey and informed pH range of 3.2-3.5. The honey samples utilized in this research seemed to be of lower pH than those used by Gidamis et al. (2004) while researching Tanzanian honey from Dodoma, Tanga, Morogoro, Same, Arusha, and Tabora and informed a pH of range of 4.4-4.87. The dissimilarity could be because of dissimilarity in soil kind and vegetation growing in areas where the samples were gathered. The honey samples in the present research might have potential medicinal attributes as their acidic nature could have yielded from organic acids, which actually produce an acidic microenvironment that inhibits pathogenic microbial growth (Aparna and Rajalakshmj 1999).

In particular, simple sugar substances, such as glucose, have been informed to have two significant roles in antimicrobial growth; one is that high sugar substances can yield a high osmotic effect that can dehydrate microbial cells (Molan 1992; Bogdanov et al. 1997). Second, gluconic acids, which actually produce an acidic micro-environment, inhibit several micro-organisms' growth (Cooper 2007). Therefore, the results of this research pose that honey of stingless honey bees from Shinyanga town, Kahama, Bukombe, and Nzega in the western area and that from Singida town and Issuna may have more medicinal attributes than that of the stinging honey bees.

Table 3.C. Effect of honey gathered from various areas on pH and total decreasing sugars substance.

Area	pH	Total decreasing sugars (mg/g)
Bahi	3.39^{a}	176.33^{b}
Bukombe	3.36^{a}	296.52^{a}
Dodoma town	3.44^{a}	199.49^{b}
Inyonga	3.54^{a}	223.07^{b}
Issuna	3.51^{a}	283.13^{a}
Kahama	3.45^{a}	296.09^{a}
Kibaigwa	3.44^{a}	172.04^{b}
Manyoni	3.48^{a}	245.01^{b}
Nzega	3.44^{a}	212.52^{b}
Shinyanga town	3.44^{a}	322.03^{a}
Singida town	3.47^{a}	195.78^{b}
Tabora town	3.46^{a}	255.23^{b}
Overall mean	3.45	239.77
SE	±0.04	±22.49
Significance	NS	*

Note: Means bearing the same superscript within the same column are not statistically dissimilar according to pdiff (P>0.05). NS= Not substantial (P>0.05) *= Substantial at P<0.05

Table 3.D. The effect of zones, regions, areas, and honey kinds on pH and total decreasing sugars substance

Area	Region	Area	Honey type	pH	Total reducing sugars (mg/g)
Central	Dodoma	Bahi	Stingless	3.45^{a}	259.54^{cd}
			Stinging	3.34^{a}	93.13^{e}
		Town	Stingless	3.59^{a}	294.78^{bc}
			Stinging	3.29^{a}	104.22^{e}
		Kibaigwa	Stingless	3.50^{a}	240.26^{d}
			Stinging	3.39^{a}	103.82^{e}
	Singida	Issuna	Stingless	3.42^{a}	288.94^{bc}
			Stinging	3.59^{a}	277.32^{bc}
		Manyoni	Stingless	3.42^{a}	226.95^{cd}
			Stinging	3.55^{a}	263.07b^{cd}
		Town	Stingless	3.41^{a}	252.92^{cd}
			Stinging	3.53^{a}	138.66^{de}
Western	Tabora	Inyonga	Stingless	3.41^{a}	204.89^{d}
			Stinging	3.68^{a}	241.25^{d}
		Nzega	Stingless	3.42^{a}	310.73^{bc}
			Stinging	3.45^{a}	114.31^{e}
		Town	Stingless	3.41^{a}	303.94^{bc}
			Stinging	3.51^{a}	206.53^{d}
	Shinyanga	Bukombe	Stingless	3.30^{a}	318.77^{bc}
			Stinging	3.42^{a}	274.27^{bc}
		Kahama	Stingless	3.42^{a}	413.18^{a}
			Stinging	3.47^{a}	179.01^{d}
		Town	Stingless	3.41^{a}	320.53^{b}
			Stinging	3.46^{a}	323.53^{b}
			SE	±0.01	±0.12
			Significance	**NS**	***

Note: Means bearing the same superscript within the same column are not statistically dissimilar (p≥ 0.05) according to pdiff. NS=Not substantial (P>0.05); *** = Substantial at P<0.001

Table 4.A. Effect of zones on color grading of honey (N = 72)

Area	Colour	Percent
Central	Water white	1.4
	Extra white	5.6
	White	2.8
	Extra light amber	19.4
	Light amber	13.9
	Amber	6.9
Western	Extra white	8.3
	White	12.5
	Extra light amber	1.4
	Light amber	13.9
	Amber	13.9
Total		100

Note: Grading was performed according to USDA Honey Colour Grading Chart (USDA 1985)

Table 4.B. Effect of areas on color grading of honey (N = 72)

Region	Colour	Percent
Dodoma	Water white	1.4
	Extra white	5.6
	White	1.4
	Extra light amber	8.3
	Light amber	8.3
Singida	White	1.4
	Extra light amber	11.1
	Light amber	5.6
	Amber	6.9
Tabora	Extra white	5.6
	White	6.9
	Light amber	4.2
	Amber	8.3
Shinyanga	Extra white	2.8
	White	5.6
	Extra light amber	1.4
	Light amber	9.7
	Amber	5.6
Total		100

Note**:** Grading was done according to USDA Honey Colour Grading Chart (USDA 1985). Values in parentheses present several examinations (n)

Honey color

The present research outcomes declare that honey samples from the western area possessed color descriptions ranging from light amber to amber to honey samples from the central area, which had color descriptions ranging from water white to amber (Table 4.A). On the other hand, honey samples from the Dodoma region had a relatively light color ranging from water white to light amber (Table 4.B).

Considering honeybee kind, the honey of stingless honey bee presented color grading of extra light amber (4.2%), amber (20.8%), and light amber (25.0%). The honey of the Stinging honey bee was represented in most color grading ranging from water white (1.4%) to light amber (2.8%). The honey color allocation by honey kinds and area where the honey samples were gathered is expressed in Table 4.C.

Table 4.C. Colour grading of stinging and stingless honey bee's honey sampled from various areas in central and western areas in Tanzania (N=72)

Area	Colour	Stingless (%)	Honey kind stinging (%)
Bahi	Extra light amber	0	4.2
	Light amber	4.2	0
Bukombe	Extra light amber	0	1.4
	Light amber	0	2.8
	Amber	4.2	0
Dodoma town	Extra white	0	2.8
	Light amber	4.2	0
	White	0	1.4
Inyonga	Light amber	1.4	0
	White	0	4.2
	Amber	2.8	0
Issuna	Extra light amber	0	2.8
	Light amber	1.4	0
	White	0	1.4
	Amber	2.8	0
Kahama	Extra white	0	2.8
	Light amber	4.2	0
	White	0	1.4
Kibaigwa	Extra light amber	4.2	0
	Extra white	0	2.8
	Water white	0	1.4
Manyoni	Extra light amber	0	4.2
	Light amber	1.4	0
	Amber	2.8	0
Nzega	Extra white	0	4.2
	Amber	4.2	0
Shinyanga town	Light amber	2.8	0
	White	0	4.2
	Amber	1.4	0
Singida town	Extra light amber	0	4.2
	Light amber	2.8	0
	Amber	1.4	0
Tabora town	Extra white	0	1.4
	Light amber	2.8	0
	White	0	2.8
	Amber	1.4	0

Note**:** Grading was done according to USDA Honey Colour Grading Chart (USDA,1985)

Results presented that honey of stingless honey bees had more of light amber to amber color. Honey color has been related to the nutritional value of the honey, where deeply pigmented honey (darkly colored) honey is superior in nutritional value to the one with light color. The darker the honey, the higher the mineral substance (Root 1980). Observations in this study suggest that stingless honey bees had relatively higher nutritional and medicinal value than stinging honey bees. It has also been informed that honey with dark color possesses a higher total phenolic substance and, consequently, higher anti-oxidant capacity (Montenegro et al. 2006). These examinations further comply with earlier findings (Table 2) that the stingless honey bee's honey had more medicinal attributes than the stinging honey bee's honey.

Mineral profile of stinging and stingless honey bee

The results presented that the mineral substance of honey samples did not vary importantly (P>0.05) between central and western zones for Zn, Fe, and Mg. However, Ca was importantly (P<0.001) higher in honey gathered from the central area, while K was higher in honey from the western area (Table 5.A). The dissimilarities in the mineral substance of honey could probably be because of dissimilarity in soil kinds that possess an influence on the floral mineral substance, which bees extract pollen and nectar (Seif and Alfadil 2009).

Results in Table 5.B declare the variability of the mineral substance of honey between areas. It was observed that there was no substantial (P>0.05) dissimilarity in the Mg substance of honey gathered from the four areas. However, the Ca substance was importantly (P<0.05) higher in honey samples gathered from Singida than that achieved from Shinyanga, Tabora, and Dodoma. A dissimilar trend was observed for K, where honey gathered from Tabora, Shinyanga, and Singida areas was a more important (P<0.05) substance than in the Dodoma region.

Table 5.C presented that honey gathered from Issuna had a higher Zn substance, followed by Nzega, Manyoni, Bahi, Dodoma, and Singida. Iron was higher in honey from Inyonga, Shinyanga town, Singida town, and Issuna. Calcium was important (P<0.05), highest in honey gathered from Singida town, and lowest in honey gathered from Kahama (P<0.05). Nzega honey yielded the highest Mg, whereas Kibaigwa honey had the lowest Mg substance. Potassium was importantly higher in Inyonga honey and lowest in Dodoma town honey (P<0.05).

The results in Table 5.D presented that potassium had the highest concentration, which was importantly (P<0.05) dissimilar from the rest in both kinds of honey. Honey sampled from Dodoma town had the lowest concentration. Kibaigwa honey had the least Ca substance (P<0.05). Zinc was the lowest in both kinds of honey, with a minimum value in Kibaigwa honey and a high value in Issuna honey. The dominance of K substance in honey is similar to the scrutinies made by Rodrigues-otero et al. (1994), who informed that K was the abundant and predominant element in honey. Also, thyme honey from Spain contained a relatively higher K concentration of 679 mg/kg (Terrab et al. 1994). Seif and Elifadili (2009) informed diversity in K substance because of floral kinds, whereby a range of 17.60 mg/kg for *Helianthus annus* to 74.66 mg/kg for *Acacia seyal* was informed. The overall mean concentration of K observed in this study is within the range reported by other workers (Terrab et al. 1994). However, in the present study, the diversity of K concentration between areas and honey kinds (stinging honey bee from Dodoma town and stingless honey bee from Singida town) could be attributed to bee kinds and diversity in floral types.

Stingless honey presented a relatively higher Zn concentration, whereas honey from Issuna was highest and, importantly, (P<0.05) dissimilar from all other honey samples (Table 5.D). The other honey of stingless honey bees from Nzega, Manyoni, and Singida towns had higher Zn substances that did not differ importantly (P>0.05). Stinging bees' honey had lower values importantly for Zn for all samples than stingless bees' honey. It was further examined that stinging bees' honey from Kibaigwa had the lowest Zn concentration. Generally, Zn was present in the lowest concentrations in relation to other minerals. These results are in accordance with Rodrigues-otero et al. (1994), who found Zn to be relatively lower while working with stinging honey bees coming from various floral kinds.

Table 5.A. The Effect of honey gathered from western and eastern zones on mineral substance

Area	Mineral substance (mg/kg)				
	Zn	Fe	Ca	Mg	K
Central	2.59^{a}	7.17^{a}	237.56^{a}	154.17^{a}	379.0^{b}
Western	2.02^{a}	8.55^{a}	162.0^{b}	143.91^{a}	531.56^{a}
SE	±0.23	±0.82	±19.55	±18.96	±154.15
Significance	NS	NS	**	NS	*

Note: Means bearing dissimilar superscripts along the same column are importantly dissimilar according to pdiff P<0.05

Table 5.B. Effect of honey gathered from various areas of Tanzania on mineral substance.

Region	Mineral substance (mg/kg)				
	Zn	Fe	Ca	Mg	K
Dodoma	2.24^{a}	3.68^{b}	169.55^{b}	134.41^{a}	281.79^{b}
Shinyanga	1.78^{b}	6.88^{ab}	131.36^{b}	130.43^{a}	504.82^{a}
Singida	2.93^{a}	10.67^{a}	305.57^{a}	173.94^{a}	476.22^{a}
Tabora	2.27^{a}	10.23^{a}	192.65^{b}	157.38^{a}	558.31^{a}
Significance	*	*	***	NS	*
Overall mean	2.31	7.86	199.78	149.04	455.29
SE	±0.33	±0.98	±24.97	±26.91	±76.34

Note: Means bearing similar superscripts along the same column are not importantly dissimilar according to pdiff (P>0.05)

Table 5.C. Effect of honey gathered from various areas on mineral substance.

Area	Mineral substance (mg/kg)				
	Zn	Fe	Ca	Mg	K
Bahi	2.63^{a}	7.12^{c}	215.09^{b}	155.34^{a}	447.89^{a}
Bukombe	1.12^{b}	4.65^{d}	119.82^{c}	127.79^{a}	436.35^{a}
Dodoma town	2.62^{a}	1.61^{e}	159.84^{c}	155.53^{a}	111.85^{c}
Inyonga	1.57^{b}	15.34^{a}	216.45^{b}	114.95^{a}	602.47^{a}
Issuna	3.29^{a}	10.80^{ab}	238.17^{b}	175.48^{a}	343.55^{a}
Kahama	1.67^{b}	4.22^{d}	91.72^{d}	141.23^{a}	496.56^{a}
Kibaigwa	1.47^{b}	2.32^{e}	133.73^{c}	92.38^{b}	285.61^{b}
Manyoni	2.89^{a}	10.38^{ab}	274.27^{b}	166.02^{a}	551.59^{a}
Nzega	2.95^{a}	8.44^{b}	190.71^{b}	189.29^{a}	531.22^{a}
Shinyanga town	2.55^{a}	11.76^{ab}	182.54^{b}	122.26^{a}	581.55^{a}
Singida town	2.62^{a}	10.81^{ab}	404.26^{a}	180.32^{a}	533.52^{a}
Tabora town	2.29^{a}	6.92^{c}	170.80^{b}	167.90^{a}	541.25^{a}
Overall mean	2.31	7.86	199.78	149.04	455.29
SE	±0.57	±1.31	±41.46	±48.62	±134.91

Note: Means bearing similar superscripts along the same column are not importantly dissimilar according to pdiff (P>0.05)

Table 5.D. Effect of zones, regions, areas, and honey kind on mineral substance

Area	Region	Area	Honey type	Mineral substance (mg/kg)				
				Zn	Fe	Ca	Mg	K
Central	Dodoma	Bahi	Stingless	4.12^{c}	7.84^{dc}	282.50^{cd}	259.57^{bc}	758.41^{d}
			Stinging	1.13^{g}	6.39^{de}	147.67^{d}	51.10^{d}	137.37^{f}
		Town	Stingless	3.90^{c}	1.34^{g}	228.42^{d}	292.41^{bc}	143.28^{c}
			Stinging	1.34^{g}	1.88^{g}	91.25^{f}	18.65^{f}	80.42^{f}
		Kibaigwa	Stingless	2.56^{e}	2.23^{g}	237.50^{d}	177.08^{c}	470.76^{e}
			Stinging	0.38^{h}	2.40^{g}	29.95^{g}	7.67^{f}	101.29^{f}
	Singida	Issuna	Stingless	5.29^{a}	11.67^{bc}	287.62^{c}	273.92^{bc}	423.37^{c}
			Stinging	1.28^{g}	9.93^{c}	188.73^{d}	77.04^{d}	260.73^{f}
		Manyoni	Stingless	4.55^{b}	12.36^{b}	442.0^{b}	277.46^{bc}	931.39^{c}
			Stinging	1.22^{g}	8.41^{c}	106.53^{ef}	54.57^{d}	171.81^{f}
		Town	Stingless	4.43^{bc}	12.10^{b}	508.70^{a}	281.98^{bc}	951.10^{c}
			Stinging	0.82^{h}	9.52^{c}	299.82^{c}	78.65^{d}	115.94^{f}
Western	Tabora	Inyonga	Stingless	1.56^{f}	12.53^{b}	221.53^{cd}	164.82^{c}	751.85^{d}
			Stinging	1.57^{f}	18.15^{a}	211.36^{cd}	65.07^{d}	453.09^{e}
		Nzega	Stingless	4.56^{b}	10.80^{bc}	319.46^{c}	350.52^{a}	894.72^{c}
			Stinging	1.34^{g}	6.08^{de}	61.96^{f}	28.07^{e}	167.72^{f}
		Town	Stingless	3.22^{d}	8.32^{c}	275.81^{cd}	310.76^{b}	961.38^{b}
			Stinging	1.36^{g}	5.52^{e}	65.79^{f}	25.04^{e}	121.12^{f}
	Shinyanga	Bukombe	Stingless	1.35^{g}	7.05^{d}	133.50^{e}	185.23^{c}	719.64^{a}
			Stinging	0.88^{h}	2.26^{g}	106.14^{ef}	70.36^{d}	153.05^{f}
		Kahama	Stingless	1.42^{g}	3.93^{fe}	94.76^{f}	244.01^{bc}	792.59^{b}
			Stinging	1.92^{f}	4.50^{e}	88.68^{f}	38.45^{e}	200.54^{f}
		Town	Stingless	3.31^{d}	19.87^{a}	274.02^{cd}	205.87^{c}	911.48^{a}
			Stinging	1.80^{f}	3.64^{fe}	91.06^{f}	38.66^{e}	251.63^{f}
			SE	±0.039	±0.182	±4.346	±3.138	±23.822
			Sign. level	***	***	***	***	***

Note: Means bearing the same superscript within the same column are not statistically different (p≥0.05) according to pdiff

The concentration of Zn observed in this study was relatively lower than that observed by Rodrigues-otero et al. (1994). They informed a minimum Zn concentration of 4.86 mg/kg to 9.61 mg/kg. The dissimilarity could probably be because of the dissimilarity in vegetation and soil kinds. Soil and vegetation possess an influence on the mineral substance of honey. Root (1980) informed that minerals such as K, Cl, S, Na, Zn, and Ca originate from the soil and get into honey via the plants. Furthermore, the plant mineral uptake varies with plant species.

Total iron (Fe) substance was slightly higher (Table 5.D) in stingless honey bees than that contained in stinging honey bees. Stingless honey bees from Shinyanga town had the highest Fe substance (P<0.05) that did not differ from the stinging honey bees from Inyonga. There were no substantial dissimilarities (P>0.05) in the Fe substance of stingless honey bees from Inyonga, Issuna, Manyoni, Singida town, and Nzega. The stingless honey bee from Dodoma town had the lowest Fe substance. According to this study, the observed iron concentration range of honey from Dodoma and Shinyanga towns had a lower minimum value of 2.05mg/kg informed by Seif and Alfadil (2009). They worked on the honey originating from *Azadirachta indica* and *Acacia seyal*. The dissimilarity in iron substance could be attributed to dissimilarities in vegetation kinds where honey was extracted.

Generally, the total levels of Ca, Mg, and K were importantly (P<0.05) higher in stingless honey bees than that of stinging honeybees gathered from various areas (Table 5.D). The reasons contributing to the dissimilarity in the mineral profile between the two bee kinds could probably be because of the smaller size of stingless honey bees that enable them to penetrate deeper into the flower and extract more nutrients, also the behavior of stingless bees of not mixing pollen with honey, unlike a stinging bee that blends the two could contribute to the mineral superiority of stingless honey bee (Cortopassi et al. 2006). The concentration of calcium observed in the present study was dissimilar from the results informed by Seif and Elifadil (2009). They tested honey and found the concentration of Ca (mg/kg) to range from 42.37 to 82.92. The dissimilarity could probably be because of dissimilarity in soil that influences floral kinds and floral substances (Bereta et al. 2005).

The values of magnesium recorded in the current study (Table 5.D) are similar to the results informed by Seif and Elifadil (2009). They tested honey from dissimilar vegetation (*Ziziphus spina, Acacia nilotica, Acacia seyal, Helianthus annus*, and *Azadirachta indica*) and found magnesium concentration to range between23.67 to 177.15 mg/kg. Also, Parmas et al. (2000) specified a mineral mixture of honey from western Spain by flame photometric method and informed mg concentration of 23.9 mg/kg.

Antioxidant, total phenolic compounds, and Vitamin C content of stinging and stingless bee honey

Tables 6.A, 6.B, and 6.C present the effect of area, areas, and areas where samples were gathered on antioxidants, total phenols, and vitamin C substance of honey. Table 6.d shows the interaction effect of area, areas, and areas on the honey antioxidant, total phenols, and vitamin C substance.

Results in Table 6.A declare that there were no substantial (P>0.05) dissimilarities in antioxidant attributes (FRAP values) and total phenols of honey between the two areas. However, honey from the central area had importantly (P<0.05) higher vitamin C than the western area.

Looking at the effect of areas (Table 6.B), there were variations in FRAP values, total phenols, and vitamin C between areas. The diversity could be attributed to dissimilarity in soil kinds that influence the area's vegetation (Terrab et al. 2004). Antioxidant attributes (FRAP values) were importantly (P<0.05) higher in Shinyanga honey but did not differ from that of Singida and Tabora (P>0.05). The higher FRAP values of Shinyanga honey may be because of its stronger antioxidant attributes than all the other honey from other areas, indicating a greater reduction of Fe^{3+} to Fe^{2+} ions. Honey from Singida had an importantly (P<0.05) higher vitamin C substance than that gathered from Tabora, Shinyanga, and Dodoma. These scrutinies suggest that honey from Singida could be a rich provenance of this vitamin than the ones gathered from Tabora, Shinyanga, and Dodoma.

Results in Table 6.C declare that there were no substantial (P>0.05) variations in total phenols in honey between areas where honey samples were gathered. Honey from Singida town yielded the highest vitamin C (P<0.05) that did not differ from honey gathered from Dodoma, Shinyanga, Manyoni, Issuna, and Bukombe. The results of the present study presented that honey from Inyonga had an importantly (P<0.05) higher FRAP value that did not differ from that from Bukombe, Issuna, and Shinyanga towns, suggesting that honey from these areas could be utilized as a potential provenance of these compounds that are significant in removing the free radicals from the body that play a role in body immunity (Organic Facts, 2016). The honey gathered from Kibaigwa had the lowest FRAP value content.

The results of the present study presented that area where honey samples were gathered, honey kind, and interactions between the two factors importantly (P<0.01) influenced the FRAP value, total phenols, and vitamin C substance of the tested honey samples (Table 6.D).

The present research results have presented that FRAP values, total phenols, and Vitamin C substance were importantly (P<0.001) higher in the honey of stingless honey bees than that of the stinging honey bees in all sampled areas from all the areas in respective zones. The FRAP values range of 72.14-973.57 µM Fe (II)/100 g examined in the present research are higher than the range of Bengal honey informed by Das et al. (2013) (101.6-622.1 µM Fe (II)/100 g) and that informed by Moniruzzaman et al. (2014) working with monofloral Bangladeshi honey who examined a FRAP range of 116-786.3 µM Fe (II)/100 g. The dissimilarity could be attributed to dissimilarity in vegetation kinds. However, the lower FRAP value (of Kibaigwa honey) suggests that the honey possesses relatively lower antioxidant attributes than all the other gathered honey samples, which might be connected to a less reduction of Fe^{3+} to Fe^{2+} ions (Moniruzzaman et al. 2013).

Table 6.A. Effect of honey gathered from western and central areas on antioxidant, total phenol, and vitamin c substance of honey.

Area	FRAP values (µM Fe (II)/100g)	Total phenol (mg/100g)	Vitamin C (mg/100g)
Central	539.90^{a}	18.41^{a}	4.77^{a}
Western	611.58^{a}	15.24^{a}	3.51^{b}
Mean	575.74	16.82	4.14
SE	±44.14	±1.30	±0.36
Significance	NS	NS	*

Note: Means bearing the same superscript within the same column are not statistically different (p≥ 0.05) according to pdiff. NS = Not substantial (P>0.05)

Table 6.B. Effect of the region on antioxidant, total phenol, and vitamin C substance of honey

Region	FRAP values (µM Fe (II)/100g)	Total phenol (mg/100g)	Vitamin C (mg/100g)
Dodoma	440.47^{b}	15.71^{b}	3.88^{b}
Shinyanga	711.11^{a}	13.36^{b}	3.94^{b}
Singida	639.33^{a}	21.10^{a}	5.66^{a}
Tabora	512.04^{ab}	17.12^{ab}	3.07^{b}
Significance	*	*	*
Overall mean	575.74	16.82	4.14
SE	±58.56	±1.78	±0.50

Note: Means bearing the same superscript within the same column are not statistically different (p≥ 0.05) according to pdiff. ***** = Substantial at P<0.05

Table 6.C. Effect of honey gathered from various areas on antioxidant, total phenol, and vitamin C substance of honey.

Area	FRAP values (µM Fe (II)/100g)	Total phenol (mg/100g)	Vitamin C (mg/100g)
Bahi	625.42^{b}	17.91^{a}	3.83^{b}
Bukombe	815.57^{a}	12.88^{a}	4.25^{ab}
Dodoma town	431.92^{c}	17.21^{a}	6.29^{a}
Inyonga	836.37^{a}	22.39^{a}	3.92^{b}
Issuna	795.66^{a}	22.10^{a}	4.66^{a}
Kahama	613.15^{b}	14.11^{a}	2.07^{c}
Kibaigwa	264.06^{d}	12.0^{a}	1.51^{c}
Manyoni	541.49^{bc}	20.20^{a}	5.44^{a}
Nzega	415.95^{c}	15.36^{a}	3.06^{b}
Shinyanga town	704.61^{a}	13.08^{a}	5.52^{a}
Singida town	580.86^{bc}	21.0^{a}	6.86^{a}
Tabora town	283.81^{d}	13.61^{a}	2.24^{c}
Overall mean	575.74	16.82	4.14
SE	±56.59	±3.11	±0.80
Significance	*	NS	*

Note: Means bearing the same superscript within the same column are not statistically dissimilar according to pdiff (P>0.05); NS = Not substantial (P>0.05); * = Substantial atP<0.05

The observed total phenol (5.13-33.55 mg/100 g) in this research was lower than the range informed by Meda et al. (2005). They observed 27 honey samples from 18 multiflora, 2 honeydews, and 7 unifloral honey in Burkina Faso and informed total phenols of 32.59 to 114.5 mg/100 g. However, the total phenols in the present research were greater than those informed by Lihu et al. (2005), working on several kinds of honey using HPLC and achieved total phenols ranging from 2.13 to 12.11 mg/100 g. The diversity in phenol substance between these tested honey samples could be connected to dissimilarity in floral kinds, method of analysis, geographic origin as well as climatic characteristics of the sampled areas. Honey with high non-peroxidic substances, such as polyphenolic contents, is anticipated to have higher antimicrobial characteristics (Meda et al. 2005).

Correlation between honey color, total phenols, and minerals

Table 7 shows the correlation coefficient between honey colors, total phenols, and mineral substance in the honey samples. There was a positive and substantial (P<0.001) correlation between honey color and total phenolic compounds in the honey samples. These scrutinies suggest that the deeper the honey, the higher the phenolic compounds contained in the honey. Likewise, a substantial (P<0.01) and positive correlation existed between honey color and mineral substance, and total phenols contained in honey. Mineral substances, total phenols, and anti-oxidants have been positively related (Montenegro et al. 2006). Therefore, honey with deep color is assumed to possess high anti-microbial activities; thus, the deeper the color the honey possesses, the broader the spectrum of anti-microbial, fungal, and yeast (Kumar et al. 2010).

Table 6.D. The effect of zones, regions, areas, and honey kinds on antioxidants, total phenols, and vitamin C substances.

Area	Region	Area	Honey type	FRAP values (µMFe (II)/100g)	T phenol (mg/100g)	Vitamin C (mg/100g)
Central	Dodoma	Bahi	Stingless	816.07^{a}	24.69^{bc}	5.45^{d}
			Stinging	434.76^{c}	11.14^{h}	2.22^{ef}
		Town	Stingless	640.71^{b}	26.39^{b}	10.99^{a}
			Stinging	223.12^{d}	8.03^{i}	1.60^{f}
		Kibaigwa	Stingless	455.99^{c}	17.07^{ef}	2.54^{ef}
			Stinging	72.14^{e}	6.93^{i}	0.47^{g}
	Singida	Issuna	Stingless	970.48^{a}	25.23^{b}	7.69^{c}
			Stinging	620.83^{b}	18.98^{de}	1.63^{f}
		Manyoni	Stingless	612.03^{b}	27.23^{b}	8.67^{bc}
			Stinging	470.95^{c}	13.18^{gh}	2.21^{ef}
		Town	Stingless	645.90^{b}	25.67^{b}	9.80^{b}
			Stinging	515.82^{c}	16.35^{ef}	3.92^{e}
Western	Tabora	Inyonga	Stingless	822.50^{a}	33.55^{a}	5.48^{d}
			Stinging	850.24^{a}	11.24^{h}	2.35^{ef}
		Nzega	Stingless	557.26b^{c}	24.07^{bc}	3.95^{e}
			Stinging	274.64^{d}	6.64^{i}	2.17^{ef}
		Town	Stingless	322.16^{d}	22.09^{c}	2.74^{ef}
			Stinging	245.45^{d}	5.13^{i}	1.73^{f}
	Shinyanga	Bukombe	Stingless	848.90^{a}	15.13^{f}	5.51^{d}
			Stinging	782.23^{a}	10.65^{h}	2.97^{e}
		Kahama	Stingless	871.07^{a}	13.87^{gh}	2.53^{ef}
			Stinging	355.24^{d}	14.36^{fg}	1.61^{f}
		Town	Stingless	973.57^{a}	19.96^{d}	9.25^{b}
			Stinging	435.64^{c}	6.19^{i}	1.78^{f}
			SE	±61.79	±0.26	±4.70
			Significance	*	***	***

Note: Means bearing the same superscript within the same column are not statistically different (p≥ 0.05) according to pdiff. NS =Not substantial (P>0.05). *** = Substantial at P<0.001

Table 7. The correlation coefficient between honey color, total phenols, and mineral content

	Colour	Total phenols	Zn	Fe	Ca	Mg	K
Colour	1						
Total phenols	0.7729***	1					
Zn	0.6739***	0.6928***	1				
Fe	0.4225***	0.4295***	0.3462**	1			
Ca	0.6505***	0.7549***	0.7554***	0.5456***	1		
Mg	0.8587***	0.7821***	0.8279***	0.2916*	0.7238***	1	
K	0.7598***	0.4045***	0.4133***	0.3644**	0.3644***	0.6969***	1

Note: *** = P< 0.001 ** =P<0.01 * =P<0.05

In conclusion, honey samples gathered from the western area had more capability to inhibit microbial growth than those gathered from the central area. In addition, honey from Shinyanga in the western area had the highest capability to inhibit microbial growth, followed by Singida, Dodoma, and Tabora. On the other hand, honey samples from Nzega and Inyonga had the highest capability to inhibit microbial growth, followed by honey samples from Shinyanga town, Bukombe, and Kahama. *C. albicans* was more sensitive to impediment by the honey of stingless honey bees, followed by *Staphylococcus saprophyticus and S. typhi*, whereas *E. coli* was not influenced by the honey of stinging honey bees. Honey of Stinging honey bee was more effective in inhibiting the growth of *A. flavus* and *C. albicans.* It was generally examined that honey of stingless honey bees had higher values for total phenols, antioxidants, and vitamin C contents than that of the stinging honey bee. Honey from Inyonga, Manyoni, Dodoma town, Issuna, Singida town, Shinyanga town, and Nzega showed relatively higher total phenol, antioxidant, and vitamin C substance. There was no substantial ($P>0.05$) dissimilarity in pH between stinging and stingless honey samples. However, total decreasing sugars and mineral substances were higher in honey samples of a stingless honey bee than that of a stinging honey bee. Total decreasing sugar was higher in honey from Kahama, Shinyanga town, Bukombe, Tabora town, Nzega, Issuna, Dodoma town, and Bahi. High-decreasing sugars were also examined in the honey of stinging honey bees from Shinyanga town. A similar trend was examined for mineral substance, where honey of stingless honey bees bore more minerals. Thus, it possessed darker color (light amber to amber) than the honey of a stinging honey bee. There was a positive and substantial ($P<0.05$) correlation between total phenols, honey color, and mineral substance of honey, suggesting that the deeper the color of the honey, the higher the mineral and total phenol substance. Therefore, honey with deep color is assumed to possess high anti-microbial activities; thus, the deeper the color the honey possesses, the broader spectrum of anti-microbial growth. Generally, honey of stingless honey bees is superior to honey of stinging honey bees in antimicrobial and antioxidant attributes, total decreasing sugars, total phenol, vitamin C, and mineral substance.

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Characterization of flavonoids from candidate *striga* grass in controlling diet legumes *Cicer arietinum* and *Vigna radiata*

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Abstract. *Akumu AA, Nyambaka H, Kuate SP, Torto B.2018. Characterization of flavonoids from candidate* striga *grass in controlling diet legumes* Cicer arietinum *and* Vigna radiata*. Biofarmasi J Nat Prod Biochem 16: 83-98.* In Africa, intercropping of some legumes with cereal plants has been noticed to generate a remarkable reduction of *Striga* invasion and enhanced cereal crop production. *Desmodium uncinatum* administers *Striga* invasion in intercrop with cereals through an allelopathic technique, including post-sprouting accretion inhibitors emanating from the roots. Having the potential to be a new technique for grass administering, Allelopathy is a natural and environmentally friendly method. A standardized profiling technique based on Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) and Liquid Chromatography coupled with Mass Spectrometry (LC/MS) was utilized to determine flavonoids in extracts of chickpea (*Cicer arietinum*) and mung bean (*Vigna radiata*), potential trap plants for *Striga* grass. Nine flavonoids involving mono-and diglycosyl derivatives of fisetin, baicalein, rhamnetin, isorhamnetin, formononetin, quercetin, isosakuranetin, and sakuranetin were temporarily distinguished. The obtained phenolics were available at concentrations higher than 0.001% of the dry materials. Many of these phenolic mixtures have been claimed to possess human health advantages. These glycosylated flavones are noted for the first time in these two species. The comprehensive analysis of the polar secondary metabolites in these leguminous plants was useful for comprehending their inhibitory chemistry and proposed biosynthesis by C-glucoside characteristics. All the segregated and described mixtures in the diet legume *C. arietinum* were O-glucosylated. At the same time, Food legume *V. radiata* should be observed further to see if it can attain *Desmodium's* allelopathic ability since it possesses inhibitory chemistry and proposed biosynthesis by C-glucosylation.

Keywords: *Cicer arietinum*, control, flavonoids, legumes, Striga, *Vigna radiata*

INTRODUCTION

Striga is one of the most significant pests that affect food production in the tropics (Esilaba 2006). The grass problem is aggravated by its reproductive capability; a single plant can deliver over 50,000 grains, which can still be practicable in the soil for 15-20 years (Kureh et al. 2003). The parasite is a serious pest that primarily impends maize production in Kenya, resulting in harvest losses of between 65 to100%. Degradation in soil fertility is one of the principal reasons for the increment in *Striga* incidence (Esilaba 2006). *Striga hermonthica* (Del.) Benth is most common on heavy soil, particularly in the densely populated parts of the Lake Victoria region of western Kenya; simultaneously, *S. asiatica* is noticed in the coast province and seriously damages upland rice (Esilaba 2006).

Research efforts have distinguished several administering options that effectively reduce *Striga* damage and emergence. These include host plant resistance, utilization of trap plants, and the improvement and maintenance of soil fertility through cereal-legume rotation/intercropping or application of organic or inorganic nitrogen (Kureh et al. 2003). However, because of ecological concerns, environmental health problems, and an increment in herbicide-resistant grass resulting from synthetic herbicides, considerable efforts in designing grass administering strategies of utilizing allelopathic mixtures as bioherbicides is considerable subdue grass is receiving examination (Zaji 2011).

Trap plants are non-host plants that induce Striga grains sprouting but are not parasitized by the grass (Kureh et al., 2003). They could generate suicidal grass sprouting, which decreases the soil's grain bank or attachment to the host when intercropped in maize (Othira et al. 2008). Some legume diversities, for example, chickpea, mung beans, cowpea (*Vigna unguiculata* L.), groundnut (*Arachis hypogaea* L.), and soybeans **(***Glycine max* Merrr.), possess the potential to generate suicidal sprouting of *S. hermonthica*, decrease attachment to host and increase the fertility of the soil (Kureh et al. 2003) by adding to the soil with nitrogen and organic matter.

The life cycle of *Striga* ssp. consists of five stages; sprouting, haustorium initiation, penetration of host tissue, physiological compatibility, and parasite accretion and maturation. Apart from normal grain sprouting requisites, the grass needs a chemical stimulation for sprouting and a second chemical signal to trigger haustorium formation, connecting *Striga* roots to its host for fount procuration (Othira et al. 2008). However, trap plants release chemicals that induce *Striga* ssp. Grain sprouting does not produce haustorium signals, nor are they attacked by the parasite (Othira et al. 2008).

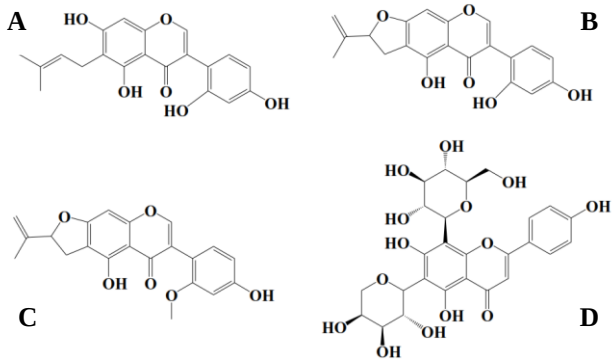

Figure 1. Segregated mixtures from *D. uncinatum* I.5,7, 2',4'-tetrahydroxy-6-(3-methylbut-2-enyl) isoflavanone (**A**), 4", 5"-dihydro-5,2',4'-trihydroxy-5"-isopropenylfurano-(2",3",7,6)-isoflavone (**B**), 4",5"-dihydro-2"-methoxy-5,4'-dihydroxy-5"-isopropenylfurano-(2",3",7,6)-isoflavone (**C**) and isochaftoside (**D**)

Even though the witch-grass, *Striga* ssp., is competent for photosynthesis once it grows, it depends on the host plant for an important portion of its carbon provision. Beyond the encumbrance of dispossessing diet and water to these parasites, host plants undergo a characteristic malady resembling severe drought symptoms, involving leaf scorching and increased root: shoot ratios as if a hex had befallen the crop the result of hormonal perturbation or toxic produced by the parasite. Invasion is generally much less severe, where water and soil fertility are optimal for crop accretion (Rich and Ejeta 2008).

Some understanding has been gained of the secondary metabolism included in the technique by which *desmodium* subdues *Striga* (Tsanuo et al. 2003). Phytochemical studies of various plants of *Desmodium* species have progressively segregated phenolic components (Tsai et al., 2011). From *Desmodium uncinatum* root, 2''-O-glucosylvitexin, vitexin, isovitexin, and apigenin have been segregated (Tsanuo et al. 2003; Guchu 2007).

Tsanuo et al. (2003) and Hooper et al. (2010) segregated three isoflavanones, 5, 7, 2',4'-tetrahydroxy-6-(3-methylbut-2-enyl)-isoflavanone, (**A**) 4",5"-dihydro-5,2',4'-trihydroxy-5"-isopropenyl furano-(2",3",7,6)-isoflavanone (**B**) and 4", 5"-dihydro-2'-methoxy-5,4'-dihydroxy-5"-isopropenylferano-(2",3",7,6)-isoflavone (**C**) from the root exudates of *D. uncinatum*. Segregated fractions comprising mixture B induced sprouting of *Striga* grains, while fractions comprising mixture C impeded radical accretion. Pickett et al. (2007) and Hooper et al. (2009) described another key haustorium accretion impeder, di-C-glycosylflavone-6-C-α-L-arabinopyranosyl-8-C-β-D-glucopyranosylapigenin, which is also known as isoschaftoside (**D**) from a polar fraction of *D. unicinatum* root exudates (Khan et al. 2010) (Figure 1).

The active components in *desmodium* root exudates described are suspected of having existed in traditional diet legumes. If resemblant characteristics were noticed in the diet legumes, then conventional breeding could be utilized to pick out and increase the characteristics. On the other hand, if these characteristics were lacking, a gene could be transferred directly from *Desmodium* into diet legumes via a genetic modification to allow legumes to subdue *Striga* spp. In the long term, it is likely to transfer the same characteristic to cereal plants through heterologous gene expression, especially to the open-pollinated diversities, rather than hybrids for more convenient availability to little scale farmers (Pickett et al. 2010).

The goals of this research were (i) to fractionate and segregate the secondary metabolites from leaf and root tissues of *C. arietinum* and the root of *V. radiata* extracts, utilizing column chromatography, TLC, and RP-HPLC; (ii) to describe the segregated mixtures from *C. arietinum* and *V. radiata* utilizing RP-HPLC and LC-MS.

MATERIALS AND METHODS

Study design

This was an experimental design in which chemical mixtures in aqueous extracts of leaves and roots of the legumes *C. arietinum* and *V. radiata* were segregated utilizing column chromatography and RP-HPLC and then described utilizing the RP-HPLC and LC-MS.

Samples and sampling procedure

Certified grains of *Cicer arietinum* L. (Desi (SCP 2), Kabuli (SCP 1) types), and *Vigna radiata* L. (KS 20) was gained from Simlaw Seeds Co., Nairobi, Kenya. The grains were cultivated in July 2011 at the Duduville campus of ICIPE, Nairobi. The plants were uprooted after 45 days to gain leaves and roots. The samples were cleaned in streaming water and then dried by air in a ventilated hood in the lab to wipe the moisture before extraction.

Chemicals and reagents

All the analytical reference mixtures involving isoschaftoside, 2-glucosylvitexin, 6-glucosylvitexin-, isovitexin, vitexin, naringenin, and apigenin were of analytical grade. Other utilized analytical chemical reagents were naturstoffreagenz A, normal silica gel, C_{18} silica gel, silica gel/TLC-plates, and silica gel C_{18} TLC-plates. While organic solvents, namely; methanol, ethyl acetate, formic acid, sulphuric acid, hexane, and butanol, were all gained from Sigma Aldrich, USA.

Instrumentation

A high-performance liquid chromatography (HPLC) comprising a quaternary pump, column compartment, auto-sampler, and variable wavelength photodiode array detector (Data system, version 1.24 ACE, Shimadzu Corporation England) was utilized. An analytical ACE, RP-C_{18} column (4.6mm i.d. × 2500 mm, 5µm particle size) and a preparative ACE, RP-C_{18} column (10mm i.d. × 2500 mm, 5µm particle size) (Hichrom LTD, England) were utilized on the process of analysis and separation. The mobile phase comprised formic acid in water (solvent **A**) and methanol (solvent **B**). A seven-step gradient analysis for a total run time of 65 min was utilized as follows: beginning with 95% solvent A and 5% solvent **B**, enhancing to 15% solvent **B** over 3min, then 25% solvent

B over 10 min, to 30% solvent **B** over 20 min, to 95% solvent **B** over 13 min and finally for 7 min 5% solvent **B**. The column compartment was kept at 20ºC utilizing 10-50µl injection volumes. The analytes were observed with PDA detection at between 225-350 nm.

The Liquid Chromatography-Mass Spectrometer utilized a quaternary LC pump (Model 1200) coupled to an Agilent MSD 6120-Single quadrupole mass spectrometer with an electrospray source. A Zorbax Eclipse Plus C_{18} column (4.6 × 100mm i.d., 3.5µm particle size) and a Zorbax narrow bore SB-C_{18} RRHT (2.1 × 50 mm i.d, 1.8µm particle size) (Agilent Technologies, USA) were applied in the analysis. The mobile phase was solvent **A**: 1% formic acid in H_2O and solvent **B**: 1% formic acid in MeOH. Analysis conditions, including a three-step isocratic analysis for a total run time of 29.91 min, were utilized as follows; beginning with 75% solvent **A** and 25% solvent **B**, enhancing to 100% solvent **B** over 25 min, then back to 25% solvent **B** over 2.9min. Injection volumes were 10-30 µl, and signals were needed in full-scan positive-ion mode utilizing a 100 to 800 *m/z* scan range.

Nine seemingly uncontaminated samples making concentrations higher than 0.001% in the plants were examined by LC-MS to ascertain their respective masses. LC-MS data were output utilizing a Hewlett-Packard 1090 photodiode array detector (Agilent Technologies) and a Perkin-Elmer SCIEX API III triple-quadrupole mass spectrometer (Perkin-Elmer, Toronto, Ontario, Canada) completed with an ion spray fount (ISV=5500, orifice voltage=50) in positive ion mode. Multiple reactions observing mode was utilized, observing the mass of the molecular (parent) ions in the first quadrupole (Q1) and scanning for the fragmented ions and flavonoids (daughter ions) in the third quadrupole (Q3). Argon was utilized as the collision gas, and nitrogen was utilized as the nebulizer gas and orifice curtain. The system was completed with a Synergi 4µ Hydro-RP 80 Å (250×2 mm) column fitted with a 4.0× 3.0 mm i.d. guard column (Phenomenex) at a flow rate of 1ml/min. The mobile phase was Solvent **A**: 0.1% formic acid in H_2O and Solvent **B**: 0.1% formic acid in MeOH. The analysis included a five-step gradient analysis for a total run time of 25 min and was utilized as follows; beginning with 90% solvent **A** and 10% solvent **B**, enhancing to 50% solvent **B** over 15 min, then 80% solvent **B** over 5 min to 95% solvent **B** over 2min and finally isocratic for 3 min. The injection volume was 20 µl, and the scanning wavelength ranged from 190-800nm. Both positive and negative ions between *m/z* 100 and *m/z* 1415 were monitored.

Laboratory procedures

Extraction

Utilizing a WTC binder 7200 drying oven, air-dried roots and leaves of *C. arietinum*L. and *V. radiata* L. were further dried at temperatures of 40º C for 48 hours. Each plant's brittle, dry material was ground in Retsch (GmbH 5667 HAAN West-Germany) grinder, weighed (Table 1), then distilled with 50:50 MeOH/CH_2Cl_2 at room temperature for 3 days in the ratio of one gram herbage: 7.5 ml solvent. Filtered to separate the filtrates from the residue, then the residual extraction was repeated twice utilizing the same solvent system. The filtrates were evaporated *in vacuo* and weighed. Each distillate was examined utilizing TLC and HPLC and then purified utilizing HPLC.

Fractionation and isolation

TLC analysis was implemented on silica plate (DC-Alufolien-Kieselgel 25F_{254nm}), and C_{18} (Alugram RP-18w/UV_{254}) utilizing mobile phase consisting of EtOAC-H_2O-HCO_2H (18:1:1), Hex-EtOAC (20:80 and 80:20). The mixtures were viewed under UV light and visualized after spraying with 5% sulphuric acid in methanol to increase spot detection. Then the TLC plates were dried at oven temperatures ranging between 80-100ºC.

Other solvent systems were examined in a bid to build up a column chromatography system to fractionate the constituents present in the distillate, and the mobile phase representing a better separation was picked out. The purifications of fractions were further performed by high-performance liquid chromatography (HPLC).

Preliminary experiments

Fingerprint chromatographic profiles of the two types of *C. arietinum*, Kabuli and Desi Chickpeas, were examined utilizing TLC plates and HPLC instruments. A comparison of the biochemical composition of aqueous extracts in the roots and leaves of the two plants was carried out based on the variation in concentration and existence or absence of secondary metabolites. Also, HPLC fingerprint profiles of *V. radiata* leaves and roots were gained.

A medium normal phase column of *C. arietinum* leaves was eluted utilizing other solvent systems of various concentrations and polarity from hexane, EtOAc, CH_2Cl_2, MeOH then Water to ascertain an appropriate solvent system for extracting polar mixtures from the legumes. TLC and HPLC examined the results. The solvent system at which polar secondary metabolites were eluted at substantial concentration was recommended for utilization in extraction.

On the other hand, the extraction of *C. arietinum* from leaves was performed by suspending the leaves in water and partitioning them with hexane, ethyl acetate, then butanol. Each distillate was examined utilizing TLC and HPLC to determine the best solvent giving a better separation of the mixtures, and utilized to assess the proportional quantities of various elements in the extracts.

Description of segregated mixtures

Isolates from each plant part were given codes in the order of their elution time/retention time. For example, CAL 1 is the first segregated peak from the crude distillate of *C. arietinum* leaf. The first two letters are the abbreviation of the plant's botanical name, and the third letter is the abbreviation of the part of the plant from which the segregation was gained.

The segregated mixtures were described utilizing the HPLC by analyzing the peak fineness of the segregated peaks, comparing their UV maximum wavelength

absorbance with standards and one to another, and utilizing retention time proportional to that of known standards. LC-MS analysis was utilized to gain molecular ions and characteristic fragments utilizing MS data of the legumes' pure extractable.

A chemical test for flavonoid glycosides was carried out utilizing filter papers dipped in an ethanol solution of the individual mixture and then exposed to ammonia vapor. The formation of yellow spots on filter paper showed the existence of flavonoid glycoside (Ahmed 2007).

Utilizing the HPLC, the segregated mixtures' retention times and UV λ_{max} were compared with known flavonoid glycosides to determine their nature at a LOD of 0.25 µg/ L compared to the chemical tests for flavonoid glycoside with a higher LOD.

Spectrophotometric description

The initial spectral scan of phenolics from nine samples of the leaves and roots of *C. arietinum* and *V. radiata* was carried out by combining the data from PDA and MS to permit easy and efficient identification. Three screening schemes were utilized as follows:

Firstly, where reference mixtures were accessible as standards, the existence or absence of a particular biophenol was appraised by comparing retention time and UV–visible spectra with that of the reference. Where the retention times were noticed to be alike, co-injection and comparison of the UV-visible spectra were carried out. Molecular mass data further verified the existence.

Secondly, when reference mixtures were not accessible, the TIC traces in positive mode at soft ionization and strong ionization conditions were monitored for relevant, suitable pseudomolecular ions $[M+H]^+$. Verification was carried out by analyzing UV–visible spectra and mass spectral fragmentation data at the expected elution time, depending on pre-identified mixtures in the sample, the structure of the target mixture, and the literature data.

Thirdly, major peaks in TIC and UV chromatograms that were not distinguished by the other screening processes were screened for novel mixtures by generating mass spectra. In all, screening was performed for nine biophenols from different classes. The principal objective was to show the power of mixed utilization of HPLC–PDA and ESI-MS in profiling legume extracts.

Samples were injected into an LC-MS system, allowing spectral analysis followed by mass ascertainment to ascertain the moieties attached to the glycoside.

RESULTS AND DISCUSSION

Method validation and preliminary results

Sample harvest

The ground leaves and roots of the *C. arietinum* and *V. radiata* plants were weighed and noted. Further, the parts of plants were distilled utilizing 50/50 MeOH/CH_2Cl_2 and evaporated *in vacuo*, then noted (Table 1).

The harvest was not optimal for *V. radiata* because of the attack of cat worms on the plantation. The distillate weights above were utilized to ascertain the capacity and amount of packing material used in column chromatography.

Fingerprints

Two different chickpea grains were grown separately on the field; the plantations were similar in the first 30 days. But on flowering, the Desi (SCP 1) chickpea owned purple flowers, while Kabuli (SCP 2) chickpeas had white flowers. Figure 2 shows TLC and HPLC chromatograms of the other parts of the plants.

Table 1. Harvest of the crude sample and extracts

Sample name	Powder (grams)	Distillate (grams)	% harvest
C. arietinum leaves	346.3	71.6	21
C. arietinum roots	59.0	6.3	11
V. radiata leaves	275.8	25.8	10
V. radiata roots	67.7	3.2	5

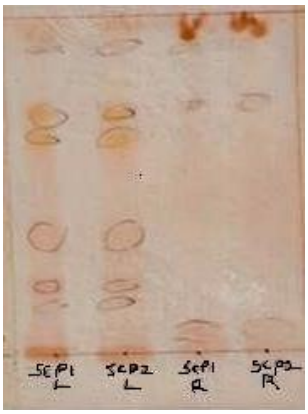

Figure 2.A. TLC card I, comparison of composition in *Cicer arietinum* leaves and roots (SCP 1 & SCP2)

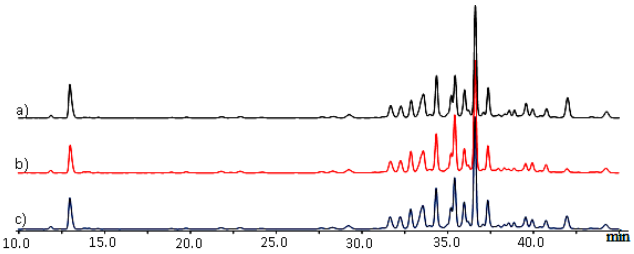

Figure 2.B. HPLC *Cicer arietinum* leaves profiles of a) Kabuli (SCP 2);b) Desi (SCP1); c) Mixture

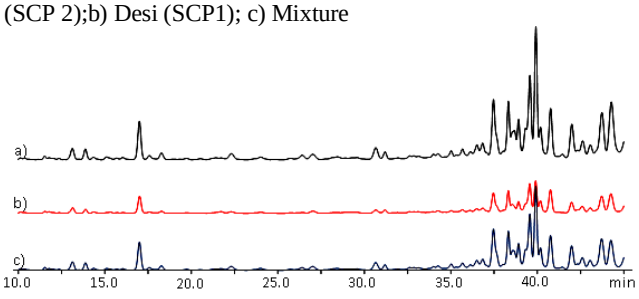

Figure 2.C. HPLC *Cicer arietinum* roots profiles of; a) Kabuli (SCP 2); b) Desi (SCP1); c) mixture

There were varieties in the amounts of mixtures found in the two plants, similarity in the TLC retention factors of eluted mixtures, and fingerprint HPLC profiles between the two types of plants showed the same aqueous chemical elements. Therefore, the resembling plant parts were mingled and examined as leaves and roots of *C. arietinum.*

Likewise, to view the aqueous secondary metabolites found in the *V. radiata* plant, see the HPLC profile of leaves and roots at 270 nm and 350 nm absorbance below (Figures 3.A and B).

Obviously, few are the polar mixtures in substantial amounts in the two parts of the plant; *V. radiata*. Even though it was likely to segregate the present mixtures utilizing the HPLC, the process was complicated because the plant possesses a raged profile (especially the root profile; the 270 nm profile is very dissimilar from the 350 nm one) mixture is in a very small amount.

Solvent system

Other solvent systems were examined for extraction to ensure maximum collection of the polar secondary metabolite from the plants. A 9.6 g portion of *C. arietinum* leaves had been distilled utilizing 80/20 MeOH/H_2O. 1 g of the distillate was suspended in water and partitioned with hexane, ethyl acetate, and butanol. The weights of fractions were noted (Table 2).

Alongside the crude sample, the separation in four fractions was examined on silica gel C_{18} TLC-plate (Figures 4.A), mobile phase; EtOAc-H_2O-HCO_2H 18:1:1. Further, HPLC chromatograms (Figure 4.B) were gained.

Likewise, 1.4 g of *C. arietinum* leaves distillate was liquefied in 50/50 MeOH/CH_2Cl_2. The soluble part of the sample (1.0 g) was fixed in 2 g of silica gel. Utilizing a 1/30 sample: silica gel ratio, a 42.3 g capacity column was packed with 31.0 g silica gel liquefied in hexane. The mobile phase varied from hexane, ethyl acetate, dichloromethane, and methanol to water. 30 fractions of 20 ml each were gained. A TLC C_{18} card was spotted after fractionation and utilizing the mobile phase; EtOAc-H_2O-HCO_2H 18:1:1 spots were developed (Figure 5.A). Eight fractions were pooled as shown on the TLC card below and then examined utilizing the HPLC (Figure 5.B).

These experiments found no separation of mixtures utilizing neither fractionating funnel nor normal phase column chromatography. From the hexane fraction, no polar mixtures were noticed. While in the butanol and water fractions, all the mixtures existing in the crude sample were found at low amounts. Ethyl acetate yielded an enhanced concentration of some mixtures existing in the original sample.

From the column chromatography, it was examined that methanol dichloromethane solvent systems liquefy most polar mixtures. Therefore, extraction of the samples was carried out utilizing 50/50 MeOH/CH_2Cl_2, and column chromatography was utilized for fractionation and purification of the samples. It was suggested that before utilizing the HPLC for the segregation of peaks, column chromatography should be carried out at least twice for every sample for purification, enhanced amounts of present mixtures, and higher resolution.

Spectroscopic study of reference biophenols

A series of flavonoid standards (Figure 6), representative of other flavonoid groups previously reported in allelopathic *D. uncinatum* and other legumes, utilizing HPLC-PDA and RP-LC-ESI-MS, were observed to determine their structure/spectra. From HPLC-PAD, various correlations between structure and UV spectrum were distilled (Table 3).

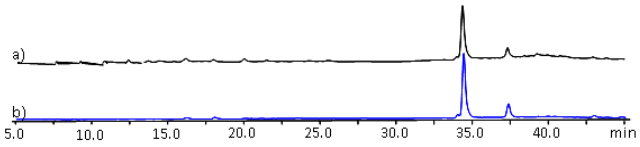

Figure 3.A. HPLC profiles of V. radiata leaves at a) at 270 nm absorbance, b) at 350 nm absorbance.

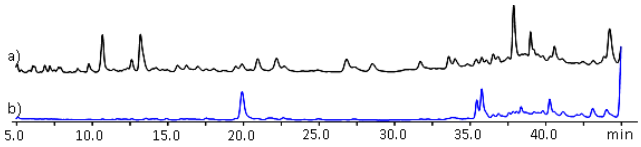

Figure 3.B. HPLC profiles of V. radiata roots at a) at 270 nm absorbance, b) at 350 nm absorbance.

Table 2. Weights of 1g *Cicer arietinum* leaf partitioned

Fraction	Weight in (mg)	Percentage weight (%)
Hexane	27.8	2.8
Ethyl Acetate	50.0	5
Butanol	20.0	2
Water	25.1	2.5

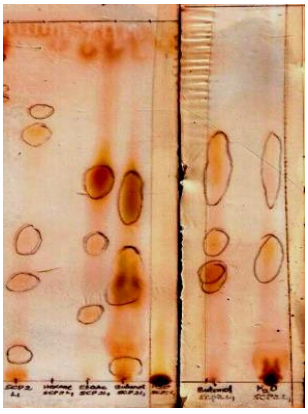

Figure 4.A. TLC card II, *C. arietinum* leaf fractionation utilizing a fractionating funnel

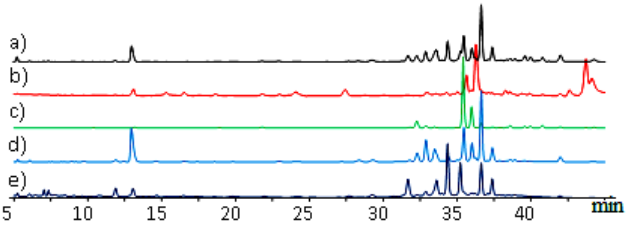

Figure 4.B. Fractionating funnel's HPLC profile at 270 nm of *Cicer arietinum* leaf a) Crude sample; b) Hexane fraction; c) Ethyl acetate fraction; d) Butanol fraction; e) Water fraction.

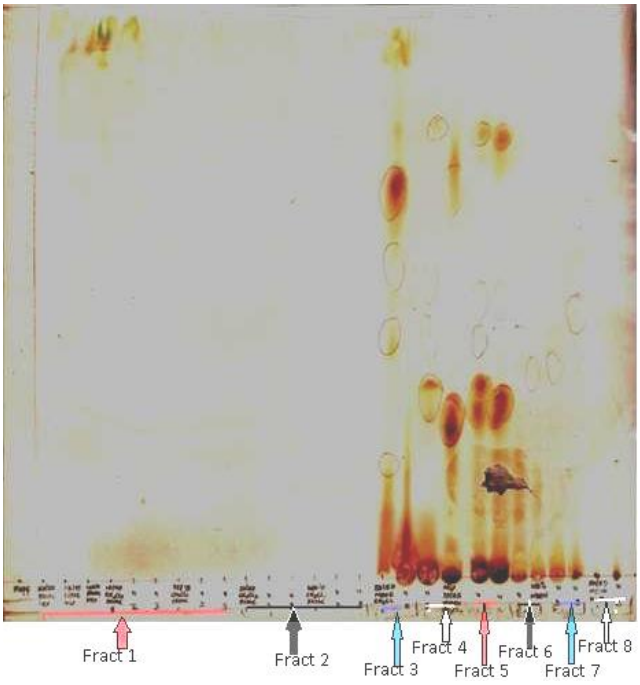

Figure 5.A. TLC card III, Preliminary column chromatography fractionation of *Cicer arietinum* leaf

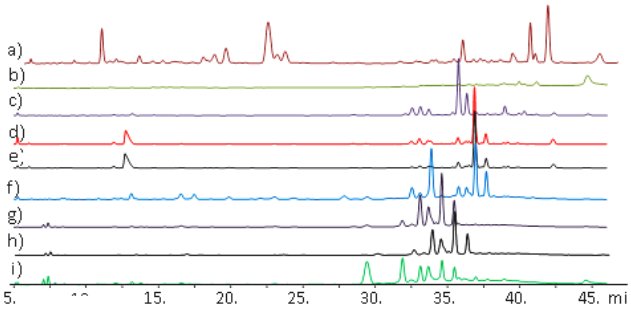

Figure 5.B. Preliminary analysis utilizing HPLC profiles at 270 nm of *Cicer arietinum* leaves fractions a) Crude sample; b) Fraction 1 c) Fraction 2; d) Fraction 3; e) Fraction 4; f) Fraction 5; g) Fraction 6; h) Fraction 7; Fraction 8

Table 3. Reference biophenols

Cording	Flavonoid standards	Retention time T_R (min)	Maximum absorbance (λ_{max}) (nm)	Molecular weight $[M]^+$
1	Genistein	8.89	212, 225, 229, 280	270
2	6-glucosylvitexin	17.83	219, 239, 334	594
3	Isoschaftoside	21.99	218, 239, 270, 336	564
4	Vitexin	24.64	214, 267, 343	432
5	2-glucosylvitexin	26.80	207, 239, 268, 334	594
6	Isovitexin	30.51	214, 239, 267, 343	432
7	Naringenin	36.68	213, 288	273
8	Luteolin	38.93	225, 256, 292, 349	286
9	Apigenin	41.74	212, 238, 267	270
10	Quercetin dihydrate	50.98	238, 258	302

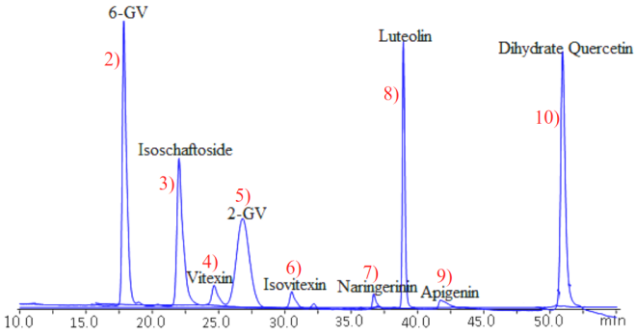

Figure 6. A simultaneous HPLC chromatogram was obtained with a photodiode array detector set at 350nm (A); Peak identification was listed in Table 3 (B)

The most influential flavonoids are the λ_{max} (a band I), and it increases in the series: flavones (apigenin) < flavonol (quercetin). The λ_{max} also indicated the substitution within the same class for glucosylvitexins, vitexin, and isovitexin. The permeation λ_{max} was not affected in isomers of the same group (e.g., vitexin and isovitexin) Table 3.

Fractions and isolates of secondary metabolites

Cicer arietinum roots

Column chromatography I. Flash fractionation column chromatography was utilized to fractionate *C. arietinum* roots (Table 4). The fractions were examined by HPLC (Figure 7) obtained with a photodiode array detector set at 270 nm.

No polar mixtures were examined using mobile phases (d, e, f, and g). The separation was poor in fraction (h), where the mixtures were not concentrated. Still, where the concentration was better, isolation of individual peaks would be complicated because the mixtures were very close to each other. In fractions (I, j, k, and l), the clarity and concentration of the mixtures were good. Therefore, it was suggested that a smaller column comprising a mixture of the four fractions should be mounted to purify the fractions further.

Table 4. Fractionation on the 1st column of *C. arietinum* roots

Fraction	Solvent System	% Conc. of extraction solvent	Weight of dry Sample in (g)	Percentage weight (%)
Crude	-	-	5.5	
0	EtOAc	100	1.77	32.2
1	EtOAc	100	0.18	3.3
2	$EtOAc/CH_2Cl_2$	75	0.06	1.1
3	$EtOAc/CH_2Cl_2$	50	0.05	0.9
4	$EtOAc/CH_2Cl_2$	25	0.03	0.5
5	CH_2Cl_2	100	0.05	0.9
6	CH_2Cl_2/MeOH	95	0.24	4.4
7	CH_2Cl_2/MeOH	90	0.86	15.6
8	CH_2Cl_2/MeOH	80	1.27	23.1
9	CH_2Cl_2/MeOH	50	1.40	25.5
10	CH_2Cl_2/MeOH	25	0.30	5.5
11	MeOH	100	0.17	3.1
12	$MeOH/H_2O$	50		
13	H_2O	100		

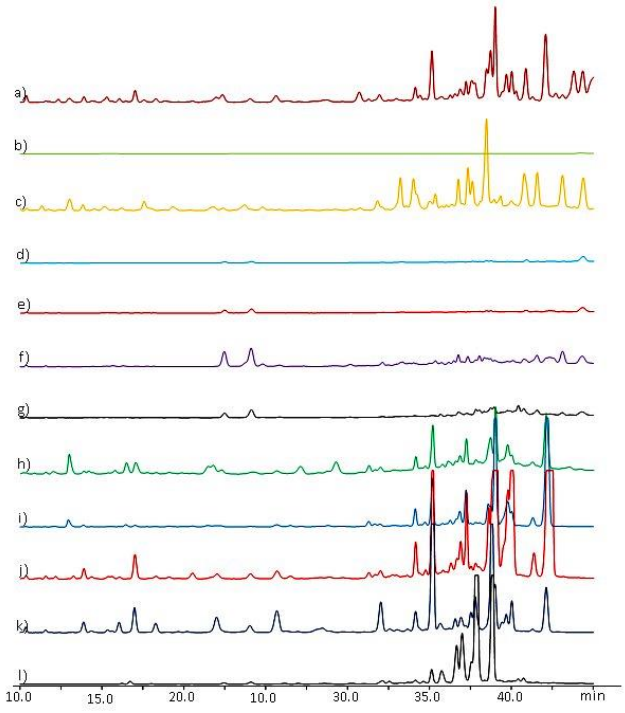

Figure 7. HPLC profiles of *C. arietinum* roots fractions from 1st column. a) Crude sample; b) 100% EtOAc-0; c) 100% EtOAc-1; d) EtOAc/CH_2Cl_2 75/25; e) EtOAc/CH_2Cl_2 50/50; f) EtOAc/CH_2Cl_2 25/75; g) 100%CH_2Cl_2;h) MeOH/CH_2Cl_2 25/75; i) MeOH/CH_2Cl_2 50/50; j) MeOH/CH_2Cl_2 80/20;k) MeOH/CH_2Cl_2 90/10; l) MeOH/CH_2Cl_2 95/5

Column chromatography II. A second 600 ml column was packed, and 4 fractions each of 20 ml were eluted utilizing 95/5, 90/10, 85/15, 80/20, then 50/50 MeOH/CH_2Cl_2, respectively. Each fraction was spotted on silica gel/TLC cards (Figure 8). The spots were developed utilizing a mobile phase comprising EtOAc-H_2O-HCO_2H (18:1:1). Further HPLC profiles were obtained with a photodiode array detector set at 270 nm (Figure 9) of 6 fractions pooled as shown on the TLC card were gained on the PDA detector. The better resolution was examined in fractions d), e), and f). Utilizing HPLC, well-resolved peaks of substantial concentration in the fractions were suggested for isolation.

Description of isolates. Utilizing an HPLC on a PDA detector at 270 nm and a preparative RP-C_{18} column (10 mm i.d. × 250 cm, 5 µm particle size), six fractions were gained, and weights were noted (Table 5). HPLC profiles on the analytical column were obtained with a photodiode array detector set at 270 nm (Figure 10).

Utilizing the LC-MS, TIC traces in positive mode at soft ionization and strong ionization conditions were monitored, and relevant pseudomolecular ions $[M+H]^+$ were gained. Results are presented in Table 5.

Analysis utilizing the RP-HPLC showed that all the six extracts; CAR 1, CAR 2, CAR 3, CAR 4, CAR 5, and CAR 6, presented characteristics of single peaks at their respective retention times. Meaning that they were good enough to be described utilizing the LC-MS. CAR 5 and CAR 6 were enough for further analysis.

On further analysis, utilizing the LC-MS, three of the mixtures that TIC scans presented with single peaks could be distinguished. Still, only CAR 5had a flavonoid aglycone based on the Phenol-Explorer database (Anon 2012) was distinguished in this study.

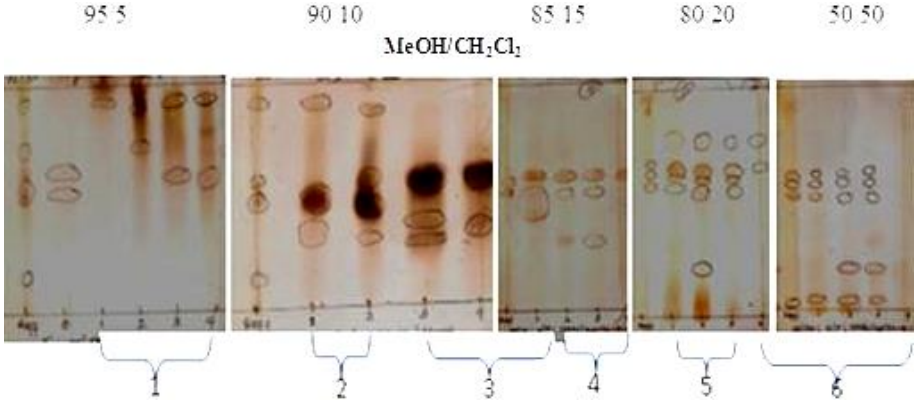

Figure 8. TLC card IV, C. arietinum root fractions from 2nd column chromatography

Table 5. HPLC, LC-MS, and Flavonoid test analysis of *Cicer arietinum* root distillate

Fraction	Weight in (mg)	% of distil-late	Retention Time (min)	UV λmax (nm)	$[M]^+$	Purity	Ammo-nia test
CAR 1	0.34	0.005	16.6~17.0	222,273,318	470/590	Impure	-ve
CAR 2	0.70	0.010	27.5~27.9	224,272	484	Impure	-ve
CAR 3	0.30	0.005	27.8~28.6	223,239,262	472	Pure	-ve
CAR 4	0.22	0.004	31.5~31.8	228,260	484	Pure	-ve
CAR 5	13.54	0.215	34.6~35.0	236,254	430	Pure	+ve
CAR 6	13.06	0.207	38.2~38.6	224,239,260,325	390/468	Impure	-ve

Cicer arietinum leaves

Column chromatography I. A 1020 ml capacity column was packed, and 40 fractions, 200 ml each, of *C. arietinum* leaves were collected (Table 6) with the varying polarity of the eluting solvent from hexane to methanol. The mobile phase was altered only at the time there was no more examinable decomposition following analysis by TLC.

Utilizing mobile phase EtOAc-H_2O-HCO_2H 18:1:1 (Figure 11.A), the spots were spread, then a repeat of the first 27 fractions was carried out utilizing EtOAc: Hex; 80:20 (Figure 11.B).

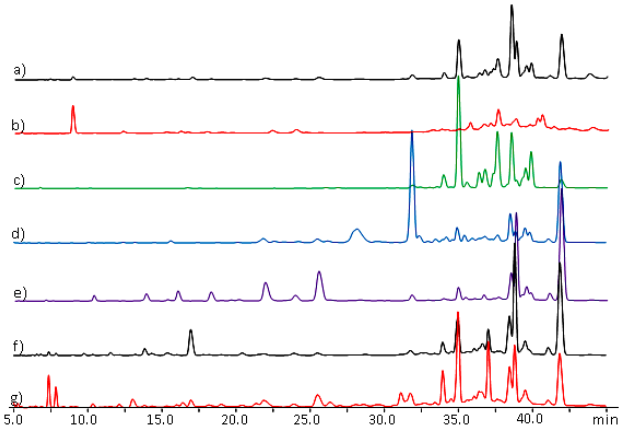

Figure 9. HPLC roots profiles of fractions from *Cicer arietinum* 2nd columncrude sample; b) Fraction 1; c) Fraction 2; d) Fraction 3; e) Fraction 4;f) Fraction 5; i) Fraction 6

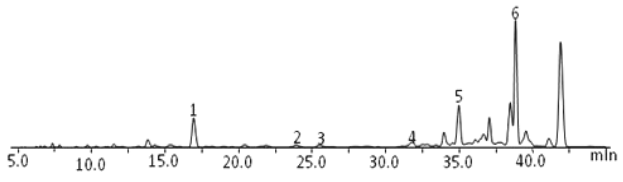

Figure 10. Segregated mixtures from *Cicer arietinum* roots

Table 6. Fractionation on the 1st column of *Cicer arietinum* leaf

Fraction	Solvent system	Percentage conc.	Weight of dry sample	Percentage harvest
CRUDE			54.33	
1	Hexane	100	1.23	2.30
2	Hexane	100	0.50	0.90
3	Hexane/EtOAc	90	0.23	0.40
4	Hexane/EtOAc	90	0.25	0.50
5	Hexane/EtOAc	90	0.15	0.30
6	Hexane/EtOAc	90	0.22	0.40
7	Hexane/EtOAc	50	0.13	0.20
8	Hexane/EtOAc	50	2.22	4.10
9	Hexane/EtOAc	50	0.38	0.70
10	Hexane/EtOAc	50	0.36	0.70
11	Hexane/EtOAc	50	0.03	0.05
12	Hexane/EtOAc	50	0.02	0.04
13	Hexane/EtOAc	50	0.14	0.30
14	EtOAc	100	0.05	0.09
15	EtOAc	100	0.04	0.07
16	EtOAc	100	0.08	0.15
17	EtOAc	100	0.12	0.20
18	EtOAc	100	0.23	0.40
19	EtOAc	100	0.34	0.60
20	EtOAc/CH2Cl2	50	0.30	0.55
21	EtOAc/CH2Cl2	50	0.25	0.50
22	EtOAc/CH2Cl2	50	0.14	0.30
23	CH2Cl2	100	0.04	0.07
24	CH2Cl2	100	0.03	0.05
25	CH2Cl2	100	0.01	0.02
26	CH2Cl2/MeOH	90	0.02	0.04
27	CH2Cl2/MeOH	90	0.04	0.07
28	CH2Cl2/MeOH	90	3.63	6.70
29	CH2Cl2/MeOH	80	4.70	8.70
30	CH2Cl2/MeOH	80	5.21	9.60
31	CH2Cl2/MeOH	80	5.31	9.80
32	CH2Cl2/MeOH	80	5.40	9.90
33	CH2Cl2/MeOH	80	2.74	5.0
34	CH2Cl2/MeOH	50		
35	CH2Cl2/MeOH	50		
36	CH2Cl2/MeOH	50		
37	MeOH	100		
38	MeOH	100		
39	MeOH	100		

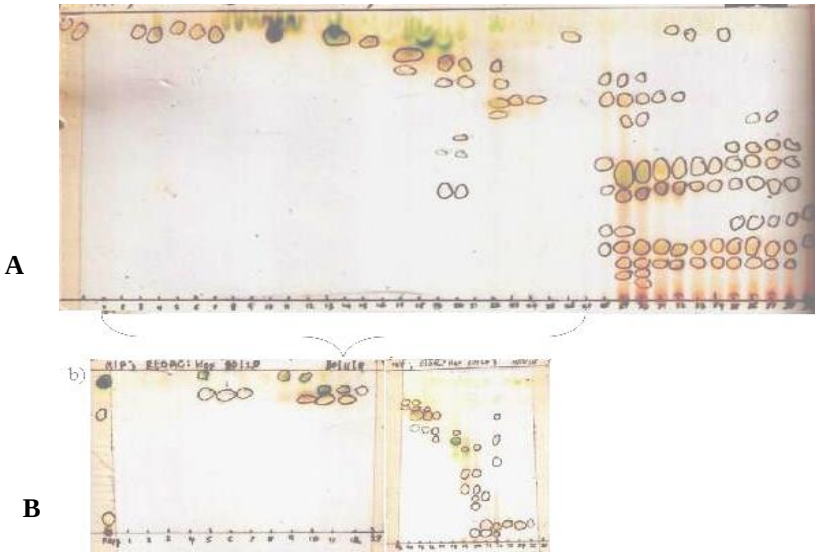

Figure 11.A-B. TLC cards V; Fractions from the 1st column chromatography of *Cicer arietinum* leaf

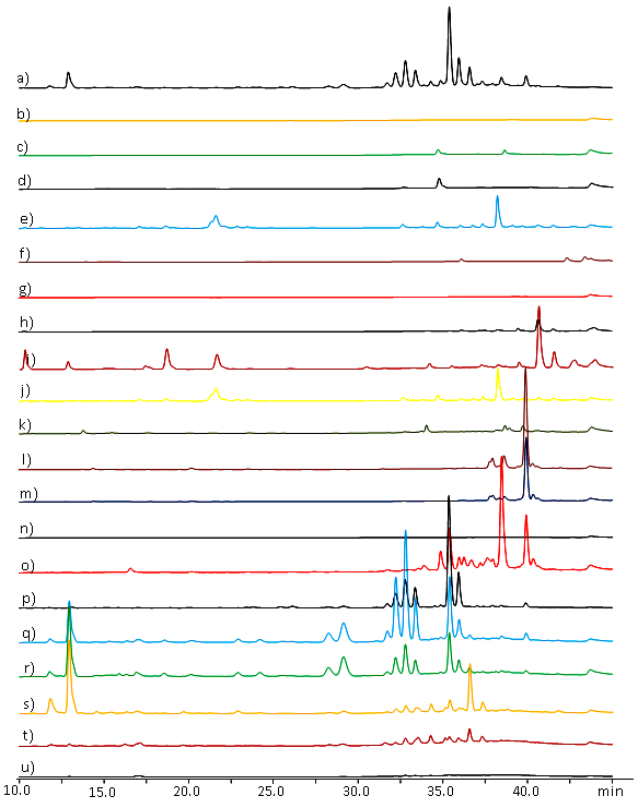

Figure 12. HPLC profiles of 1st column chromatography of *Cicer arietinum* leaf. a) Crude sample; b) Fraction 2; c) Fraction 4; d) Fraction 6; e) Fraction 8; f) Fraction 10; g) Fraction 12; h) Fraction 14; i) Fraction 16; j) Fraction 18; k) Fraction 20; l) Fraction 22; m) Fraction 24; n) Fraction 26; o) Fraction 28;p) Fraction 30; q) Fraction 32; r) Fraction 34; s) Fraction 36; t) Fraction38;u) Fraction 40

Only the even-numbered fractions on the TLC card were examined on HPLC and were obtained with a photodiode array detector set at 270 nm (Figure 12).

The mixtures were primarily eluted in fractions 29-33. Therefore, these fractions were pooled, dried, and a reversed-phase column packed and eluted with ethyl acetate, dichloromethane and methanol while slowly changing the percentage concentrations of solvents (Table 7).

Column chromatography II. The sample from fractions 29-33 weighing 23.4 g was fixed in C_{18} silica gel and a reversed-phase column packed and then eluted with ethyl acetate, dichloromethane and methanol while slowly varying the percentage concentrations of the solvent system. Results are presented in Table 7.

Fourteen fractions were spotted on silica gel TLC card IV, and spots were expanded utilizing mobile phase; EtOAc-H_2O-HCO_2H 18:1:1 (Figure 13).

HPLC analysis of four was relatively similar to the 14 fractions, as shown on the TLC card (Figure 14).

The majority of the mixtures concerning the crude sample were accessible in fraction D with better separation. The fractions involving fractions 12, 13, and 14 had been eluted utilizing a mobile phase of concentration varying from 30/70 to 40/60 MeOH/CH_2Cl_2. This fraction was suggested for further fractionation utilizing the HPLC.

Description of isolates. A shorter gradient elution technique was optimized from the original 65 min technique that lasted 49 minutes. 11 extracts were gained; Figure 15 shows the HPLC peak profile obtained with a photodiode array detector set at 270 nm.

Table 7. Fractionation of *Cicer arietinum* leaf on the 2nd column chromatography

Fraction	Solvent System	Percentage Conc.	Weight of dry sample	Percentage Harvest
1	EtOAc	100	0.52	2.2
2	EtOAc	100	0.71	3.0
3	EtOAc/CH_2Cl_2	50	0.33	1.4
4	CH_2Cl_2	100	0.10	0.4
5	CH_2Cl_2	100	0.12	0.5
6	CH_2Cl_2	100	0.02	0.09
7	CH_2Cl_2/MeOH	90	2.15	9.2
8	CH_2Cl_2/MeOH	90	1.34	5.7
9	CH_2Cl_2/MeOH	90	2.07	8.8
10	CH_2Cl_2/MeOH	80	1.37	5.9
11	CH_2Cl_2/MeOH	80	0.86	3.7
12	CH_2Cl_2/MeOH	70	1.49	6.4
13	CH_2Cl_2/MeOH	70	1.55	6.6
14	CH_2Cl_2/MeOH	60	0.21	0.9

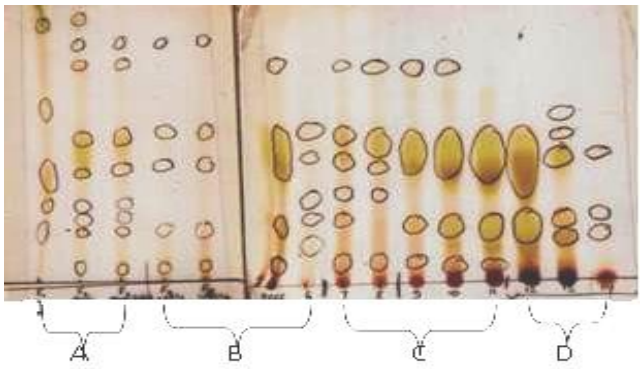

Figure 13. TLC cards VI; Fractions from the 2nd column of *Cicer arietinum* leaf

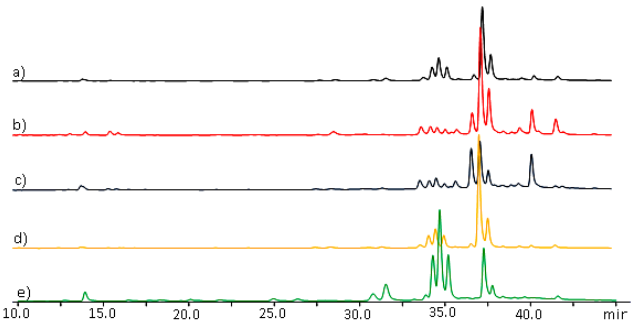

Figure 14. HPLC profiles of fractionations from 2nd column chromatography of *Cicer arietinum* leaf II. a) Crude sample; b) Fraction A; c) Fraction B; d) Fraction C; e) Fraction D

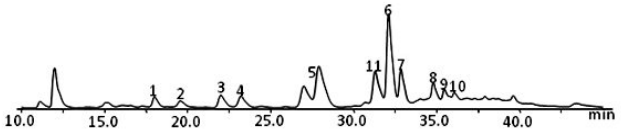

Figure 15. The segregated peaks from *Cicer arietinum* leaf's fraction D

Utilizing the LC-MS, TIC traces in positive mode at soft ionization (25ºeV) and strong ionization (40ºeV) conditions were monitored, and relevant pseudomolecular ions $[M+H]^+$ were gained. Weights of purified samples and their respective molecular ions are shown in Table 8.

Analysis utilizing the RP-HPLC showed nine extracts; CAL 1, CAL 2, CAL 3, CAL 4, CAL 7, CAL 8, CAL 9, CAL 10, and CAL 11, which were substantial concentrations and gave characteristics of single peaks at their respective retention times. It meant that they were good enough to be described utilizing the LC-MS. But CAL 5 and CAL 6 each produced twin peaks that were inseparable utilizing HPLC, and therefore, they were not good enough to be described utilizing the LC-MS.

Utilizing the LC-MS, seven of the above mixtures, which TIC scans gave single peaks each and had flavonoid aglycone based on the Phenol-Explorer database (Anon 2012), were distinguished in this research. Though pure, CAL 1 and CAL 10 were not distinguished in this research since their aglycone is not of any reported flavonoid.

Vigna radiata roots

Column chromatography. A 270.7 ml normal phase column was packed, and 2.5 g of *V. radiata* root distillate was eluted utilizing solvents of varying polarity from hexane to methanol. The results are presented in Table 9.

In gathering the fractions, the samples were spotted on pieces of silica gel TLC cards. Utilizing relevant mobile phases, 20:80, 80:20 Hex: EtOAc and EtOAc-H_2O-HCO_2H 18:1:1, the spots were expanded (Figure 16).

Comparing the number of fractions per spot and the pigmentation of the eluted spots per sample (Figure 16) above, the fractions were pooled into seven fractions. Then utilizing the HPLC set at 270 nm, the samples were examined (Figure 17). The peaks in fraction F were segregated utilizing the HPLC instrument.

Description of isolates. Utilizing a short technique that lasted 30 minutes, eight peaks were each segregated from the roots of *V. radiata* (Table 10). Utilizing the LC-MS, TIC traces in positive mode at soft ionization and strong ionization conditions were monitored, and relevant pseudomolecular ions $[M+H]^+$ were gained.

Table 9. Fractionation of *Vigna radiata* root utilizing column chromatography

Combi-ned fraction	Fraction	Solvent system	% Conc.	Combined weight (g)	% Harvest
A	1	Hexane/EtOAc	50	0.16	6.4
	2	Hexane/EtOAc	50		
	3	Hexane/EtOAc	50		
B	4	Hexane/EtOAc	50	0.06	2.4
	5	Hexane/EtOAc	50		
	6	Hexane/EtOAc	50		
	7	EtOAc	100		
	8	EtOAc	100		
C	9	EtOAc	100	0.24	10.0
	10	EtOAc/CH_2Cl_2	50		
	11	EtOAc/CH_2Cl_2	50		
	12	EtOAc/CH_2Cl_2	50		
	13	CH_2Cl_2	100		
	14	CH_2Cl_2	100		
D	15	CH_2Cl_2	100	0.0012	0.05
	16	CH_2Cl_2/MeOH	90		
	17	CH_2Cl_2/MeOH	90		
	18	CH_2Cl_2/MeOH	80		
	19	CH_2Cl_2/MeOH	80		
	20	CH_2Cl_2/MeOH	80		
E	21	CH_2Cl_2/MeOH	80	0.33	13.2
	22	CH_2Cl_2/MeOH	80		
	23	CH_2Cl_2/MeOH	80		
	24	CH_2Cl_2/MeOH	80		
	25	CH_2Cl_2/MeOH	80		
	26	CH_2Cl_2/MeOH	50		
	27	CH_2Cl_2/MeOH	50		
	28	CH_2Cl_2/MeOH	50		
	29	CH_2Cl_2/MeOH	30		
	30	CH_2Cl_2/MeOH	30		
	31	CH_2Cl_2/MeOH	30		
	32	CH_2Cl_2/MeOH	30		
F	33	CH_2Cl_2/MeOH	30	1.2	48.0
	34	CH_2Cl_2/MeOH	30		
	35	MeOH	100		
	36	MeOH	100		
	37	MeOH	100		
	38	MeOH/H_2O	50		

Table 8. HPLC, LC-MS, and Flavonoid test analysis of *Cicer arietinum* leaf extracts

Fraction	Weight in (mg)	%age of extract	RT (min)	UV λ_{max} (nm)	$[M]^+$	Purity	NH_3test
CAL 1	0.54	0.0008	17.9~18.3	224, 239,265, 345	610	Pure	-ve
CAL 2	1.80	0.0025	19.3~19.7	222, 238,255, 352	640	Pure	+ve
CAL 3	1.70	0.0024	21.7~22.1	225, 238,257, 353	626	Pure	+ve
CAL 4	4.00	0.0056	22.9~23.3	230, 265,325, 337	610	Pure	+ve
CAL 5	4.50	0.0063	25.6~26.0	236,265,347	-	Impure	+ve
			26.0~26.4	244, 351			
CAL 6	1.83	0.0026	29.1~29.5	232, 254,354	-	Impure	+ve
			29.8~30.2	236, 265,347			
CAL 7	1.70	0.0024	32.5~32.9	238, 255,352	609	Pure	+ve
CAL 8	2.40	0.0034	34.5~34.9	230, 265,337	448	Pure	+ve
CAL 9	0.33	0.0005	36.5~36.9	220, 244,264, 348	448	Pure	+ve
CAL 10	0.09	0.0001	37.7~38.1	224, 239,265, 348	-	Impure	-ve
CAL 11	0.25	0.0004	29.4~29.8	218, 244,348	610	Pure	-ve

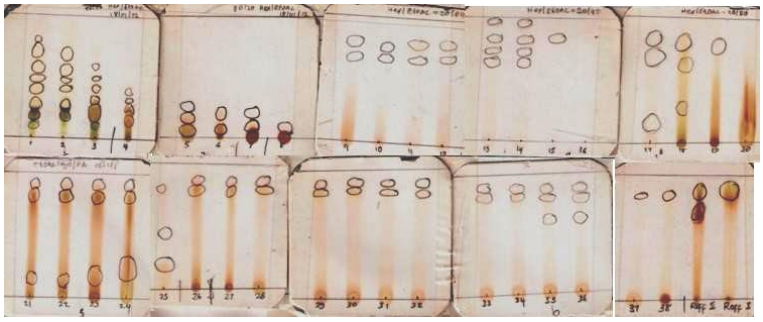

Figure 16. TLC card VII; fractionation of *Vigna radiata* root by column chromatography

Table 10. HPLC, LC-MS, and flavonoid test analysis of *Vigna radiata* root extracts

Fraction	Weight in (mg)	% of distillate	RT (min)	UVλmax (nm)	Molecular mass [M]+	Purity	NH3test
VRR 1	1.64	0.05	5.1~5.5	227,257,324	-	Impure	-ve
VRR 2	1.50	0.05	9.8~10.2	226,244,272	-	Impure	-ve
VRR 3	2.90	0.09	10.5~10.9	230,247,295	322, 484,623	Impure	-ve
VRR 4	1.70	0.05	12.8~13.2	229,257	-	Impure	-ve
VRR 5	2.60	0.08	13.9~14.3	226,250	-	Impure	-ve
VRR 6	1.70	0.05	16.4~16.8	219,256,282,319	594	Pure	-ve
VRR 7	1.75	0.06	18.0~18.4	226,314	146, 311	Impure	-ve
VRR 8	2.41	0.08	19.7~21.0	240,299,335	382, 461, 487, 562,592	Impure	-ve

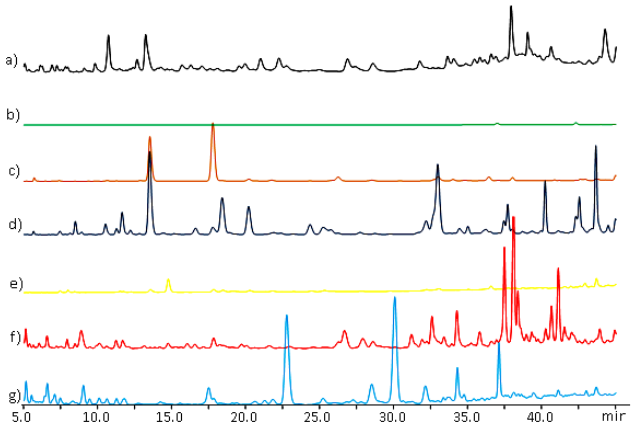

Figure 17. HPLC profiles of *Vigna radiata* root's fractions a) Crude sample; b) Fraction A; c) Fraction B; d) Fraction C; e) Fraction D; f) Fraction E;g) Fraction F

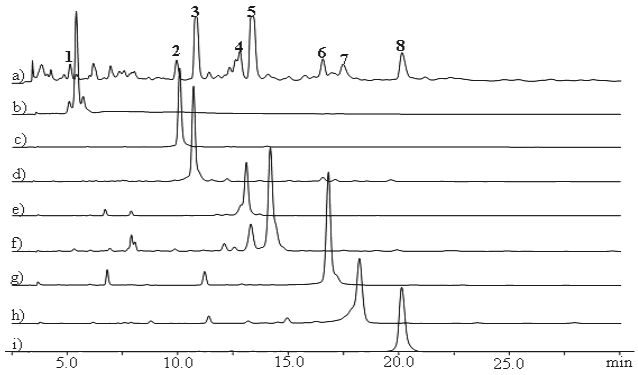

Figure 18. HPLC profile of segregated peaks from *Vigna radiata* roots' fraction F. a) Fraction F; b) VRR 1; c) VRR 2; d) VRR 3; e) VRR 4; f) VRR 5; g) VRR 6; h) VRR 7; i) VRR 8

Analysis utilizing the RP-HPLC showed all the eight fractions; VRR 1, VRR. 2, VRR 3, VRR 4, VRR 5, VRR 6, VRR 7, and VRR 8, and they were of substantial concentration and gave characteristic of single peaks at their respective retention times (Figure 18). It meant that they were all good enough to be described utilizing the LC-MS.

Though all these fractions showed negative results on the ammonium test, VRR 6 had flavonoid aglycon based on the database.

Utilizing the LC-MS, only VRR 6 TIC scans showed a single peak and had flavonoid aglycone based on the Phenol-Explorer database (Anon 2012). It was the only one of the fractions distinguished in this research. The rest of the isolates' TIC scans showed multiple peaks; hence several molecular masses were examined for these fractions.

Though unidentified, VRR 3 showed 3 peaks on the TIC scans, in which one of the peaks had a molecular mass of 484, portraying characteristics of C-Glucosylation characteristics. Fragmentation patterns divulged by the MS technique (Figure 19) supplied structural information about inter glycosidic linkages and aglycone replacement (Andersen 2006). Usually, fragments 162 and 132 amu are gained when the sugar is connected to the aglycone through an O-link. But a direct connection to the aglycone shows characteristic fragmentation of 120 or 90 amu (Andersen 2006). The integrity of the aglycone structure is kept at the expense of the glycan for C-glucosides (Raymond et al. *20*06). Two fragmentations were examined on this sample's glycan.

Identity of the mixtures

Some of the peak elution times were stable with that of a diglycoside accessible in the *icipe*'s (BCED) library. For this reason, MS scans were arranged up to 1415 mass/charge (*m/z*) because most diglycosides possess an *m/z* of 431 to 700, and a single moiety had 269 to 317 *m/z*. Parent peaks were distinguished at 641, 627, 611, 610, 595, 449, and 431 *m/z*, with daughter peaks at 317, 312, 303, 287, 271, and 269 *m/z*. A subsequent, direct injection MS-electron scan gained these peaks as well as several others in lesser quantities. These masses were compared with all combinations of known diet flavonoid and glycosyl moieties.

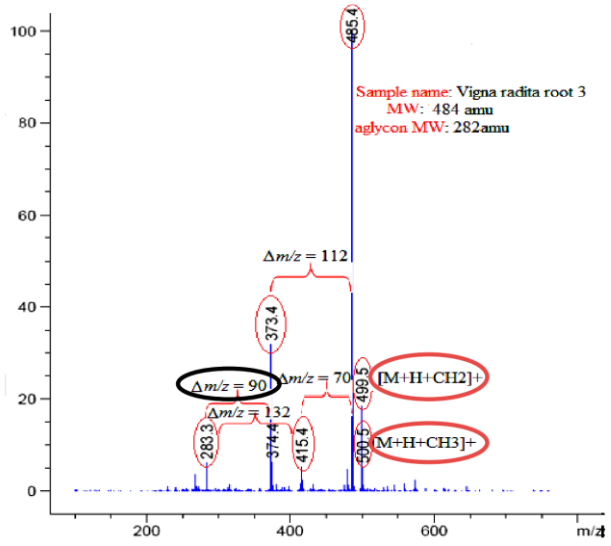

Figure 19. MS fragmentation of C-Glucosylated VRR 3

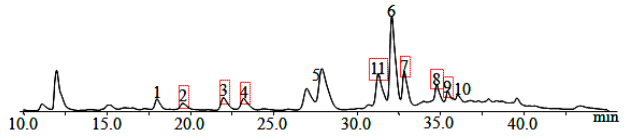

Figure 20.A. Segregated and distinguished mixtures from *Cicer arietinum* leaf

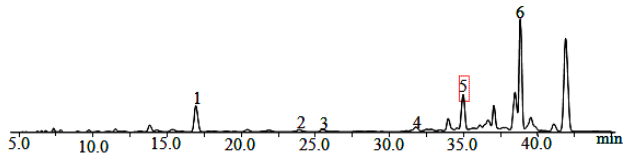

Figure 20.B. The segregated and recognized mixture from *Cicer arietinum* root

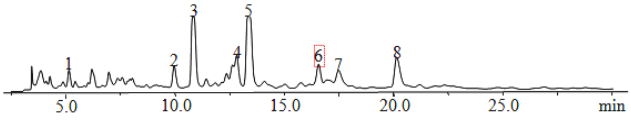

Figure 20.C. The segregated and distinguished mixture from *Vigna radiata* root

Though eleven fractions were gained from *C. arietinum* leaf (Figure 20.A), only the outlined seven mixtures were distinguished from the plant (CAL 2, CAL 3, CAL 4, CAL 7, CAL 8, CAL 9, and CAL 11) (Figure 20.A). Six fractions were segregated from *C. arietinum* root (Figure 20.B). But only (CAR5) outlined in Figure 20.B was recognized in this study.

In Figure 21.A, a white solid CAR 5 possessed a retention time of 34.1-34.4 min on HPLC. It presented $[M + H]^+$ at *m/z* 431 and $[M + Na]^+$ at *m/z* 453, in the positive ion mass spectrum. Therefore, its molecular mass was inferred to be 430. Its UV spectrum exhibited maximum permeation at 254 nm and a shoulder peak at 300 nm with a weak band I (236 nm) and strong band II (254 nm). The positive MS spectrum of *m/z* 431 indicated ions at *m/z* 269 representing a loss of 162 amu recommending the existence of a sugar molecule. In the Phenol-Explorer database (Anon 2012), Formononetin-7-*O*-glucoside owned a molecular mass of 430 amu. Based on these data, CAR 5 was temporarily recognized as Formononetin-7-*O*-glucoside.

In Figure 21.B, CAL 2, a yellow solid, possessed a retention time of 19.4~19.7 min on HPLC. It exhibited $[M+Na]^+$ at *m/z* 663 and an intense *m/z* 700 ions in the normal mass spectra, which disconnected to harvest an *m/z* 641 in the ms2 spectra. The resulting 59 amu neutral loss indicated the existence of an acetate $[M+H+CH_3COO^{\cdot}]^+$ radical ion (confirming *m/z* 641 as the molecular ion). Further disconnection of the *m/z* 641 ions yielded an intense *m/z* 479 in the ms3 spectra, which shows the existence of a terminal galactose sugar (because of the 162 amu neutral loss). An additional *m/z* 317 ion fragment was noticed in the ms3 ion spectra, showing the existence of another terminal glucose sugar (because of the 162 amu neutral loss). The positive MS spectrum of *m/z* 641 gave fragmentation ions at *m/z* 503, 479, and 317.

Its UV spectrum displayed maximum permeation at 255 and 352 nm, a shoulder peak at 222 nm, and minimum permeation at 245 nm. These data showed that its first aglycon had a molecular mass of 316. From the precursor ion in the molecule, the existence of two sugar molecules of molecular weights 162 amu is shown. Rhamnetin-3-O-galactoside-4'-O-glucoside had a molecular mass of 640 amu in the Phenol-Explorer database (Anon 2012). Based on these data, the coalition of the various chromatographic and spectroscopic methods (Table 11), and comparison, CAL 2 was temporarily recognized as rhamnetin-3-O-galactoside-4'-O-glucoside.

Figure 21.C presented that yellow solid CAL 3 got a retention time of 21.7~22.1 min on HPLC. It showed $[M+H]^+$ at *m/z* 627 and $[M + Na]^+$ at *m/z* 650 in the positive ion mass spectrum, representing that its molecular mass was 626. Its UV spectrum displayed maximum permeation at 238, 258, and 353 nm, a shoulder peak at 225 nm, and minimum permeation at 246 nm.

The positive MS spectrum of *m/z* 627 gave fragmentation ions at *m/z* 465, 446, and 303. These data showed that CAL 3 was a diglucoside, and the aglycon had a molecular mass of 302. In the Phenol-Explorer database (Anon 2012), quercetin-3,7-O-diglucoside had a molecular

mass of 626. Based on these data, the coalition of the various chromatographic and spectroscopic methods (Table 11), and comparison, CAL 3 was temporarily recognized as Quercetin-3,7-O-diglucoside.

Figure 21.D indicated that a yellow solid CAL 4 owned a retention time of 22.9~23.2 min on HPLC. It showed $[M + H]^+$ at *m/z* 611 and $[M + Na]^+$ at *m/z* 633 in the positive ion mass spectrum, representing that its molecular mass is 610. Its UV spectrum showed maximum permeation at 265, 325, and 337 nm, a shoulder peak at 230 nm, and minimum permeation at 245 nm.

The positive MS spectrum of *m/z* 611 gave fragmentation ions at *m/z* 449, 430, and 287. These data showed that CAL 4 possessed an aglycon of molecular mass of 286. From the precursor ion, the existence of two sugar molecules of molecular weights 162 amu were in the molecule. Sakuranetin-5,4'-O-diglucoside had a molecular mass of 610 on the Phenol-Explorer database (Anon 2012). Based on these data, the coalition of the various chromatographic and spectroscopic methods (Table 11) and comparison CAL 4 was temporarily recognized as Sakuranetin-5,4'-O-diglucoside.

Figure 21.E indicated that a yellow solid CAL 7 had a retention time of 32.6~33.0 min on HPLC. It showed $[M +Na]^+$ at *m/z* 633, $[M]^+$ at *m/z* 610, [M+H]+ at *m/z* 611 and$[M + 132]^+$ at *m/z* 742 in the positive ion mass spectrum. Therefore, its molecular mass was ensured to be 610. Its UV spectrum showed maximum permeation at 255 and 352 nm, a shoulder peak at 238 nm, and minimum permeation at 245 nm, recommending that it was an isomer of CAL 2.

The positive MS spectrum of *m/z* 611 represented fragment ions at *m/z*, 479, 461, 317, and 302; sequential losses of 132 amu, 132+H_2O amu, and 162 amu showed the existence of a pentosyl and a glycosyl moiety respectively. The sugar molecules were most likely enclosed in the aglycon, whose molecular mass is 316 amu. Based on these and data from the Phenol-Explorer database (Anon 2012), CAL 7 was temporarily recognized as Isorhamnetin-3-O-xyloside-7-O-glucoside.

In Figure 21.F, CAL 8, a yellow solid, had a retention time of 34.6~35.0 min on HPLC. It indicated $[M +Na]^+$ at *m/z* 471 and $[M+H]^+$ at *m/z* 449 in the positive ion mass spectrum. Therefore, its molecular mass was ensured to be 448.

Its UV spectrum showed maximum permeation at 265 and 337 nm, a shoulder peak at 230 nm, and minimum permeation at 246 nm, recommending an isomer of CAL 4. The positive MS spectrum of *m/z* 449 exhibited fragment ion at *m/z* 287; the loss of 162 amu pointed to a glucosyl enclosed to the aglycon, which molecular mass is 286. Based on these data from the Phenol-Explorer database (Anon 2012), CAL 8 was temporarily recognized as Isosakuranetin-5-O-diglucoside.

In Figure 21.G, CAL 9, a yellow solid, bore a retention time of 36.6~36.9 min on HPLC. It exhibited $[M +H+3CO]^+$ at *m/z* 533, $[M+H+CO]^+$ at *m/z* 476 and [M+H]+ at *m/z* 449 in the positive ion mass spectrum.

The molecular mass of the mixture was assumed to be 448. Its UV spectrum showed maximum permeation at 244, 264, and 348 nm, a shoulder peak at 220 nm, and minimum permeation at 245 nm, recommending that CAL 9 is neither an isomer of CAL 4 nor of CAL 8.

The positive MS spectrum of *m/z* 449 represented fragment ions at *m/z* 287. The loss of 162 amu pointed to the existence of a glucosyl fragment and recommended that the glucosyl be enclosed to the aglycon, which molecular mass was 286. In the Phenol-Explorer database on polyphenol content in foods (Anon 2012), luteolin, kaempferol, scutellarein, and fisetin owned a molecular mass of 286. This mixture was neither luteolin nor scutellarein because it was an isomer or the same mixture as CAL 11. Based on these data, CAL 9 was temporarily recognized as; either fisetin-3-O-glucoside, fisetin-7-O-glucoside, fisetin-4'-O-glucoside kaempferol-3-O-glucoside or Kaempferol-7-O-glucoside.

CAL 11 in Figure 21.H, a yellow solid, owned a retention time of 29.4~29.8 min on HPLC. It exhibited $[M +Na]^+$ at *m/z* 633 and $[M+H]^+$ at *m/z* 611 in the positive ionization mode. Therefore, its molecular mass was assumed to be 610.

Its UV spectrum showed maximum permeation at 244 and 348 nm, a shoulder peak at 218 nm, and minimum permeation at 238 nm recommending that it was an isomer of CAL 9 but not an isomer of CAL 4. The positive MS spectrum of *m/z* 449 indicated fragment ions at *m/z* 449 and 287; sequential losses of 162 amu, and 2 (162) amu, and showed the existence of two sugar molecules recommending the two glucosyls adhered to the aglycon whose molecular mass is 286. Compared with published data, O-diglucosides of scutellarein is yet to be reported in the diet. Based on these data from the Phenol-Explorer database (Anon 2012), CAL 11 was temporarily recognized as either Fisetin 3,7-O-diglucoside or Kaempferol 3,7-O-diglucoside.

Out of eight fractions segregated from *V. radiata* root (Figure 20.C), only VRR 6 is outlined in Figure 20.C was recognized in this study.

Figure 21.I represented that VRR 6, a red solid, held a retention time of 15.1~15.4 min on HPLC. It pointed $[M +Na]^+$ at *m/z* 617 and $[M +H]^+$ at *m/z* 595 in the positive ion mass spectrum. Therefore, its molecular mass was assumed to be 594.

Its UV spectrum represented maximum permeation at 256, 282, and 319 nm, a shoulder peak at 219 nm, and minimum permeation at 245 nm. The positive MS spectrum of *m/z* 432 represented fragment ions at *m/z* 271, sequential losses of 162 amu. It showed that two glucosyls adhered to the Aglycon molecular mass of 270.

Based on these data and the Phenol-Explorer database (Anon 2012), Apigenin, Baicalein, Genistein, and Galangin possess a molecular mass of 270. But a comparison of the UV results of the mixture with Apigenin and Genistein standards indicated that the aglycone of VRR 6 cannot be Apigenin or Genistein. Also, from the database, glucosides of Galangin are yet to be reported. Therefore, VRR 6 was temporarily recognized as Baicalein-7-O-diglucoside.

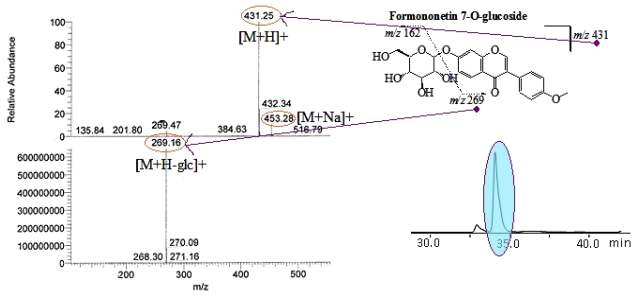

Figure 21.A. HPLC profile, LC-MS fragmentation, and likely structure of CAL 5

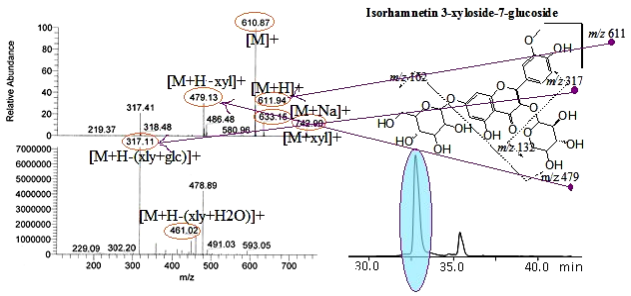

Figure 21.E. HPLC profile, LC-MS fragmentation, and likely structure of CAL 7

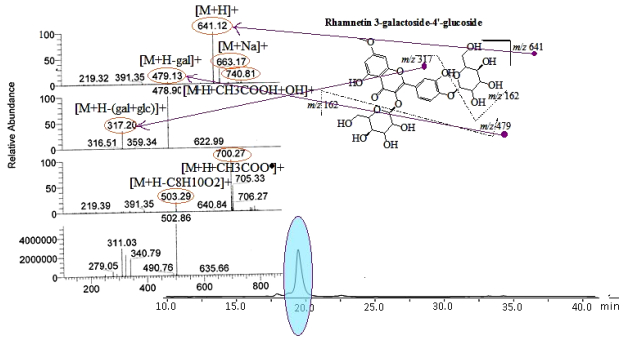

Figure 21.B. HPLC profile, LC-MS fragmentation, and likely structure of CAL 2

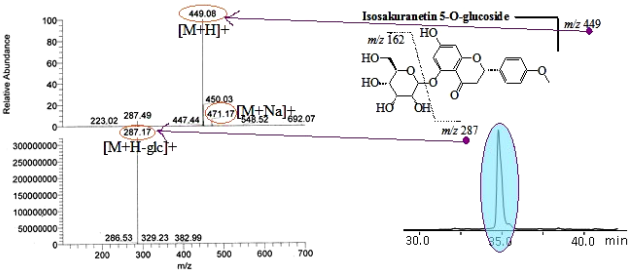

Figure 21.F. HPLC profile, LC-MS fragmentation, and likely structure of CAL 8

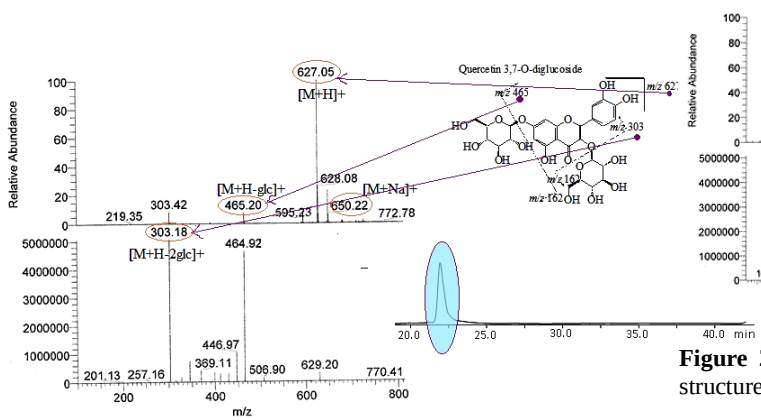

Figure 21.C. HPLC profile, LC-MS fragmentation, and likely structure of CAL 3

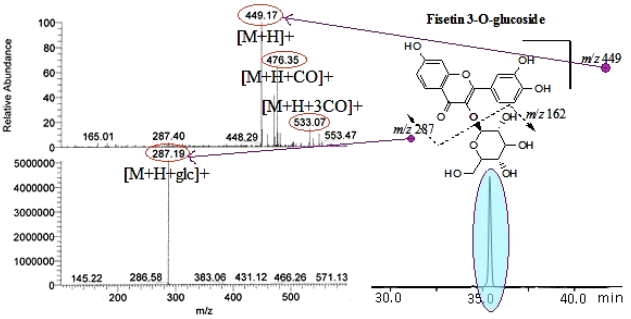

Figure 21.G. HPLC profile, LC-MS fragmentation, and likely structure of CAL 9

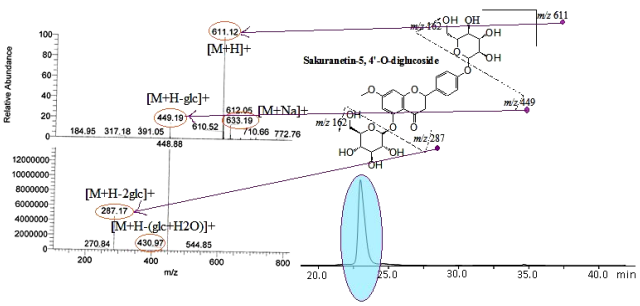

Figure 21.D. HPLC profile, LC-MS fragmentation, and likely structure of CAL 4

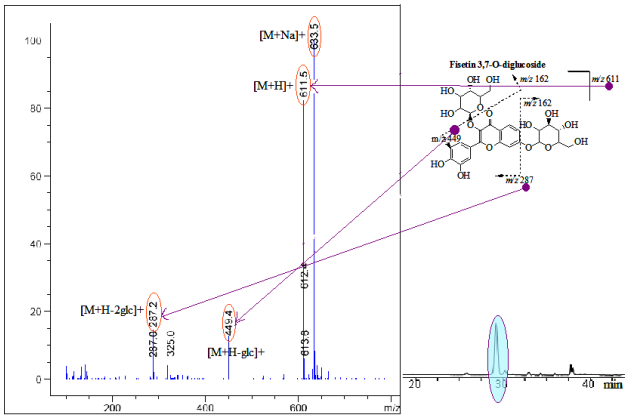

Figure 21.H. HPLC profile, LC-MS fragmentation, and likely structure of CAL 11

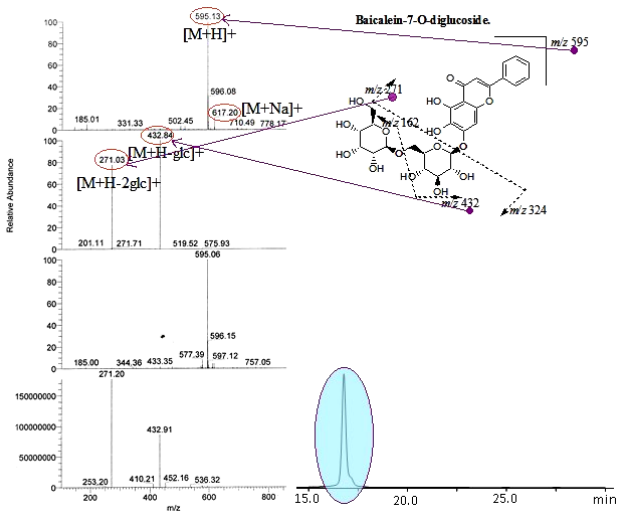

Figure 21.I. HPLC profile, LC-MS fragmentation, and likely structure of VRR 6

Summary of identified mixtures

Finally, the coalition of the various chromatographic and spectroscopic methods and comparison with published data (Anon 2012) allowed the temporary identification of several mixtures, as summarized in Table 11.

In conclusion, the secondary metabolites segregated from the leaf of *C. arietinum* L. were eleven, and those from the root tissues were six. Description of these metabolites utilizing HPLC and LC-ESI-MS represented that they are all O-glucosylated. It had no inhibitory chemistry and proposed biosynthesis by C-glucosylation (Pickett 2011). The plant gathered several unidentified secondary metabolites in its leaf and root since all the mixtures described and recognized in this research have never been reported before. The ones noticed in the leaf were not replicated in the root or vice versa. Eight secondary metabolites were segregated from the root of *V. radiata* L. Out of these, HPLC and LC-ESI-MS described four mixtures. One mixture, VRR 6, was recognized as Baicelein-7-O-diglucoside. The compound of VRR 3 indicated the characteristic of C-glucoside in MS fragmentation though it was not recognized in this research. This signified that *V. radiata* bore inhibitory chemistry and proposed biosynthesis by C-glucosylation (Pickett 2011).

Table 11. Summary of the temporarily recognized mixtures

Compound/ possible identification	Retention time (min)	UVMaximum absorbance (λmax) (nm)	ESI-MS fragment ions (% proportional intensity)	[M]+
CAR 5 Formononetin 7-O-glucoside	34.1~34.4	236, 254	m/z 453 [M+Na]+ (10) and431[M+H]+ (100).m/z269 [M+H-162]+ (<5)	430
CAL 2 Rhamnetin 3-O-galactoside-4'-O-glucoside	19.4~19.	222,238,255, 352	m/z740[M+CH3COOH+OH]+ (<5)663[M+Na]+ (30)and641[M+H]+ (100).m/z623 [M+H-H2O]+ (<5), 479 [M+H-162]+ (<5),317[M+H-2 (162)]+ (<5)	640
CAL 3 Quercetin 3,7-O-diglucoside	21.7~22.1	225, 238,258, 353	m/z650[M+Na]+ (<10)and627[M+H]+ (100).m/z, 465 [M+H-162]+ (5), 446 [M+H-(162+H2O)]+ (1),303[M+H-2 (162)]+ (<5)	626
CAL 4 Sakuranetin-5, 4'-O-diglucoside	22.9~23.2	230, 265,325, 337	m/z 633 [M+Na]+ (15) and611[M+H]+ (100).m/z449 [M+H-162]+ (15), 430[M+H-(162+H2O)]+ (<2),287 [M+H-2 (162)]+ (5)	610
CAL 7 Isorhamnetin 3-O-glucoside-7-O-xyloside	32.6~33.0	238, 255,352	m/z742[M+132]+ (10),633[M+Na]+ (15),611[M+H]+ (30) and 610[M]+ (100). m/z479[M+H-132]+ (30), 461[M+H-(132+H2O)]+ (12),317 [M+H-(132+162)]+ (20)	610
CAL 8 Isosakuranetin 5-O-glucoside	34.6~35.0	230, 265,337	m/z471[M+Na]+ (<10)and449[M+H]+ (100). m/z 287 [M+H-162]+ (5)	448
CAL 9 Fisetin 3-O-glucoside	36.6~36.9	220, 244,264, 348	m/z533[M+H+3CO]+ (15), 476[M+H+CO]+ (65) and 449[M+H]+ (100). m/z287 [M+H-162]+ (<5)	448
CAL 11 Fisetin 3,7-O-diglucoside or kaempherol 3,7-O-diglucoside	29.4~29.8	218, 244,348	m/z633[M+Na]+ (100)and 611 [M+H]+ (84). m/z449 [M+H-162]+ (13)and287 [M+H-2 (162)]+ (15)	610
VRR 6 Baicalein-7-O-diglucoside	15.1~15.4	219,256,282, 319	m/z 710 [M+Na+C6H5O·]+ (<5),617[M+Na]+ (15)and595[M+H]+ (100).m/z432 [M+H-163]+ (<5)and271[M+H-2 (162)]+ (<5)	594

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Nutritional composition of aquatic plants and their potential for use as animal feed: A case study of the Lower Volta Basin, Ghana

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Abstract. *Etse WJ, Annang T, Ayivor JS. 2018. Nutritional composition of aquatic plants and their potential for use as animal feed: a case study of the Lower Volta Basin, Ghana. Biofarmasi J Nat Prod Biochem 16: 99-112.* The study was conducted to determine the nutritional composition of selected dominant aquatic plants and their significant effect on the chemical and physical characteristics of the water. Aquatic plants, namely *Nymphaea lotus*, *Typha australis*, *Ipomoea aquatica*, and *Scirpus cubensis*, were collected, identified, and authenticated at the Ghana Herbarium. The proximate nutritional compositions of these plants were measured using the standard procedure outlined in the Association of Official Analytical Chemists (AOAC 2002). Water and sediment quality analyses of some physicochemical variables were also carried out using processes described in the standard water and wastewater examination methods. The results showed that nutrient composition, such as the crude protein, ether extracts, ash content, and nitrogen-free extracts, was significantly higher than the corresponding constituents in *Panicum maximum* used as a control for the study. The findings also indicated that levels of heavy metals in all plants fell within the WHO/FAO standards for metals in vegetables and food. The effects of the physicochemical parameter of water also revealed that pH, nitrate, turbidity, DO, and BOD levels were significantly different from the control site. The level of heavy metals in the sediment samples revealed significant variations in the distribution of the metals, with Zn showing the most significant difference and Pb the least with a mean level of 7.5±0.86 mg/L and 0.4±0.03 mg/L, respectively. These plant species suggest a high nutritional potential and indicate their possible use as mixed ingredients in animal feed. Exploiting these aquatic plants for animal feed would be a step towards better utilization of these plants to help manage aquatic plants within the basin.

Keywords: Aquatic plant, lower volta basin, nutritional composition

INTRODUCTION

Aquatic plants have been conventionally perceived as a nuisance rather than a useful resource for years (Shah et al. 2010) because of their environment's challenges. The aquatic vegetation can change the physicochemical characteristics of both water and hydrosoil, thereby altering water quality (Petosa et al. 2010). They can also provide habitat and food for the larval stage of animal vectors of human diseases such as malaria, posing a health hazard (El-Shinnawy et al. 2000). Besides the specific effects and the detrimental effect of the excessive growth of aquatic plants described above, aquatic plants may influence the programs of water resource utilization and management (Malik 2007).

In Ghana, several major river systems, like the Tano, Pra, Ankobra, Kakum, Ochi, Ayensu, and Densu, have been affected by severe aquatic macrophyte infestation, resulting in the improper utilization and management of the impoundments (deGraft-Johnson 1996). Annang (2008) stated that the regulation of the flow regime of the Volta River due to the generation of the Akosombo Dam in 1963 and the Kpong dam in 1981 had created an ideal situation for the quick growth of aquatic plants in the Lower Volta Basin of the Ghana (LVB). They noted that this has resulted in some of the problems mentioned above. Meanwhile, varieties of water plants, including *Nymphaea lotus*, *Ipomoea aquatica*, *Scirpus cubensis*, *Typha australis*, and *Ceratophyllum demersum* species, are abundant in the Volta basin. Aquatic vegetation in the lower Volta has contributed to the level of poverty in the basin communities specifically since it has limited the mobility of fishing boats in the waterways along with the basin communities (Annang 2008). Consequently, the Volta River Authority (VRA) purchased four mechanical weed harvesters at a total value of US$ 830,000 (Ghana Bulletin 2013), which are positioned at Kpong for the physical and mechanical harvesting of the aquatic plants at huge expense and dumped the harvested plants as waste without considering utilizing these plants. However, the cost-benefit of this initiative is subject to much controversy because elsewhere in other parts of the world, water plants are used as biofuels, compost, medicine, animal feed, and even as a source of food for humans.

Despite all these adverse effects of aquatic plants, many researchers have documented the chance of using these aquatic plants as a source of animal feed (Anon 1984). The previous study surveyed aquatic plants in Sringar and found that animals fed with the studied aquatic plants generated approximately 3 liters of milk per day per animal, more than animals fed with straw (Shah et al. 2010). This research ultimately explores all the possible ways to use these plants as an ingredient in animal feeds to continuously harvest and use the nuisance plants, which will subsequently decrease the adverse effects caused by water plants in the aquatic ecosystem.

Therefore, this study suggests that using water plants as animal feed may provide an efficient, effective, and

environmentally friendly means of controlling and managing water plants within the Lower Volta Basin. Specific objectives of this study are to assess the nutrient composition of some dominant aquatic plants using proximate analyses, to identify the phytochemicals that exist in the selected samples, to measure heavy metal levels in the water, plant material, and sediment, and to investigate the social perception on the use of aquatic plants in feeding animals.

MATERIALS AND METHODS

Study area

The study was performed on the Lower Volta Basin (LVB) in Ghana, and three sang sites were chosen (Kpong, Big Ada, and Amedeka). Ada and Kpong areas are the stretches on the Lower Volta heavily populated with diverse water plants. Water samples were brought from Amedeka, where there were no weeds, and served as a control site.

Brief description of the sampling sites

Kpong head pond

The surface area is about 37.4 km^{2}, with a maximum depth of 15m and an average depth of 5m (Ansa-Asare and Asante 1998). The Kpong head pond has about 85% of its total surface infested with aquatic weeds. Among the numerous plant species present are *N. lotus*, *T. australis*, *S. cubensis*, *I. aquatica* and *Vossia.* Human activity, mainly through fishing at the site, is high.

Big Ada

The Ada sampling site has vegetation typical of the savanna transition zone. Species present are *T. australis*, *I. aquatica*, *S. cubensis,* and some terrestrial plant species. Fishing is a significant activity in the area. Furthermore, recreational activities such as swimming and boating are common practices within the region.

Short description of plants under study

Typha australis Schumach.

Typha australis belongs to the family Typhaceae, common names as cattail, punks, reedmace, bulrus, corn dog grass, etc. The rhizomes are edible.

Typha is often found to colonize newly exposed wet mud areas with its abundant wind-dispersed seeds. Seeds can survive inside the soil for long periods. The seeds germinate best with sunlight and fluctuating temperatures, typical of many wetland plants that regenerate on mudflats. Rhizomes also spread Thypa, forming large, interconnected strands. It is considered the dominant competitor in wetlands in many regions, and it often eliminates other plant species with its large canopy. For instance, the Great Lakes bay is among the most abundant wetland plants. Different species of cattails adapted to different water depths. *Typha* can be aggressive in their competition with other indigenous species and has become a problem in many regions in North America. It may be more critical to avoid invasion by preserving water level fluctuations, including periods of drought, and to keep infertile conditions (Gott 1999).

Nymphaea lotus Linn.

Nymphaea lotus belongs to the family Nymphaeaceae, a species of water lily with lily pads that float on the water and flower blossoms above the water. The color of the flower is white and sometimes tinged with pink. It is found in ponds and prefers clear, warm, still, and slightly acidic waters. The plant can be located in association with other water plant species, such as *Utricularia stellaris*. The plant is invasive of any stretch of calm water. It has colonized parts of Volta Lake (Wiersema 1982).

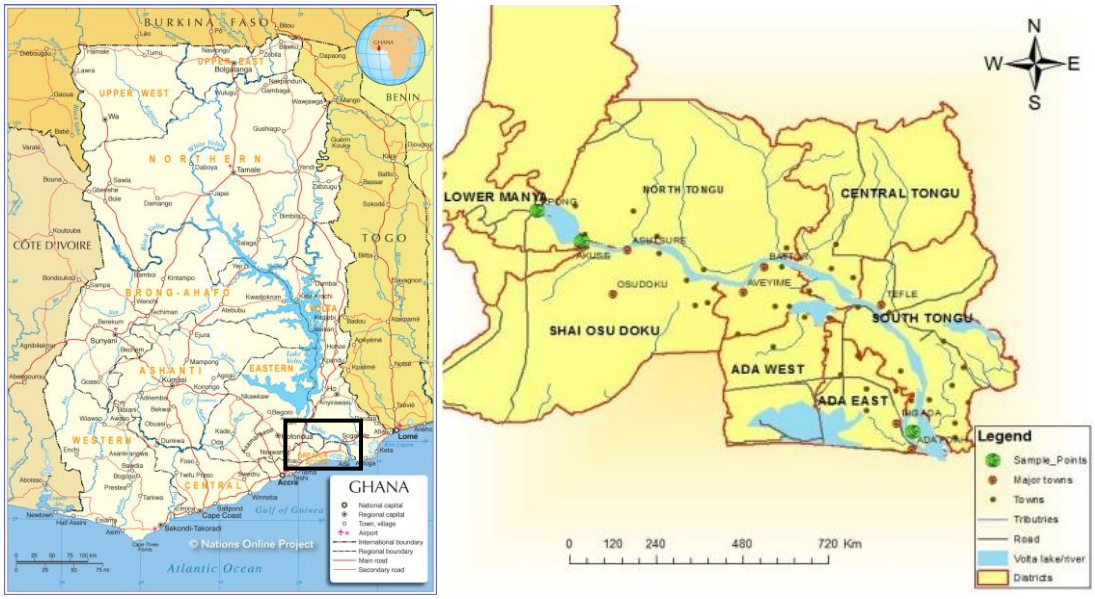

Figure 1. Map of the study area in Lower Volta Basin, Ghana

Ipomoea aquatica Forsk.

Ipomoea aquatica belongs to the family Convolvulaceae. It is a semiaquatic, tropical plant grown as a vegetable for its tender shoots and leaves. In English, this plant is called water spinach and increases in water or moist soil. They are hollow and can float. Propagation is either planting seeds from flowers or planting cuttings of the stem shoots that will root along with nodes (Prasad et al. 2008).

Scirpus cubensis Poeppig & Kunth

Scirpus cubensis is a leafy plant that belongs to the family Cyperaceae. The large colony of medium-height grasses grows in water, with spherical inflorescences only somewhat visible among the many leaves. It is a significant duck food (Junk and Piedade 1997).

General methods

The study adopted quantitative and qualitative approaches to data collection. Using appropriate protocols, plant, water, and sediment samples were analyzed quantitatively in the laboratory. Four different plant species, namely *N. lotus, T. australis, I. aquatica,* and *S. cubensis,* were taken from each sampling site from January to March. Water and sediment were also taken from the same sample locations for analysis.

Reconnaissance survey

A reconnaissance survey was performed on the 13th and 14th of January 2015 to assess the problems in the various regions. After the survey, two sampling sites were chosen using a judgment sampling technique to identify significant environmental challenges. Garman Etrex 20 Global Positioning System (GPS) recorded the coordinates of the sampling sites.

Aquatic plants

Plant samples collection

Four different plant species were collected from Ada and Kpong and transferred into black polyethylene bags from the sampling sites to the laboratory. The plants were selected based on dominance, availability, and accessibility at the two sampling sites to allow for comparison between the plant taken at the two sampling locations.

Plant sample identification

The herbarium of the Botany Department, University of Ghana, Legon identified and authenticated *T. australis, N. lotus, I. aquatica,* and *S. cubensis.*

Plant sample preparation

Plant samples were rinsed with water and then dried for one week in an oven at a temperature of 50^{o}C. The dried samples were pulverized and kept for further analysis.

Plant samples analysis

Proximate determination

Moisture content was measured by the loss in weight that occurs when the sample was dried to a constant weight in an oven. Two grams of the plant sample were weighed, and the sample was then dried in an oven for 36 hours at 65 0C cool in a desiccator and weighed. The process was continued until a stable weight was achieved.

$$\%\text{Moisture} = \frac{(\text{wt of sample + dish before drying}) - (\text{wt of sample + dish after drying})}{\text{Wt of sample taken}} \times 100$$

Ether extract

The ether extraction by the soxhlet apparatus represents the fat and oil in the plant sample. This equipment consists of 3 main components; an extractor which comprises the thimble which holds the sample; a condenser for cooling and condensing the ether vapor; and a 250 mL flask.

Procedure: 150 mL of anhydrous diethyl ether (petroleum ether) was placed in the flask. Three grams of the sample were weighed into a thimble plugged with cotton wool. The thimble with its content was put into the extractor; the ether in the flask was then heated. As the ether vapor arm of the extractor condensed to liquid from the sample in the thimble, the ether-soluble substances were dissolved and were carried into the solution through the siphon tube back into the flask. The extraction was performed for 5 hrs. The thimble was removed, and almost all of the solvent was distilled from the flask into the extractor. The flask was disconnected and placed in an oven at 65^{0}C for 4 hours, cooled in the desiccator, and weighed.

$$\%\text{Ether extract} = \frac{(\text{wt of flask + extract}) - (\text{tare wt of flask}) \times 100}{\text{wt of sample}}$$

Crude fiber

Crude fiber is determined by the organic residue left after sequential extraction of a sample with ether. The fat-free material was moved to a flask/beaker, 200 mL of pre-heated 1.25% sulphuric acid was added, and the solution was gently boiled for about 30 mins, maintaining a constant volume of acid by pouring hot water. The Buckner flask funnel is fitted with pre-heated Whatman filter paper. The boiled acid sample mixture was filtered through the funnel under sufficient suction, washed several times with boiled water (until the residue was neutral to litmus paper), and transferred back into the beaker. Following this step, 200 mL of pre-heated 1.25% sodium sulfate (Na_2SO_4) was added and boiled for 30 mins, filtered under suction, and washed thoroughly with hot water and ethanol twice. The residue was dried at 65 ^{0}C for 24 hrs and measured. The residue was moved into a crucible and placed in a muffle furnace (400-600 ^{0}C) and ash for 4hrs, then cooled in a desiccator and weighed.

$$\%\text{Crude fiber} = \frac{\text{Dry wt of residue before ashing} - \text{wt of residue after ashing}}{\text{wt of sample}} \times 100$$

Crude protein

Crude protein was calculated using a Kjeldahl method involving digestion, distillation, and titration. The nitrogen content of the plant sample was measured and multiplied by a factor of 6.25 (this factor was based on the fact that most protein contains 16% of nitrogen).

Digestion: 2g of the sample was weighed into a Kjeldahl flask, 25 mL of concentrated sulphuric acid, 0.5 g of copper sulfate, 5 g of sodium sulfate, and a speck of selenium tablet were added. The heat was applied in a fume cupboard slowly at first to prevent excessive frothing, followed by digestion for 45 mins until the digester became clear pale green. After cooling down, one hundred mL of distilled water was rapidly added to the samples.

Distillation: Markham distillation apparatus was stemmed up, and 10 mL of the digest was added to the device via a funnel and allowed to boil. Ten mL of sodium hydroxide was added from a measuring cylinder so that ammonia was not lost. It was then distilled into 50 mL of 2% boric acid containing screened methyl red indicator.

Titration: the alkaline ammonium borate created was titrated directly with 0.1N HCl. The volume of acid used was fitted into the formula below.

$$\%N = \frac{14 \times VA \times 0.1 \times w \times 100}{1000 \times 100}$$

Where:
VA = volume of acid used
w = weight of the sample
%N = Percentage nitrogen
%crude protein =%N x 6.25

Ash

Ash is the inorganic residue of the organic matter of a plant sample burnt in a muffle furnace at 400-600^{0}C for 4hrs. Two grams of the sample were weighed into a pre-heated crucible and later placed in the muffle furnace at 400-600 ^{0}C for 4hrs or until whitish-grey ash was obtained. The crucible was placed in the desiccator, allowed to cool, and weighed.

$$\%Ash = \frac{\text{wt of crucible+ash-wt of the crucible}}{\text{wt of sample}}$$

Nitrogen Free Extract (NFE)

NFE was determined by mathematical calculation by subtracting the sum of percentages of all the nutrients already calculated from 100.

$$\%NFE = 100 - (\%moisture + \%CF + \%CP + \%EE + \%Ash)$$

NFE represents soluble carbohydrates and other digestible and easily utilizable non-nitrogenous substances in the feed.

Phytochemical screening

Dried plant samples were pulverized into powder. Five grams of the powdered material was dispersed in 50 mL of methanol. The solution was left to stand for 24 hrs and filtered with Whatman No. 1 filter paper. The filtrate was assessed for the phytochemical screening using the following tests.

Test for alkaloids (Wagner's reagent test)

A fraction of the extract was treated with 3-5 drops of Wagner's reagent (1.27 g of iodine and 2 g of potassium iodide in 100 mL of water) and observed for the formation reddish-brown precipitate or coloration.

Test for flavonoids (Alkaline reagent test)

Two mL of the extract was treated with a few 20% sodium hydroxide solution drops. The formation of intense yellow color, which becomes colorless with the addition of dilute hydrochloric acid, suggests the presence of flavonoids.

Test for phenols (Ferric chloride test)

A fraction of the extracts were treated with 5% aqueous ferric chloride and observed for deep blue or black color formation.

Test for saponins (Foam test)

Six mL of water was added to 2 mL of the extract, shaken vigorously, and observed for the visible foam that confirms the existence of saponins.

Test for amino acids and proteins (1% ninhydrin solution in acetone)

Two mL of filtrate was treated with 2-5 drops of ninhydrin solution placed in a boiling water bath for 1-2 minutes and observed for the formation of a purple color.

Test for terpenoids (Salkowki's test)

One mL of chloroform was added to 2 mL of each extract, followed by three drops of concentrated sulphuric acid. A precipitate showed a reddish-brown color that produced immediately indicated the presence of terpenoids.

Test for sterols (Liebermann-Burchard test)

One mL of chloroform, acetic anhydride, and concentrated H2SO4 were dropped and observed for dark pink or red color formation.

Mineral analysis

One gram of the powdered sample was added with 25 mL of concentrated HNO_3 in a flask. The flask was then heated until the evolution of the brown fume stopped. The mixture was added with 1 cm^3 of perchloric acid and then heated to a clear solution. After that, 30 mL of hot distilled water was poured into the digest and heated to boiling. The solution was filtered hot into a clean 50 mL volumetric flask, cooled, and meshed up to the mark with distilled water. Na and K content was analyzed by flame atomic emission spectrophotometer. A spectrophotometer with standard air-acetylene flame analyzed the content of Ca, Cu, Zn, and As.

Water

Water sample collection

A 500 mL plastic bottle was used to fetch water at each sampling point. The samples were kept on ice in the chest to keep the temperature low to suppress microbial activity before transporting them to the laboratory. Nitric acid ($_3$ drops) was added to the water sampled for heavy metal analysis.

Physicochemical analysis of water samples

Water at each sampling point was sampled in a 500 mL plastic bottle. This was later used in the laboratory for further investigation. The samples were kept on ice in the chest to keep the temperature low (about 10ºC) to avoid microbial activity before transporting it to the laboratory. Physicochemical parameters of the water samples were measured at the Ecological laboratory, University of Ghana.

pH

The pH of water samples was determined in situ using a portable pH meter.

Temperature

The temperature was measured in situ to a depth of about four inches for nearly a minute. The readings were allowed to stabilize and noted.

Turbidity

Turbidity was measured using HACH 2100Q. The turbidity meter was powered on after 20 NTU (Nephelometric Turbidity Unit) cell was filled with the sample, cleaned, and placed in the cell holder and covered with the lid. Reading was then done and recorded.

Total Dissolved Solids (TDS)

A glass fiber disc was prepared by putting it on a membrane filter, and a vacuum was applied to it. A clean dish was heated at 120ºC for one hour in an oven and allowed to cool in a desiccator. The disc was weighed and noted before being used. The water samples in the plastic bottles were vigorously shaken. One hundred milliliters of the water sample was transferred into a volumetric flask through a graduated measuring cylinder. The sample was filtered through the glass fiber disc using a suction pump. The total filtrate was moved to a previously weighed evaporating dish and evaporated to dryness in a water bath. The sample was dried for 2 hours and cooled in a desiccator, and the constant weight was calculated. The drying cycle was repeated until weight loss was less than 0.0005 g. The value was counted using the formula below.

$$TDS\ (mg/L) = (A-B) / Cx10^6$$

Where,
A = weight of residue + dish
B = weight of dish
C= Volume of water sample

Dissolved Oxygen (DO) by the sensor method

The electrode end of the DO meter was dipped into the water in the area. The temperature of the water was taken at the same time.

Biochemical Oxygen Demand (BOD)

BOD was determined immediately after determining the DO content. This process was carefully done to prevent air bubbles by tilting the BOD bottles and gradually submerging them into the water. The bottle was allowed to overflow and covered with a stopper. Each bottle was kept in an ice chest and transported to the laboratory. The BOD bottles were held in a dark cupboard to prevent light from contacting the containers and their content for five days in the laboratory. The DO of the water was calculated with the same meter after the fifth day. The difference in DO between day one and day five marked the BOD.

$$BOD\ in\ mg/L = DO_1 - DO_5$$

Nitrate (NO_3^-)

Cadmium Reduction Method measured nitrate level in each sample using Nitrate Powder Pillows in direct reading Hach Spectrophotometer Model DR. 2010. Ten mL of the sample were placed into the sample cell of the Spectrophotometer and added with one Nitraver 5 Nitrate Reagent powder pillow. The solution was shaken, then placed in the cell holder to determine the nitrate concentration in mg/L at 500 nm.

Phosphate (PO_4^{3-})

Ten mL of the water sample was placed in the sample cell. Phos Ver 3 Phosphate pillow was added to the cell content and immediately swirled to mix. The mixture was allowed to settle.

The spectrophotometer displayed the results in mg/L PO_4^{3-}at 890 nm reading.

Sulfate (SO_4^{2-})

100 mL of the water sample was placed into a 250 mL Erlenmeyer flask, and 5 mL of conditioning reagent was added and mixed by stirring. One g of $BaCl_2$ was added and shaken for 60 seconds. The reading was carried out at a wavelength of 420 nm.

Determination of heavy metals in water samples

Concentrated HNO3 (5 mL) was added to 100 mL of a water sample and evaporated on a hot plate to the lowest volume before precipitation occurred. Digestion was completed after the appearance of the clear light-colored solution. The solution was filtered through 0.45 µm filter paper and moved into a flask, cooled, and top to the mark for analysis. The concentration of Copper (Cu), Cadmium (Cd), Arsenic (As), lead (Pd), and Zinc (Zn) were determined using 240 FS Atomic Absorption Spectrometer by direct aspiration of water samples into an air-acetylene flame, and all into nitrous oxide-acetylene flame.

Sediment

Sample collection

Using a trowel, sediments from the sampling sites were collected under the aqueous layer. The residue was placed in a plastic container and kept in an ice chest before being transported to the laboratory for analysis.

Sediment digestion and analysis

Sediment samples (0.4 g) were digested in Teflon tubes. Four mL of concentrated nitric acid (HNO_3) was added to the content slowly. The tubes were sealed and placed in stainless steel bombs, then put on a hot plate and heated at150 ºC for 7 hours, then cooled down to ambient temperature before carefully opening the bombs to release pressure. The samples were moved into the graduated polypropylene tubes, and the Teflon tubes were rinsed

three times with distilled water and then added to the content of the polypropylene tube. The material was diluted to the 50 mL mark of the machine with distilled water and mixed well. Determination of heavy metals from sediments was carried out using the cold vapor atomic absorption according to Milner and Whiteside (1981).

Social survey

Sociological data and other relevant information on the ethnobotanical use of aquatic plants and their effects on inhabitants' livelihood were investigated using a questionnaire. One hundred twenty questionnaires were conveniently administered to targeted members within the two communities from the 25th of February to the 6th of March 2015. Targeted members included fishers and women, farmers, travelers on the lake, Traders along the lake, and other community members very close to the lake. All respondents were 18 years and above. Questions were created based on background information and general information on the lake and aquatic plants. The objective was to assess the inhabitant' perception of the use of aquatic plants in feeding animals within the communities. Sixty questionnaires were administered in each community.

Statistical analysis

The data were subjected to single-factor analysis of variance (ANOVA) using SPSS software version 16 for windows. Differences were declared significant at $p \leq 0.05$, and means found to be significantly different were separated using the least significant difference LSD (Post hoc test) at $p \leq 0.05$. The analyzed data were expressed as means with their standard deviation (X±SD).

Experimental precautions

All glassware was thoroughly cleaned before use. (i) Identifiable and fixed landmarks were used to locate the same spot for sampling throughout the study. (ii) Plant samples for phytochemical analysis were air-dried at ambient temperature. (iv) BOD bottles were used to collect water for BOD calculation. (v) The bottles were carefully filled to the brim to remove air bubbles.

RESULTS AND DISCUSSION

Proximate analysis of the plant samples

Moisture content

The average moisture content of the plant is shown in Table 1. The result ranged from a minimum of 3.1% in *T. australis* to a maximum of 19.6% in *I. aquatica*. Analysis of variance (ANOVA) showed a significant moisture content difference at 95% family. The Least significant difference (LSD) multiple comparison tests showed no significant difference between the average between *T. australis, S. cubensis,* and *N. lotus.* Still, they were however significantly different from *I. aquatica*. The mean moisture content of ranking are as follows; *I. aquatica* > *N. lotus* > *S. cubensis* > *T. australis.* The average moisture content in the plant samples from Kpong also ranged from 2.1% in T. australis to 19.1% in *I. aquatica*. The analysis data show similar trends to the plants sampled at Ada.

Table 1. Nutritional composition of aquatic plants from Ada

Aquatic plants		Moisture (%)	Ether extract (%)	Crude protein (%)	Crude fiber (%)	NFE (%)	Ash (%)
N. lotus	Min	4.50	4.5	13.45	9.78	41.07	9.70
	Max	7.90	14.75	20.10	20.13	55.52	12.56
	Mean	6.30	8.08	15.17	16.53	46.06	11.27
	S.e	1.70	5.78	3.62	5.84	8.20	1.45
I. aquatica	Min	15.67	3.20	11.23	17.87	22.11	6.54
	Max	23.45	13.67	17.98	23.78	37.74	12.45
	Mean	19.56	9.92	14.96	20.40	26.20	8.95
	S.e	1.71	5.83	3.43	3.04	4.64	3.10
T. australis	Min	3.10	3.45	19.78	9.78	43.07	4.50
	Max	3.20	11.56	20.23	17.89	54.41	8.86
	Mean	3.17	7.27	20.04	15.15	48.06	6.32
	S.e	0.06	4.08	0.23	4.65	5.79	2.32
S. cubensis	Min	2.20	5.6	11.45	7.98	45.59	4.50
	Max	9.40	7.67	18.64	19.87	61.07	8.86
	Mean	5.37	6.29	16.53	14.51	52.24	7.09
	S.e	3.68	1.20	3.39	6.03	7.09	2.32

Table 2. Nutritional composition of aquatic plants from Kpong

Aquatic plants		Moisture (%)	Ether extract (%)	Crude protein (%)	Crude fiber (%)	NFE (%)	Ash (%)
N. lotus	Min	6.20	5.63	15.10	13.60	31.41	12.31
	Max	7.10	6.31	18.62	17.76	40.53	15.32
	Mean	6.60	6.05	16.68	15.69	36.91	19.96
	S.e	0.45	0.36	1.79	2.08	4.84	1.53
I. aquatica	Min	17.63	11.62	12.30	19.64	10.15	9.63
	Max	20.36	13.16	15.63	24.30	25.64	10.73
	Mean	19.14	12.40	13.92	22.19	19.29	10.06
	S.e	1.39	0.77	1.67	2.36	8.11	0.59
T. australis	Min	1.80	5.30	18.43	11.61	45.40	6.32
	Max	2.60	6.21	26.30	14.63	49.32	12.31
	Mean	2.17	5.40	22.78	13.15	47.37	8.78
	S.e	0.41	0.80	4.0	1.51	1.96	3.14
S. cubensis	Min	1.80	4.70	14.30	10.20	52.89	6.32
	Max	3.20	6.30	16.32	12.70	58.20	12.31
	Mean	2.53	5.54	15.64	11.84	55.77	8.67
	S.e	0.70	0.80	1.16	1.42	2.68	1.41

Table 3. Mineral composition in plant samples from Ada

Aquatic plants		Na (mg/L)	Ca (mg/L)	K (mg/L)	P (mg/L)
N. lotus	Min	0.10	0.35	0.23	0.11
	Max	0.23	0.45	0.43	0.20
	Mean	0.16	0.40	0.33	0.16
	S.e	0.06	0.05	0.10	0.05
I. aquatica	Min	0.20	0.02	0.23	0.11
	Max	0.32	0.41	0.43	0.34
	Mean	0.28	0.31	0.33	0.23
	S.e	0.07	0.11	0.10	0.11
T. australis	Min	0.21	0.21	0.16	0.14
	Max	0.30	0.34	0.27	0.20
	Mean	0.25	0.28	0.22	0.17
	S.e	0.05	0.07	0.06	0.03
S. cubensis	Min	0.31	0.23	0.28	0.31
	Max	0.43	0.43	0.32	0.65
	Mean	0.36	0.33	0.31	0.54
	S.e	0.06	0.10	0.02	0.19

Table 4. Mineral composition in plant samples from Kpong

Aquatic plants		Na (mg/L)	Ca (mg/L)	K (mg/L)	P (mg/L)
N. lotus	Min	0.01	0.27	0.19	0.03
	Max	0.16	0.41	0.43	0.11
	Mean	0.10	0.35	0.28	0.08
	S.e	0.08	0.07	0.13	0.04
I. aquatica	Min	0.16	0.41	0.42	0.05
	Max	0.23	0.56	0.51	0.16
	Mean	0.20	0.46	0.45	0.11
	S.e	0.04	0.08	0.05	0.05
T. australis	Min	0.10	0.32	0.34	0.05
	Max	0.23	0.56	0.52	0.16
	Mean	0.15	0.45	0.42	0.11
	S.e	0.07	0.12	0.09	0.05
S. cubensis	Min	0.23	0.46	0.12	0.54
	Max	0.43	0.65	0.32	0.67
	Mean	0.33	0.55	0.21	0.62
	S.e	0.10	0.09	0.10	0.07

Table 5. The concentration of heavy metal in plant samples from Ada

Aquatic plants		Cu (mg/L)	As (mg/L)	Zn (mg/L)	Cd (mg/L)	Pb (mg/L)	Cr (mg/L)
N. lotus	Min	2.34	0.01	1.65	0.07	0.57	0.08
	Max	3.45	0.18	2.0	0.16	0.75	0.12
	Mean	2.71	0.08	1.8	0.12	0.66	0.10
	S.e	0.64	0.08	0.17	0.05	0.09	0.02
I. aquatica	Min	0.45	0.02	0.13	0.14	0.23	0.01
	Max	0.57	0.19	0.23	0.17	0.24	0.06
	Mean	0.52	0.12	0.18	0.15	0.23	0.03
	S.e	0.06	0.08	0.05	0.02	0.00	0.02
T. australis	Min	0.34	0.23	0.20	0.13	0.18	0.08
	Max	0.43	0.28	0.32	0.32	0.23	0.15
	Mean	0.39	0.25	0.27	0.23	0.21	0.12
	S.e	0.05	0.03	0.06	0.10	0.03	0.04
S. cubensis	Min	0.23	0.01	0.05	0.36	0.25	0.06
	Max	0.25	0.23	0.19	0.52	0.37	0.21
	Mean	0.24	0.14	0.11	0.44	0.32	0.15
	S.e	0.01	0.11	0.07	0.08	0.06	0.08

Table 6. Compositions of heavy metal in plant samples from Kpong

Aquatic plants		Cu (mg/L)	As (mg/L)	Zn (mg/L)	Cd (mg/L)	Pb (mg/L)	Cr (mg/L)
N. lotus		2.61	0.01	0.72	0.08	0.67	0.01
	Max	6.41	0.06	2.81	0.15	0.97	0.23
	Mean	4.07	0.03	1.91	0.12	0.80	0.14
	S.e	2.04	0.02	1.10	0.04	0.15	0.11
I. aquatica	Min	0.26	0.06	0.06	0.10	0.23	0.07
	Max	0.43	0.20	0.21	0.13	0.30	0.16
	Mean	0.34	0.13	0.15	0.11	0.25	0.12
	S.e	0.09	0.02	0.08	0.01	0.04	0.05
T. australis	Min	0.17	0.14	0.26	0.13	0.17	0.13
	Max	0.63	0.20	0.42	0.23	0.23	0.32
	Mean	0.44	0.18	0.36	0.19	0.20	0.23
	S.e	0.24	0.03	0.09	0.05	0.03	0.10
S. cubensis	Min	0.08	0.16	0.25	0.32	0.25	0.02
	Max	0.12	0.34	0.63	0.47	0.38	0.19
	Mean	0.10	0.23	0.40	0.38	0.32	0.12
	S.e	0.02	0.09	0.20	0.08	0.06	0.08

Table 7. Physicochemical parameters of water samples from Ada, Kpong, and Amedeka

Parameter		Ada	Kpong	Amedeka (Control)
pH	Min	6.4	6.20	6.5
	Max	7.0	6.53	6.9
	Mean	6.5	6.37	6.67
	S.e	0.1	0.16	0.15
	Min	28.40	30.10	29.2
Temperature (°C)	Max	30.20	32.60	32.3
	Mean	29.17	31.73	30.93
	S.e	0.93	1.42	0.68
	Min	2	13	2
Turbidity (NTU)	Max	17	17	3
	Mean	5.40	14.67	2.33
	S.e	1.28	2.08	0.58
	Min	17.6	8.6	22.3
DO (mg/L)	Max	19.2	12.7	26.6
	Mean	18.43	10.77	24.42
	S.e	0.80	2.06	2.50
	Min	6.5	1.6	0.93
BOD (mg/L)	Max	8.4	4.5	1.87
	Mean	7.57	3.10	1.13
	S.e	0.97	1.45	0.21
	Min	1.25	2.2	0.76
Nitrate (mg/L)	Max	1.84	2.4	1.23
	Mean	1.47	2.30	0.97
	S.e	0.32	0.10	0.01
	Min	0.43	0.71	0.53
Phosphate (mg/L)	Max	0.71	0.92	0.87
	Mean	0.55	0.81	0.73
	S.e	0.14	0.11	0.11
Sulphate (mg/L)	Min	4.0	5.0	4.12
	Max	4.0	6.0	4.67
	Mean	4.0	5.33	4.33
	S.e	0.00	0.14	1.53

Ether extract

The average percentage of ether extract of the plant samples from Ada fell from 6.3% in *S. cubensis* to 9.9% in *I. aquatica*. ANOVA at a 95% family-wise confidence level showed that the percentage of ether extract in the various plant samples showed no significant difference ($P>0.05$). However, *I. aquatica* shows a higher variation in percentage ether extract, followed by *N. lotus*, *T. australis*, and *S. cubensis*.

However, the average percentage of ether extract in the plant sample from Kpong ranged from 5.5% in *S. cubensis* to 12.4% in *I. aquatica*. ANOVA at a 95% family-wise confidence level indicated that the percentage of ether extract in the various plants' samples was statistically significant ($P<0.05$). However, the LSD revealed that the means of *T. australis*, *S. cubensis*, and *N. lotus* are not significantly different, but there were, however, considerably different from *I. aquatica*.

Ash content

The average percentage ash content of the plant sample from Ada ranged from 6.3% in *T. australis* to 11.3% in *N. lotus*. *S. cubensis* and *I. aquatica* also documented an ash content of 7.0% and 8.9%, respectively. Analysis of variance (ANOVA) at a 95% family-wise confidence level

showed that the ash content in the sampled plants was not statistically significant (P>0.05).

The average percentage ash content of the plant sample from Kpong also ranged from 8.6% in *S. cubensis* to 13.9% in *N. lotus*. Analysis of variance (ANOVA) at a 95% family-wise confidence level suggested that the ash content in the sampled plants was statistically significant. However, the least significant difference showed no significant differences in the average between T. australis, S. cubensis, and I. aquatica. Still, each was significantly different from N. lotus. The average percentage ash content in the studied plants in decreasing order are as follows; *N. lotus* > *I. aquatica* > *T. australis* > *S. cubensis*.

Crude fiber

The average percentage crude fiber content of the plant sample from Ada ranged from 14.5% in *S. cubensis* to 20.4% in *I. aquatica*. The *T. australis* and *N. lotus* also showed crude fiber content of 15.1% and 16.5%, respectively. Analysis of variance (ANOVA) at a 95% family-wise confidence level showed that the crude fiber content in the sampled plants was not statistically significant (P>0.05).

The average percentage crude fiber content of the plant sample from Kpong also ranged from 11.8% in *S. cubensis* to 22.2% in *I. aquatica*. The *T. australis* and *N. lotus* also demonstrated crude fiber content of 13.1% and 15.6%, respectively. Analysis of variance (ANOVA) at a 95% family-wise confidence level indicated that the crude fiber content in the sampled plants differs significantly (P<0.05). The least significant difference revealed no significant differences in means between *S. cubensis*, *T. australis*, and *N. lotus*, but there were yet significantly different from *I. aquatica*.

Nitrogen-free extract

The average percentage NFE content of the plant sample from Ada ranged from 26.2% in *I. aquatica* to 52.0% in *S. cubensis*. The *T. australis* and *N. lotus* also showed mean NFE content of 48% and 46%, respectively. ANOVA at a 95% family-wise confidence level showed that the NFE content in the sampled plants was not statistically significant (P>0.05).

The average percentage NFE content of the plant sample from Kpong also ranged from 19.2% in *I. aquatica* to 55.7% in *S. cubensis*. The *T. australis* and *N. lotus* also showed mean NFE content of 47.3% and 36.9%, respectively. Analysis of variance (ANOVA) at a 95% family-wise confidence level indicated that the NFE content in the sampled plants was highly statistically significant. The least significant difference revealed no substantial differences in the average of NFE between *T. australis* and *S. cubensis* but was yet different from *N. lotus* and *I. aquatica*. The average percentage NFE content in the studied plants in decreasing order of value are as follows; *S. cubensis* > *T. australis* > *N. lotus* > *I. aquatic*.

Crude protein

The average percentage crude protein content of the plant sample from Ada ranged from 14% in *S. cubensis* to 20% in *I. aquatica*. The *N. lotus* and *T. australis* also showed mean percentage crude protein content of 16.5% and 15.1%, respectively. ANOVA at a 95% family-wise confidence level revealed that the crude protein content in the sampled plants was not statistically significant (P>0.05)

The average percentage crude protein content of the plant sample from Kpong also ranged from 13.9% in *I. aquatica* to 22.7% in *T. australis*. The *N. lotus* and *S. cubensis* also showed mean percentage crude protein content of 16.6% and 15.6%, respectively. ANOVA at a 95% family-wise confidence level indicated that the crude protein content in the sampled plants was statistically significant (P>0.05). The LSD, however, showed that there were no significant differences in the average crude protein among *S. cubensis*, *N. lotus*, and *I. aquatica* but were somehow significantly different from *T. australis*.

Mineral composition

The sodium levels in plants sampled at the Ada sampling location fell from 0.10-0.23 mg/L, 0.20-0.32 mg/L, 0.21-0.30 mg/L, and 0.31-0.43 mg/L for *N. lotus*, *I. aquatica*, *T. australis*, and *S. cubensis* respectively while that sampled at Kpong sampling location fell from 0.01-0.16 mg/L, 0.16-0.23 mg/L, 0.10-0.23 mg/L and 0.23-0.43 mg/L for *N. lotus*, *I. aquatica*, *T. australis*, and *S. cubensis* respectively.

The calcium levels in plants taken at the Ada sampling location fell from 0.35-0.45 mg/L, 0.20-0.41 mg/L, 0.21-0.34 mg/L, and 0.20-0.46 mg/L for *N. lotus*, *I. aquatica*, *T. australis*, and *S. cubensis* respectively whereas that taken at Kpong sampling location fell from 0.27-0.41 mg/L, 0.41-0.56 mg/L, 0.32-0.56 mg/L and 0.46-0.65 mg/L for *N. lotus*, *I. aquatica*, *T. australis*, and *S. cubensis* respectively.

The potassium levels in plants taken at the Ada sampling location fell from 0.23-0.43 mg/L, 0.23-0.43 mg/L, 0.16-0.27 mg/L, and 0.28-0.32 mg/L for *N. lotus*, *I. aquatica*, *T. australis*, and *S. cubensis* respectively whereas that sampled at Kpong sampling location fell from 0.19-0.43 mg/L, 0.42-0.51 mg/L, 0.34-0.52 mg/L and 0.12-0.32 mg/L for *N. lotus*, *I. aquatica*, *T. australis*, and *S. cubensis* respectively.

The phosphorus levels in plants taken at the Ada sampling location also fell from 0.11-0.20 mg/L, 0.11-0.34 mg/L, 0.14-0.20 mg/L, and 0.31-0.65 mg/L for *N. lotus*, *I. aquatica*, *T. australis*, and *S. cubensis* respectively while that sampled at Kpong sampling location fell from 0.03-0.11 mg/L, 0.11-0.21 mg/L, 0.05-0.16 mg/L and 0.54-0.67 mg/L for *N. lotus*, *I. aquatica*, *T. australis*, and *S. cubensis* respectively.

Heavy metal concentration in plant samples

All six selected heavy metals were detected in the plant samples at the Ada and Kpong sampling sites. These metals include copper (Cu), Zinc (Zn), Cadmium (Cd), Arsenic (As), and Lead (Pb). The copper concentration in plants sampled at the Ada sampling site ranged from 2.3-3.45 mg/L, 0.45-0.57 mg/L, 0.34-0.45 mg/L, and 0.23-0.25

mg/L for *N. lotus, I. aquatica, T. australis,* and *S. cubensis* respectively while that sampled at Kpong sampling sites ranged from 0.261-6.41 mg/L, 0.26-0.43 mg/L, 0.17-0.6 mg/L and 0.08-0.12 mg/L for *N. lotus, I. aquatica, T. australis,* and *S. cubensis,* respectively.

The arsenic concentration in plants sampled at the Ada sampling site ranged from 0.01-0.18 mg/L, 0.02-0.19 mg/L, 0.23-0.28 mg/L, and 0.01-0.23 mg/L for *N. lotus, I. aquatica, T. australis, and S. cubensis* respectively while that sampled at Kpong sampling sites ranged from 0.01-0.06 mg/L, 0.06-0.20 mg/L, 0.14-0.20 mg/L and 0.16-0.34 mg/L for *N. lotus, I. aquatica, T. australis,* and *S. cubensis* respectively.

The zinc concentration in plants sampled at the Ada sampling site ranged from 1.65-2.0 mg/L, 0.13-0.23 mg/L, 0.20-0.32 mg/L, and 0.05-0.19 mg/L for *N. lotus, I. aquatica, T.australiss, and S. cubensis* respectively, while that sampled at Kpong sampling sites, ranged from 0.72-2.81 mg/L, 0.06-0.21 mg/L, 0.26-0.42 mg/L and 0.25-0.63 mg/L for *N. lotus, I. aquatica, T. australis,* and *S. cubensis* respectively.

The Cadmium concentration in plants sampled at the Ada sampling site ranged from 0.07-0.16 mg/L, 0.14-0.17 mg/L, 0.13-0.32 mg/L, and 0.36-0.52 mg/L for *N. lotus, I. aquatica, T. australis, and S. cubensis* respectively while that sampled at Kpong sampling sites ranged from 0.08-0.15 mg/L, 0.10-0.13 mg/L, 0.13-0.23 mg/L and 0.32-0.47 mg/L for *N. lotus, I. aquatica, T. australis,* and *S. cubensis* respectively.

The lead concentration in plants sampled at the Ada sampling site ranged from 0.57-0.75 mg/L, 0.23-0.30 mg/L, 0.17-0.23 mg/L, and 0.25-0.38 mg/L for *N. lotus, I. aquatica, T. australis, and S. cubensis* respectively while that sampled at Kpong sampling sites ranged from 0.67-0.96 mg/L, 0.06-0.21 mg/L, 0.26-0.42 mg/L and 0.25-0.63 mg/L for *N. lotus, I. aquatica, T. australis,* and *S. cubensis* respectively.

Physicochemical parameters of water

Temperature and pH

The water temperature sampled at the Ada stretch of the river ranged from 28.4 to 30.2ºC with an average value of 29±0.9ºC, and that of the Kpong stretch of the river varied from 30.1-32.6ºC with an average temperature of 31.7±1.0ºC. At the same time, that of Amedeka fell from 29.2-32.3ºC with a mean temperature of 30.93± 0.68ºC.

Statistical analysis using ANOVA displayed that there were statistically no significant differences in temperature between the three sampling locations ($t=42.2$, $P<0.05$) at a 5% level of significance (95% family-wise confidence level).

Pearson's product-moment correlation matrix performed to determine the degree, direction, and strength of the interrelationship between the physicochemical parameters and heavy metal concentration in the water sample at the locations indicated that temperature had a strong significant positive relationship with cadmium with a correlation coefficient (r) of the 0.854. There was, however, a weak correlation between temperature and the following metals Zn, Cu, Cr, and As.

The water pH sampled at the Ada stretch of the river ranged from a minimum of 6.8 to a maximum of 7.0 pH units with an average value of 6.9±0.1; that of Kpong ranged from 6.2-6.5 pH units with a mean pH of 6.37±0.2 and that of Amedeka range from 6.3 to 6.9 with a pH of 6.70±0.15.

Statistical analysis using ANOVA indicates no significant difference in the pH average from the three sampling locations. Pearson's product-moment correlation matrix, nevertheless, showed that pH had a highly significant negative correlation with cadmium and zinc at 5% and 1% levels of substantial, respectively (pH-Cd, $r=-0.776$, $p<0.05$), pH-Cr, $r=-0.669$, $P<0.01$). There was, yet, a strong positive correlation between pH and Zn.

The DO in the water sampled at Ada fell from 17.6 mg/L to 19.2 mg/L with an average value of 18.4±0.8 mg/L. Meanwhile, the Kpong stretch of the river also ranged from 8.6 mg/L-12.7 mg/L with an average of 10.7±2.0 mg/L, and that of Amedeka ranged from 22.3 mg/L to 26.6 mg/L with a mean DO value of 24.40±2.50 mg/L.

Statistical analysis using ANOVA indicates a significant difference in means of DO from the three sampling sites. Pearson's product-moment correlation matrix stated that there was generally a weak correlation between DO and the studied metals; however, DO have a strong positive relationship with zinc with a correlation coefficient of $r=0.770$ and a negative correlation with cadmium with a correlation coefficient of $r=-0.805$ at 5% level of significance.

The BOD in the water sampled at Ada ranged from 6.5 mg/L to 8.4 mg/L with an average value of 7.5.4±0.9 mg/L, and that of Kpong ranged from 1.6 mg/L-4.5 mg/L with an average value of 3.1±1.4 mg/L, and that of Amedeka ranged from 0.93 mg/L to 1.87 mg/L with an average DO of 1.13±0.21 mg/L. ANOVA test ranged revealed no statistically significant differences in dissolved oxygen between the water at Kpong and Amedeka. There was, however, a statistically significant difference in dissolved oxygen content between (Kpong and Amedeka) and (Amedeka and Kpong).

Pearson's product-moment correlation matrix revealed that BOD negatively correlates with cadmium and Zinc. (BOD-Cd, $r=-0.831$, $P<0.05$), (BOD-Zn,$r=-0.763$,$P<0.05$).

Nitrate, sulphate, and phosphate

The nitrate level of the water sampled at the Ada stretch of the river ranged from 1.25 mg/L to 1.84 mg/L with an average value of 1.47±0.3 mg/L, and that of Kpong ranged from 2.2-2.4 mg/L with an average value of 2.3±0.1 mg/L, and that of Amedeka ranged from 0.76 mg/L to 1.23 mg/L. ANOVA revealed no statistical difference between water from Amedeka and Ada sampling locations, but a significant difference was shown between (Amedeka and Kpong) and (Ada and Kpong) sampling locations ($P<0.05$).

The sulfate concentration of the water sampled at the Ada stretch of the river recorded an average value of 4 mg/L, that of Kpong fell from 5-6 mg/L with an average value of 5.3±0.5 mg/L, and that from Amedeka fell from 4.12 mg/L to 4.67 mg/L with an average 4.33 mg/L. There

was, however, no statistically significant differences in sulfate levels between the three-sampling area (P>0.05).

The Phosphate level of the water sampled at the Ada stretch of the river ranged from 0.43 mg/L to 0.71 mg/L with an average reading of 0.5±0.1 mg/L; that of Kpong fell from 0.71-0.92 mg/L with an average value of 0.8±0.1 mg/L and that of Amedeka ranged from 0.53 mg/L to 0.87 mg/L with a mean value of 0.73±0.11 mg/L.

There were no statistically significant differences in phosphate levels between the three sampling locations using ANOVA (P<0.05).

Results of heavy metal analysis in water and sediment

Heavy metals detected at the three sampling sites include copper (Cu), lead (Pb), Cadmium (Cd), Arsenic (As), and Zinc (Zn). The total heavy metals concentrations in sediments and water at Ada sampling location fell from (3.73-4.82 mg/L, 0.34-0.50 mg/L), (0.44-0.49 mg/L, 0.1-0.2 mg/L), (1.2-1.7 mg/L, 0.02-0.06 mg/L), (6.8-8.5 mg/L, 0.14-0.19 mg/L), (0.9-3.2 mg/L, 0.01-0.03 mg/L) and 0.9-1.5 mg/L, 0.01-0.02 mg/L) for copper (Cu), Zinc (Zn), lead (Pb), Chromium (Cr), Cadmium (Cd), and Arsenic (As) respectively. The total heavy metal level in sediment and water sample at Kpong sampling locations also fell from 4.6-5.7 mg/L, 0.38-0.46 mg/L (Cu), 0.4-0.5 mg/L, 0.112-0.113 mg/L (Pb), 0.5-0.9 mg/L, 0.05-0.06 mg/L (Cr), 0.9-2.5 mg/L, 0.05-0.06 mg/L (Cd), 5.3-6.8 mg/L, 0.09-0.14 mg/L (Zn) and 0.1-0.6 mg/L, 0.01-0.04 mg/L (As). The control site (Amedaka sampling location), however, recorded very low levels of heavy metal level in the sediments and water. It fell from (0.8-1.2 mg/L, 0.35-0.41 mg/L), (0.1-0.18 mg/L, 0.01-0.02), (0.9-1.2 mg/L, 0.2-0.3), (4.1-4.8 mg/L, 1.2-1.3), (0.1-1.5 mg/L, 0.01-1.2 mg/L) and 0.2-0.8 mg/L, (0.01-0.04 mg/L) for copper (Cu), Cadmium (Cd), lead (Pb), Zinc (Zn) and Arsenic (As) respectively. The average values and the standard deviations of the heavy metals concentration are illustrated in Table 8.

Correlation between Physico-chemical parameters and heavy metals in sediments

Considerable numbers of significant positive and adverse correlations were observed between the following physicochemical variables and heavy metals levels in the sediment sample; Temperature and Cadmium (r=-0.960, P<0.01), Temperature and Zinc (r=-0.722, P<0.05), pH and Cr (r=-0.543, P < 0.05), As and Zn (r=0.719, P<0.05), Cr and As (r=0.935, P<0.01), Pb and Cd (r =-0.632, P<0.05). Table 9 displays the person's product-moment correlation matrix of the significant physicochemical and heavy metal levels in the lower Volta basin sediments.

Table 8. Mean values of heavy metals (mg/L ±SD) in sediment samples in the study area

Site	Cd	As	Pb	Cr	Zn	Cd
Ada	2.3±1.2	1.2± 0.3	0.47±0.02	1.4±0.25	7.5 ± 0.86	2.3 ± 1.2
Kpong	1.8±0.8	0.3±0.02	0.4±0.03	0.7 ±0.02 5	80 ± 0.8	1.8±0.8
Amedaka	0.8±0.02	0.1±0.01	0.2±0.001	0.4±0.001	2.3±0.02	1.2±0.03

Table 10. Comparison between the mean concentration of trace metals in water and sediment

Site	Sample	Parameter	Cd	As	Pb	Cr	Zn	Cd
Ada	Water	F	7.5	24.1	244.5	51.4	118.7	7.5
	Sediment	P-value	0.00*	0.00*	0.00*	0.00*	0.00*	0.10ns
Kpong	Water	F	8.5	32.3	185.9	47.2	114.3	11.2
	Sediment	P-value	0.00*	0.00*	0.00*	0.00*	0.00*	0.30ns
Amedak	Water	F	7.2	25.8	175.3	49.5	117.5	9.6
	Sediment	P-value	0.00*	0.00*	0.00*	0.00*	0.00*	0.20ns

P<0.05 probability level; ns: Not significant

Table 9. Pearson's product-moment Correlation between Physico-chemical parameters and heavy metals in sediments

VAR	pH	Temp	Cu	AS	Pb	Cr	Zn	Cd
pH	1					-0.543*		
Temp		1	-0.558	-0.503	).58	-0.5438	-0.722*	-0.960*
Cu			1		-0.692*			
As				1		).935**	).719*	
Pb					1			-0.632*
Cr						1		
Zn							1	).662*
Cd								1

Note: *Correlation is significant at the 0.05 level (2-tailed).**Correlation is significant at the 0.01 level (2-tailed). Temp; Temperature

Table 11. Results of the phytochemical analysis of four aquatic plants

Phytochemicals	*Nymphaea lotus*	*Ipomoea aquatica*	*Typha australis*	*Scirpus cubensis*
Alkaloids	+	-	+	+
Flavonoids	+	+	+	+
Saponins	+	+	+	+
Sterols	+	+	+	-
Phenols	+	+	+	+
Proteins	+	-	+	+
Terpenoids	+	+	-	+

Note: All experiments were done in triplicate. Legend: += Present, -= Absent.

Phytochemical screening

Results obtained from the qualitative screening of plant samples are indicated in Table 11. Of the seven phytochemicals analyzed, three were found in all four plant samples; saponins, flavonoids, and phenols. *N. lotus* possessed all the phytochemicals tested present. Alkaloids and proteins were not identified in *I. aquatica*. Terpenoids were not present in *T. australis*. Sterols were not present in *S. cubensis*.

Social survey

A questionnaire was given to 120 individuals in the study area's chosen communities, Kpong (50%) and Ada (50%). Concerning the sex of the individuals, 71 individuals were males, while 49 individuals were females.

Forty-seven people were between the ages of 20-29 years, 30%, 20.8% were between the ages of 30-39 and 40-49, respectively, whereas only 10% were 50 years and above at the time the interview was sought.

Similarly, thirty-five of the respondents engage in fishing while 28.3% participate in various trading activities, with 11.7%, 10%, and 15% participating in farming, office work, and handwork, respectively.

The results showed that about 47% of the respondents were indigenous while 53% were settlers. The educational degree of the respondents was sought, and it demonstrated that 39.2% of the community had a JSS education. In comparison, 15% had secondary/6th form education, Ten percent (10%) had a tertiary education with 1.7% having vocational, and 34.2% had no formal training.

On the possible use of the river, 48 individuals use the water for domestic activities, whereas 36.7% use the water for fishing. Nevertheless, 11.7% and 10.8% use the water for irrigation and swimming, respectively.

When the respondents were questioned whether the plants that grow at the sampling sites are used to feed their animals, 65.2% answered in the affirmative, while the rest said they don't use the plants to feed animals.

Similarly, 67.5% of the individuals attest that the plants harvested imposed medicinal value, while the remaining respondents thought the opposite. When the respondents' perspectives were sought to ascertain whether the plants are the habitat of some wild animals, including snakes, 92.3% answered in the affirmative. However, most of the respondents had not been bitten by those snakes during the water use for their various activities.

When the respondents were questioned whether they fish in regions densely populated with weeds, 32.7% answered in the affirmative, while the rest said they do not fish in such areas. The respondents similarly confirmed that the abundance of weeds had decreased their fish catch, as the majority representing 79.2%, answered in the affirmative.

On the possible water utilization for drinking purposes, 43.3% of the individuals drink the water directly from the river. In contrast, the remaining 56.7% use other sources, such as wells and boreholes, as significant drinking water. However, this same water used by humans is also an excellent source of drinking water for animals such as cattle, sheep, goats, and pigs, as 74.2% of the respondents affirmed that fact.

When the respondents were questioned whether the abundance of weeds had raised the cost of transportation on the river, 54.2% replied in the affirmative, while source 45.8% said it had not raised the cost of shipping. The respondents noted that the vector-borne disease currently prevalent in the area is malaria instead of Schistosomiasis, which was the most significant disease in the area.

Also, 78% of the respondent confirmed that children no longer swim in densely infested locations. When the respondents were questioned about which kind of livestock they have in their communities, 100% indicated the presence of cattle, goats, sheep, pigs, and poultry in their communities.

Discussion

Nymphaea lotus from Ada exhibited the highest ash content, a total mineral or inorganic material of the sample. This implies that *N. lotus* has the highest total mineral or inorganic content, which may be vital to animals and, subsequently, humans. The other proximate constituents (crude fiber, protein, ether extract, ash, and nitrogen-free extract) were higher than a fresh early bloom *Panicum maximum* from Tanzania. Higher protein content makes it appropriate for animal feed and human feed. For this reason, many humans around the world feed on *N. lotus* (Nordeide et al. 1994). This trend was similar to that observed for *N. lotus* at Kpong. The findings obtained in the proximate analysis of *N. lotus* were identical to that found by Shah et al. (2010) in Srinagar. The crude protein content and nitrogen-free extracts of *N. lotus* are equal to Alfalfa hay (C.P= 16.91%, NFE= 40.55%) (Banerjee and Matai 1990).

The proximate constituents (crude protein, ash content, ether extract, and nitrogen-free extracts) of *I. aquatica* from Ada were higher than the corresponding components in a Fresh early bloom *P. maximum* from Tanzania. The result implies that *I. aquatica* has a higher nutritional value than *P. maximum*. The high ash content of *I. aquatica* indicates that the plant contains the nutritionally important mineral element.

The crude protein content in *I. aquatica* was higher than that reported in *I. aquatica* leaves found in Vietnam (Ogle et al. 2001). The crude fiber was, however, lower than that of *P. maximum*. The results showed a similar trend at Kpong.

Typha australis possessed the highest crude protein content that is comparable to that of *Azolla pinnata* (21.9%) and *Pistia stratiotes* (20.5%) (Banerjee and Matai 1990) which is an edible underwater tuber. The crude protein content, ether extracts, and nitrogen-free extracts were higher than the corresponding materials in *P. maximum*. Ash content was lower in *T. australis* than *P. maximum* suggesting lower mineral or inorganic material in *T. australis*. The nutrient composition of *Typha australis* was taken from Ada was more or less similar to the samples from Kpong.

Scirpus cubensis had the highest NFE indicating the presence of more digestible carbohydrates, vitamins, and

other non-nitrogen soluble organic compounds, which are crucial for animal and human growth. Ether extracts and Nitrogen free extracts were also higher than the corresponding constituents in *P. maximum*. Crude fiber and ash content were higher in *P. maximum* than in *S. cubensis*.

The variation in the mineral composition of the four aquatic plants may be due to differences in the genus and species level of the plants (Kalita et al., 2007). Among the four species, *S. cubensis* showed a relatively higher mineral content. Calcium and phosphorus were unusually high in *S. cubensis* at Ada. Non-availability of adequate quantities of minerals in the diet affects animal growth and may cause irrecoverable deficiency diseases (De Silva and Anderson 1994).

All heavy metals analyzed in the plants were lower than the standard set by WHO/FAO, 2007 for metals in foods and vegetables. Among the species studied, Zinc and Copper were unusually high in *N. lotus*, perhaps due to the natural abundance of this element in the environment. Besides, the broad nature of the leaves of *N. lotus* gives the plant a sufficient surface area to absorb metals from the water. The presence of a relatively higher content of Copper and Zinc in *N. lotus* makes it more considerable to be incorporated into the animal diet because it will provide the animals with essential trace elements.

All the plant species except *I. aquatica* have proteins, suggesting that they can be utilized as feed for animals and their overgrowth as weeds. The presence of secondary metabolites indicates that the plants are potential sources of pharmaceutical agents. This is because secondary metabolite derived from the plant has been shown to possess pharmacological activities and are involved in disease cure, prevention, and general health of the human body, as demonstrated by the folkloric use of plant parts for medicinal purposes.

The average temperature of water sampled at Ada and Kpong was similar to the control site (Amedeka). However, there was a slight difference in temperature at the sampling sites. The small temperature differences could be attributed to solar radiation, which relies on weather conditions and water mixing. However, the water temperature at the three sampling locations was higher than WHO's standard for drinking water. The time difference during sampling also accounts for temperature changes. Temperature serves an important role in governing the seasonal succession of the biota (Dhanalakshmi et al., 2013). Ofori-Danson and Ntow (2005) made a similar observation in the Volta River.

The lower pH values at Ada and Kpong may be due to the number of plants whose photosynthetic activities released carbon dioxide into the water, forming carbonic acid and reducing the pH. The pH that ranged between 5.0 and 9.0 at the sampling locations was considered appropriate for aquatic plant growth and fish survival (Jobbling 1995). Values of pH outside the range of 5.0 to 9 should be regarded as indications of industrial pollution or some cataclysmic event. Nevertheless, the pH of the water sampled at three sites all fell within the standard of WHO guidelines of drinking water.

The low DO record at Ada and Kpong reflects the high number of living organisms in the plant in the water. The aquatic plants impeded the water flow rate within infested areas on the field. DO is affected by flow rate, mixing, and aeration due to wind action (Straskaba and Tundisi 1999). However, the DO levels recorded at the two sampling sites were higher than the WHO standards for drinking water (7.5 mg/L), indicating that the water body is much oxygenated despite the presence of numerous aquatic plants.

BOD values measured for Ada and Kpong were higher than the control location'. Moderately polluted rivers show a BOD that falls in the range of 2-8 mg/L (Thakre et al., 2010). The BOD values recorded at the two locations above fell within the scope of a moderately polluted water body. Thus, it implies that aquatic plants exert some pollution level on the water. The BOD value of water in the control area was below the range of pollution (1.13 mg/L). Asante et al. (2008) mentioned an average BOD value of 4.5 mg/L on the Weija Reservoir. Karikari et al. (2013) also described a BOD value of 3.5 mg/L on the Volta River, which agrees with this study.

Nitrate levels at Ada and Kpong were higher than those at the control location. Nutrient levels were usually low in the water body of the study area. However, the nitrate grossly exceeded the total average of 0.1 mg/L of nitrate in freshwater bodies (Meybeck and Helmer 1989), perhaps could be a result of runoff from fertilized farmlands and domestic waste. This partly accounts for the high nitrate levels in infested areas of aquatic plants. The nitrate levels of the water at the three sampling areas were, however, fell within the WHO guideline of nitrate in drinking water (10 mg/L).

The average sulfate concentration in freshwater bodies is 4.8 mg/L (Meybeck and Helmer 1989). The sulfate concentration of water from Ada and the control site (Amedeka) fell below 4.8 mg/L. Sulfate concentration in water at Kpong was higher than the average sulfate concentration in freshwater. There was, however, no significant difference in means of sulfate concentration at the three sampling points. The sulfate level in the water could be due to the natural occurrence as sulfur occurs naturally in its reduced form in igneous and sedimentary rocks (Singleton 2000). However, the sulfate concentration at the three sampling points was within the WHO guideline of 250 mg/L for drinking water.

Aquatic plants in the study area have no significant influence on the phosphate concentration of the water. This result contradicts Mironga et al. (2012) in Lake Naivasha, that phosphate concentration in infested areas is lower than in uninfested areas. The natural background reading of phosphate (P-PO43-) in inland waters usually ranges from 0.005 to 0.05 mg/L (Dunne and Leopold 1978); the mean PO43-contents at all three sampling sites were outside the range. As witnessed during the study, the result might be attributed to washing utensils, clothing and bathing children and adults in the river at each sampling area.

The turbidity values of the water taken at Kpong and Ada were higher than that of Amedeka, which served as the control site. The average turbidity of Ada and Amedeka is similar; however, significant differences appear in the turbidity between Amedeka and Kpong on the one hand

and Ada and Kpon on the other side. The relatively high turbidity measured at Ada and Kpong may be due to the high levels of suspended particles originating from debris from the numerous aquatic plants.

All the heavy metal concentrations analyzed in the water were lower than the WHO guideline for drinking water except for cadmium and lead. The relatively high cadmium concentration in the study area might be coming from runoff from farms that use phosphate fertilizers containing cadmium (De Meeus et al. 2002). The lead amount exceeded the WHO guideline of lead in drinking water at Ada and Kpong. The primary sources of lead in the environment came from lead batteries and lead paints disposed of improperly (Rhue et al. 1992).

Except for cadmium, the differences in the concentrations of Chromium (Cr), copper (Cu), lead (Pb), Zinc (Zn), and Arsenic (As) in water and sediment samples at the sampling locations were significant. The corrosion of galvanized pipes on the canoes and other deposits, erosion of natural deposits, and runoffs from waste batteries and paints from the city and nearby surroundings could be the primary sources of heavy metal contamination in the water and sediments. The natural weathering of rocks beneath sediment might also be a potential source of heavy metal released into the deposits and the water column.

The social survey findings show that some community members often used some aquatic plants to feed animals such as cattle, pigs, goats, sheep, and poultry. Regular harvesting and feeding of animals with aquatic plants could lower the detrimental effects of these weeds on the water body. None of the respondents has ever employed any of the numerous aquatic plants as food. These findings contradict what Shah et al. (2010) reported in India: humans feed on aquatic. Again, 67% of the respondent indicated that the plants have medicinal value. This was confirmed by the results obtained in the phytochemical analysis.

Other socio-economic impacts of the aquatic plants suggest a loss of earnings opportunities as fishers could not access fishing and landing sites, as well as interference of the weeds with fishing gear and clogging of pumps. The abundance of aquatic plants has subsequently led to a reduction in fish catch.

Moreover, the aquatic weeds impede the flow rate of the water and provide excellent breeding grounds for mosquitoes. The phenomena were evident in the two communities, Kpong and Ada, as respondents indicated the presence of numerous mosquitoes at night. However, the respondent stated a decline in schistosomiasis, which previously was a significant water health-related issue in the communities.

Although 92.3% of the respondent attested to the vegetation harboring wild animals such as snakes, there has not been any incidence of snake bites in the communities from the river.

Conclusion

The current study has underlined that *N. lotus*, *I. aquatica*, *T. australis*, and *S. cubensis* could be significant sources of proteins and minerals suitable for incorporation into the animal diet. The results suggest that all four plant species have higher nutritional value than *P. maximum*. The exploitation of these aquatic plants will not only be of economic importance but also would be a step towards a sustainable and effective way of managing aquatic vegetation within the Lower Volta Basin.

Nymphaea lotus, *T. australis*, and *S. cubensis*. have demonstrated the presence of phytochemicals, promising the nutritional and antioxidant properties. Harvesting these plants for animal fodder and medicinal use will drastically decrease the invasive effects of these weeds on the water body and aid in the management of aquatic plants within the LVB.

Heavy metal concentrations analyzed in the plants were lower than the standard set by WHO/FAO, 2007 for metals in foods and vegetables. Copper and Zinc were relatively higher in Nymphea lotus than in other plants. The high levels of copper and zinc in *N. lotus* make it suitable to be added to the animal diet due to the vital role of copper and zinc in animals.

The physicochemical parameters of water (pH, nitrates, DO, BOD) revealed that most of the water parameters differed significantly from the control site. The concentrations of heavy metals in the waterfall are within the WHO guideline for drinking water except for lead (Pb) and Cadmium (Cd). The measured levels of all the studied metals in the sediment samples were higher than the concentrations in the water columns. Heavy metals fractionation should be carried out to ascertain the concentrations of heavy metals in each sediment fraction.

From the survey, responses from the respondents suggested that some groups of people use some of the plants, particularly *N. lotus*, to feed animals. Consistent harvesting of this plant will aid in managing aquatic plants within the LVB.

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