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Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret.
Jl. Ir. Sutami 36A Surakarta 57126, Central Java, Indonesia. Tel./Fax.. +62-271-663375, email: rjnpb@smujo.id, rumphius.jnpb@gmail.com

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Journal:

Saharjo BH, Nurhayati AD. 2006. Domination and composition structure change at hemic peat natural regeneration following burning; a case study in Pelalawan, Riau Province. Biodiversitas 7: 154-158.

Book:

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

Chapter in book:

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

Abstract:

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

Proceeding:

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

Thesis, Dissertation:

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

Information from internet:

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

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Extraction and assessment of pectin from pumpkin peels

AWADIA ALDOW RAMADAN HAMED[1], SALMA ELGHALI MUSTAFA[2,♥]
[1]College of Graduate Studies, Sudan University of Science and Technology. Khartoum, Sudan
[2]Department of Food Science and Technology, College of Agricultural Studies, Sudan University of Science and Technology. Khartoum, Sudan.
♥email: salma-a@sustech.edu

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Abstract. *Hamed AAR, Mustafa SE. 2018. Extraction and assessment of pectin from pumpkin peels. Biofarmasi J Nat Prod Biochem 16: 1-7.* Pectin is characterized as intricate blends of polysaccharides that compose around 33% of most plants' cell wall dry substance. This study was done to extricate pectin from a completely ripened pumpkin (*Cucurbita* spp.) using two distinct techniques; the soxhlet acid extraction technique and acid extraction technique, and to find out the impact of utilizing distinctive acids on the yield of pectin; nitric and citric acids were utilized. In addition, to analyze the impact of time on pectin yield, the extraction procedure was carried out using 3 different times, namely, 30, 60, and 90 min. The chemical substances of pumpkin peel (dampness, ash, protein, fat, fiber, total sugar, and calcium) and the chemical properties of pectin (methoxyl content, acetyl content, identical weight, and level of esterification) were extracted with both nitric and citric acids were also identified. The outcomes produced by the soxhlet acid extraction technique and acid extraction technique were 7.72% nitric acid and 6.80% citric acid and 6.24% nitric acid, and 5.36% citric acid, respectively, demonstrating that the utilization of soxhlet acid extraction technique with nitric acid got the highest yield of pectin. Additionally, it was also discovered that the time for pectin extraction was 60 min at the very most. The outcome for the chemical substances of pumpkin peel (dampness, fiery remains, protein, fiber, fats, add up to sugar, and calcium) were 20.1, 7.1, 3.2, 10.15, 2.3, 57.15, and 0.308%, respectively. The examination discovered that the chemical properties of pectin extracted with both nitric and citric acid were for the equivalent weight (1250 and 1250 g/mol), methoxyl content (6.20 and 6.29%), acetyl contains (0.43 and 0.43%) and the level of esterification (66.53 and 66.57%) respectively. The outcome got from this examination showed that pectin extracted from pumpkin peel is of high quantity and quality and is promising for commercial production.

Keywords: *Cucurbita***,** pectin, pumpkin peels

INTRODUCTION

Pumpkins belong to the gourd family *Cucurbita*ceae, containing watermelons, cucumbers, gourds, marrow, and squash (Elshafie 1981). *Cucurbits* consist of 110 genera, and 640 species predominate in the tropics, mostly as herbs of very rapid growth and climbing habit with abundant sap (Trease and Evans 1989). Pumpkin is rich in polysaccharides, carotene, mineral salts, vitamins, and other substances beneficial to health, resulting in various processed food products being developed (Ptichkina et al. 1998).

Ordinarily, fruits are productized into juice, beverage, squash, and syrups. During productizing, peel presents almost 5-20% of the whole fruit. Therefore, the vast sum of by-products (55-60% peel and 5-10% seed) has contributed to the pollution of the environment. Nevertheless, by-products can be utilized as functional food components such as photochemical, pharmaceuticals, food products, essential oils, seed oil, pectin, and dietary fibers. Therefore, by-products are considered to be rich sources of edible and health-promoting agents (Ali, 2014).

Pumpkin is also a low-cost source of pectin (Murkovic et al. 2002). Pectin is an abundant multifunctional component in the cell walls of all plants (Willats et al., 2006; Ngouémazong et al., 2012). Pectin is a polysaccharide consisting mostly of polymers rich in galacturonic acid, containing significant amounts of rhamnose, arabinose, galactose, and 13 monosaccharides (Vincken et al. 2003). Waldron et al. (2003) reported that pectin's composition, structure, and physiological properties could be influenced by extrication conditions, source, location, and many other environmental factors. Therefore, the network of pectin must be broken to be extracted.

Pectins are regularly extracted from citrus fruits and apple pomace, and they are customarily utilized as gelling agents for jams and marmalades. In combination with water and other substances, it can act as a thickener, gelling agent, stabilizer, emulsifier, cation-binding agent, etc. (Bottger 1990). Once a substance has so many properties of technological interest, this makes pectin a biopolymer especially valuable for medicine, food production, and applications in drug encapsulation (Ptichkina et al., 2008; Souza et al., 2009; Benjamin et al., 2012).

The objectives of this study were: (i) To determine the chemical composition of pumpkin peels (moisture, protein, ash, fat, fiber, total sugar, and calcium), (ii) To investigate the effect of different extraction methods (soxhlet acid extraction method and acid extraction method) with different acids (nitric and citric acids) in yield of pectin, (iii) To examine the effect of time on pectin yield, (iv) To evaluate the properties of the extracted pectin.

MATERIALS AND METHODS

Materials

Pumpkin peels

Pumpkin was achieved directly from the local central market for fruits and vegetables in North Khartoum, Sudan was then stripped manually using a knife, baked at 70;49˚C for 24h in an oven and ground, and kept for further analysis.

Chemicals:

In this study, all chemicals and reagents were of analytical grade. Nitric acid, hydrochloric acid, sulphuric acid, and boric acid are from SDFCL Sd Fine Chem Ltd. (Mumbai, India). Sodium hydroxide, citric acid, methyl red, phenol red, and catalyst tablet were from Lobach Emie Pvt. Ltd. (Mumbai, India). Petroleum ether is from Alpha Chemical (India), Magnesium sulphate is from Landcech Chemical (India), while Sodium chloride is from Scharlaau (Spain), and ethanol is from Drummer and Sons, Co. Ltd. (Sham Industrial City, Syria).

Instruments

Soxhlet, protein unit, ovens, pH meter, and centrifuge are from J.P Selecta (Spain), water bath from Daiham Scientific, Co., Ltd. (South Korea), and thermometer (Omsons, India).

Methods

Analysis of the chemical composition of pumpkin peels

Moisture content. The AOAC method (1999) was used to consider the moisture content. Samples (5 g) were accurately weighed and dried to constant weight in a vacuum oven at 70˚C and 450mm Hg for three hours. The analysis was carried out in triplicate.

Moisture% = (W1-W2)/ S

Whereas:
W1 : weight before drying
W2 : weight after drying
S : weight of the sample.

Ash content. AOAC (1999) method was used to consider the ash content. Samples (5 g) were accurately weighed and put into relatively broad crucibles that had been ignited, then they were cooled in desiccators and weighed. The crucible and its content were ignited in a muffle furnace at 550 ºC until light grey ash of samples with constant weight was obtained. The analysis was carried out in triplicate samples.

The ash content was calculated as follows:

$$Ash\% = W_1\text{-}W_2 / W_1\text{-}W_0$$

Whereas:
W_0 : weight of the empty crucible
W_1 : weight before ash processing
W_2 : weight after ash processing

Protein content

The determination of Nitrogen content was done with the semi-micro Kjeldahl distillation according to AOAC (1999) method. An exact 0.2 g of the sample was assimilated in a small digestion flask using a half tablet of catalyst (each tablet contains 1 g anhydrous sodium sulphate and 0.1 g copper sulphate). 3.5 mL of concentrated sulphuric acid was added to it. Then the digest was diluted and transferred to the ammonia distillation apparatus using the minimum volume of distilled water and made alkaline with 20 mL of 40% sodium hydroxide. The ammonia was distilled into 2% boric acid solution (10 mL), plus methyl red indicator (3-4 drops) for 5-10 minutes. After lowering the receiving flask clear of the condenser, the apparatus was steam distilled for 5 minutes. The distillate was then titrated with 0.02N hydrochloric acid.

$$\mathbf{N\%= \frac{(m1HCL - m1blank) \times normality\ of\ HCL \times 14 \times 100}{sample\ (mg)}}$$

Crude protein = % N×6.25 6.25= refer to formula of protein

Fats content. The estimation of fat content was done using the method of AOAC (1999). Triplicate samples (2 g) were weighed and put into a thimble plugged with a piece of cotton wool. The thimble was then put into a soxhlet extractor. A dry and accurately weighed soxhlet flask was fitted to the extractor. Boiling petroleum with a temperature range of 60-80 ºC was poured into two-thirds of the flask. The instrument was then set up and fitted to the condenser. Water was allowed to flow through the condenser, and an electric heater was put on for reflux. The extraction was done for eight hours. Then the instrument was carefully disassembled, and the liquid in the flask was evaporated to dryness in an oven at 100 ºC to a constant weight.

$$\mathbf{Fat\%= \frac{the\ weight\ of\ ether\ extract \times 100}{Weight\ of\ sample}}$$

Fiber content. AOAC (1999) method was used to estimate the fiber content. The sample from the ether extract was air-dried and transferred to a dry conical flask. Then 200 mL of 1.25% sulphuric acid boiled within one minute were added, and the mixture then was boiled gently for 30 minutes; constant volume was maintained, and the flask was shaken every few minutes. The residue was then filtered through apices of cotton fitted to the Buchner funnel. The filtration of the solution was completed within 10 minutes. The insoluble matter was washed with hot water until it was free from acid. It was then washed back into the original flask by a washing bottle containing 1.25% sodium hydroxide (200 mL) measured at room temperature and brought to boiling. It was allowed to boil for 30 minutes, filtered immediately through an ash-less filter paper, and then washed with 1% HCL, followed by boiling water until it was free from acid. The residue was transferred to a dried weight crucible and dried at100ºC to reach constant weight. The residue was finally ashed at 600ºC in a muffle furnace. The weight of the ash was

subtracted from the weight of the dish plus insoluble residue before ashing, and the difference was expressed as crude fiber percentage.

Carbohydrate content. It was estimated according to the method of AOAC (1999).

Carbohydrate% = 100-(moisture +ash+ protein+ fat and fiber%).

Calcium content. The method described by Elmer (1993) was used to determine the content of Calcium. One gram of the material was put in a porcelain crucible, placed in a cool muffle furnace, and ashed at 500 ºC overnight. The crucible content was dissolved into 5 mL of 25% HCL. The solution was warmed to dissolve the residue and filtered through an acid-washed filter paper in a 50 mL volumetric flask. The filter paper was washed; the solution was diluted with distilled water to volume and mixed well. Using one milliliter of this solution, the amount of calcium was spectrophotometrically determined at a wavelength of 422.7 nm using an Atomic Absorption spectrophotometer (AA-6800, Japan).

Pectin extraction method

The extraction method is referred to as the method by Malviya (2010). It can be carried out in two steps.

Pectin extraction. Pectin was extracted under reflux in a condensation system using water which was acidified for 1hr with acid to pH 2. The temperature of extraction media was maintained at 80ºC, and extraction time was adjusted to about 6 h. Whatman cellulose thimble with 33 mm of internal diameter and 80 mm of external length was used as an extractor thimble. Dried powdered pumpkin peel was taken in Soxhlet, and reflux was continued for 6 h.

Pectin precipitation. Hot acid extract was pressed into a cheesecloth bag, and the cake was cooled to 4 ºC. Pectin was precipitated by alcohol treatment 2:1 (v/v), followed by continuous stirring for 15 min, and allowed to stand for 2 h for better precipitation. This allowed the filtering of pectic substances because pectin remained floating on the surface of alcohol. Floating pectin coagulate was filtered through a cheesecloth, washed with alcohol 70%, and pressed. Pressed pectin was dried to constant weight at 35-45 ºC in a hot oven. Hard pectin cake was ground and sieved through a 20 mesh size sieve and, at last, was stored in a desiccator for further use.

Acid extraction method. According to Crandall et al. (1978), the method for acid extraction has some modifications. Four hundred mL of distilled water (DW) was poured into a 2000 mL Erlenmeyer flask and kept at the desired temperature, i.e., 80 ºC, using the stirring hot plate or the shaking water bath, and 100 g peel was added to the water. Measured amounts of acid using different acids (Nitric and Citric Acids) were added to the peel-water mixture until the desired pH was 2. The mixture was stirred at a constant temperature until the desired extraction time had elapsed. The pH and temperature were recorded, and the mixture was allowed to cool in ice water until it reached 55 ºC. The mixture was then centrifuged at 5050 rpm for 10 min. The filtrate was vacuum filtered using filter paper, and the solids were resuspended in 400 mL of 60 ºC DW water for 5 min. Acid extracted pectin was kept after overnight precipitation. The pectin was separated from the alcohol solution using a double layer of cheesecloth, and the samples were washed three times with 70% diluted alcohol to remove any impurities. The resulting pectin was dried under vacuum at 50 ºC in aluminum sample dishes until all moisture was removed. Pectin was cooled, weighed, and ground using a mortar and pestle. Ground pectin was stored in small plastic sample bags. Both two extraction procedures were done twice using nitric and citric acids. The amount of pectin was calculated according to the following equation:

$$\textbf{Pectin yield} = \frac{\textbf{Weight of extracted pectin} \times 100}{\textbf{Weight of dried peel}}$$

Effect of time in pectin extraction

The effect of time on pectin yield was examined to determine whether increasing extraction time will increase pectin yield. The extraction time was set at 30, 60, and 90 min; the extraction time followed the method by Crandall et al. (1978), with little modification. Effect of time on acid extracted pectin yield was done at extraction conditions of 80ºC, pH 2 using 1 N nitric acids, and solid to liquid ratio of 1:4 g/mL using a water bath.

Chemical analysis of extracted pectin

Moisture content. The method of AOAC (1995) was used to determine the moisture content of pectin. Triplicate samples (1 g) were weighed in dried and weight aluminum dishes. Samples were then dried for 4 hours at 105 ºC (20 mmHg). They were then cooled in desiccators, and their constant weights were determined. The moisture content was calculated as follows:

Moisture% = (W_1-W_2)/ S

Whereas:
W_1 : weight before drying.
W_2 : weight after drying.
S : weight of the sample.

Ash content. The AOAC (1995) method was used to determine the ash content of pectin. Triplicate samples (1 g) were previously weighed, then heated, cooled, and weighed their crucible weight. The sample was then heated at 600 ºC for 3 hours, cooled, and determined constant weight. The ash content was calculated as follows:

Ash% = W_1-W_2 / W_1-W_0

Whereas:
W_0 : weight of empty crucible.
W_1 : weight before ashing
W_2 : weight after ash

Ash alkalinity. The method by Owens et al. (1952) was used to determine the ash alkalinity of pectin. The ash was dissolved in 25 mL of 0.1 N HCL, heated gently, and then

titrated with 0.1 N sodium hydroxide using a phenolphthalein indicator. The alkaline number of ash was calculated as the number of a millimeter of acid required to neutralize 1 g of ash.

Equivalent Weight. Ranganna's method (1995) was used to determine Equivalent weight. Triplicate samples (0.5gm) were weighed into a 250 mL conical flask and moistened with 5 mL ethanol. The product was mixed with 1 gm sodium chloride and 100 mL of distilled water. The mixture was stirred vigorously, and free acidity was determined by direct titration against 0.1 N sodium hydroxide using phenol red as an indicator. Also, a blank containing the same quantities of reagents was tested. This titre is known as the initial titre (IT) or free acid titre. The equivalent weight was calculated as follows:

Equivalent weight = weight of sample (mg) / Meg of sodium hydroxide

Meq (Miliequivalent of sodium hydroxide) = Normality × Titre volume of Noah

Methoxyl content. Ranganna's method (1995) was used to determine MeO content. The neutral solution was collected to determine the equivalent weight, then 25 mL of sodium hydroxide (0.25 N) was added. The mixed solution was stirred thoroughly and kept at room temperature for 30 min. After 30 min, 25 mL of hydrochloric acid (0.25 N) was added and titrated against 0.1 N NaOH.

The following formula calculated methoxyl content :

$$\text{Methoxyl content \%} = \frac{\text{Meq of NaOH } 3.1 \times 100}{\text{Weight of sample}}$$

Whereas:

Meq of NaOH = normality of NaOH titre figure 3.1 refers to a formula weight of methoxyl group.

Degree of esterification (DE). Based on methoxyl content (Owens et al. 1952), the DE of pectin was measured

From IT and ST obtained, the degree of esterification and Anhydrouronic acid (AUA) content was calculated as follows:

Degree of esterification (DE) = ST × 100/ ST+ Corrected IT The IT corrected the ash alkalinity

Acetyl content. Owens et al. (1952) method was used to determine Acetyl content. Triplicate samples (0.5 gm) of each pectic substance were weighed into a flask, and 250 mL of 0.1N NaOH were added. The flask was covered, shaken, and left for one night. The solution was then diluted to 50 mL, from which 20 mL were taken and placed in the distillation apparatus. Twenty milliliter magnesium sulphate sulphuric acid solution (100 g magnesium sulphate and 1.5 gm sulphuric acid diluted to180 mL) were added. The solution was then steam distilled, and 10 mL of distillate was collected. The acetic acid of the distillate was then titrated with sodium hydroxide (0.05 N) to phenol redpoint. The titre was corrected for the blank reagents. The acetyl content (formula weight 43) of the sample was calculated according to the following equation:

$$\text{Acetyl content (\%w/w)} = \frac{\text{(ml NaOH) (normality of NaOH)} \times 43}{\text{Weight of sample (gm) in the liquot}}$$

Whereas:

MI NaOH : volume required to titre distillate-volume of NaOH required to titre blank distillate run.

43 : formula weight of Acetylene.

Statistical analysis

Microsoft Excel software on the statistical page of social science was used to analyze the data of this study. Data were expressed as mean ± standard deviation (STD). The statistical significance was considered at $P< 0.05$. All experiments were organized using a completely randomized design with three replicates, repeated for reproducibility. The data obtained from the measurements were subjected to a T-test which was used to compare the difference between properties of pectin extracted by different acids. All measurements were carried out in triplicate for each sample. The experimental data were reported as the means ± SD of independent trials (Wagner, 1985).

RESULTS AND DISCUSSION

Chemical compositions of pumpkin peel

Chemical compositions expressed on a dry basis were presented in Table 1. The moisture content was 20.1%. The Ash content of pumpkin peel was 7.1 %. The protein content of pumpkin peel was 3.2%. The fiber content was 10.15 %. The fat content was 2.3. The carbohydrates of pumpkin peel were 57.15 %, and calcium was 0.308 %. This result was different from Ibrahim's study (2008), which found that the chemical compositions of pumpkin peel on a weight basis were as follows: the moisture content was 84.7-90.26%; the Ash content of pumpkin peel was 0.81-1.35%; the protein content of pumpkin peel was 1.3-1.64%; the fiber content was 3.21-4.82%; the content of fat, carbohydrates, and calcium were 0.04, 5.17 and 0.062 % respectively.

Table 1. Chemical compositions of pumpkin peels on dray basis

Parameter	%
Moisture	20.1
Ash	7.1
Protein	3.2
Crud fiber	10.15
Fats	2.3
Carbohydrate	57.15
Calcium	0.308

Note: Values are means of 3 replicates for each parameter

Effect of different methods on pectin yield

The optimal extraction conditions of this study used nitric and citric acid to adjust the pH of pumpkin peel to 2, which was then extracted for 60min at 80ºC. Soxhlet extraction was done for six hours with alcohol precipitation for two hours, but in acid extraction without soxhlet, alcohol perception was ten hours. The results were 7.72% nitric acid and 6.80% citric acid using the soxhlet acid extraction method and 6.24% nitric acid and 5.36% citric acid using the no soxhlet acid extraction method. As shown in Figure 1, the result indicated that using the soxhlet acid extraction method gave a higher yield than using the acid extraction method without soxhlet to extract pectin from pumpkin peel. The result in this study was different from the result of the study by Yeoh et al. (2008). They showed that, for microwave extraction, the greatest total amount of pectin yield was 5.27% on a dry basis for 15 minutes of extraction. However, the greatest amount of material per unit time (%/min) was obtained after 5 minutes. This amount was the same as that result extracted by the Soxhlet extraction method for three hours from orange peel.

Effect of different acids on pectin yield

In this study, the extracted pectin from pumpkin peels was 6.24% using nitric acid and 5.36% using citric acid. The study showed that nitric acid yielded higher results than citric acid, respectively, as shown in Figure 1. A significant difference of p0.05 was found between the two acids. The result was higher than the range of 0.253-0.233% for pectin extraction from alcohol insoluble pumpkin peel solids using hydrochloric acid to adjust pH to 2 and which was extracted for 60min at 80ºC (Ibrahim, 2008). It differed from Sayah et al. (2014), who extracted pectin from steam distillation and non-conditioned orange peels using different acids.

Citric acid gave the best average yield (25.71% ± 0.007), while sulfuric, hydrochloric, and acetic acids gave a very low average yield of pectin, namely 6.49% ±0.005, 7.96% ± 0.005, and 10.19% ± 0.006, respectively.

Effect of extraction time in pectin yield

The effect of extraction time on pectin yield was observed to determine the effect of the extraction time on the increase of pectin yield. The results are shown in Figure 2. There was an increase in pectin yield during 30 min and 60 min of extraction time. Since there was no noticeable trend of pectin yield increase with extraction time from 60 min to 90 min, so, the suitable extraction time was 60 min. The result of this study disagreed with (Campbell 2006). There was increasing in pectin yield between 0-45min. No noticeable on-time trend occurred with increasing extraction time from 45 min to 90 min.

Chemical composition in pectin pumpkin peels

Moisture content

The moisture content of pectin pumpkin peels extracted by nitric and citric acids was 5.54±0.01% and 5.42±0.0%, respectively, as shown in Figure 3. A significant difference of p0.05 was found between the two samples. The result is lower than the value of grapefruit peels pectin, with the range of 7.88-8.96%, as claimed by Mohamed (1999). The values were also higher than mango pulp pectin, with the range of 5.03-5.04%, as Abderahman (2002) reported.

Ash content

The Ash content of pumpkin peels pectin extracted with nitric and citric acids was 3.17±0.006 and 2.96 ±0.006%, respectively, as shown in Figure 4. There was a significant difference of p0.05 between the two samples. The result is higher than a lemon with the range of 1.56-1.65%, an orange with the range of 0.81-4.83% in orange, or an apple with the range of 0.49-8.05%, as stated by Abderahman (2002). Mohamed (1999) reported a range of 1.8% to 2.0% for grapefruit peels pectin.

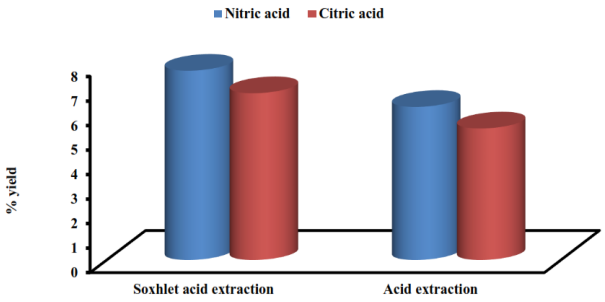

Figure 1. Pectin yield by using different methods and acid

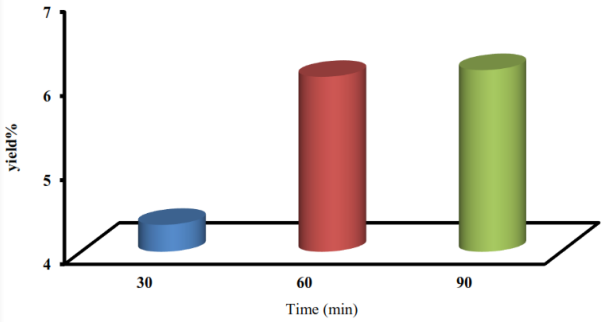

Figure 2. Effect of extraction time on pectin yield

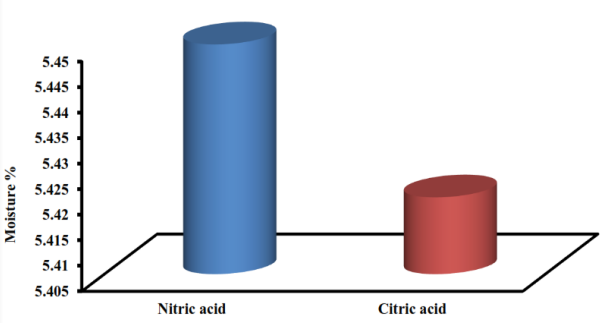

Figure 3. The moisture content of pectin in pumpkin peel

The equivalent weight

The equivalent weights of extracted pectin from pumpkin peels, using nitric and citric acid, were 1250 ± 0.0 and 1250 ± 0.0 g/mol, respectively, as shown in Figure 5. The results showed no significant difference of p0.05 between the two samples. Still, they were higher than cocoa husk pectin (510.68-645.19 g/mol), which was reported by Ramli and Asmawati (2011), and the ones reported by Mohamed (1999) for grapefruit peel pectin for citrus pectin (620-749 g/mol). The equivalent weights obtained in this study were lower than, with a range of 1389-2003 g/mol (Abderahman 2002) or apple pomace pectin (833.33-1666.30 g/mol) (Kumar and Chauhan 2010) and ambarella peels (263,000-303,000 g/mol) (Koubla 2008).

Methoxyl content

The methoxy1 contents of pumpkin peel pectin extracted by nitric and citric acids were 6.20±0.10% and 7.23±0.89%, respectively, as shown in Figure 5. No significant difference p 0.05 was observed between the two samples. In this study, the methoxyl was lower than the one found by Ali (2014) for lemon pomace (10.25±022%) and the one reported by Madhav and Pushpalatha (2002) for pomelo peel (8.57%), Lime (9.92%), passion (8.81-9.61%); it was similar to peel of mango (7.33%), banana (7.03%) (Madhav and Pushpalatha, 2002) but it was higher than dragon fruit pectin (2.98% to 4.34%) (Ismail et al. 2012). Methoxyl content was an important factor in controlling pectin's setting time and the pectin's ability to form gels (Constenla and Lozano 2003).

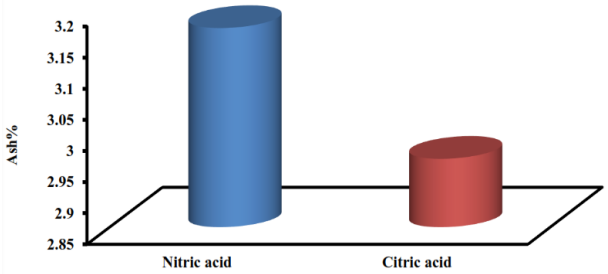

Figure 4. Ash content of pectin of pumpkin peel

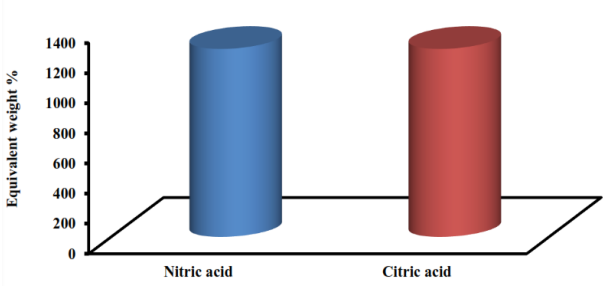

Figure 5. The equivalent weight of pectin of pumpkin peel

Acety1 content

The acety1 contents of pumpkin peel pectin extracted with nitric and citric acids were 0.43±0.01 and 0.43±0.03%, respectively (Figure 6). There was no difference of p0.05 between the two samples. The result was in accordance with the finding of Mohamed (1999), which showed that 0.46-1.63% for grapefruit peels pectin, and it was also higher than the result reported by Abderahman (2002) for acety1 content in mango pulp pectin (0.117% to 0.314%). The acety1 group in pectin materials has an important role in their effect on the jelly formatting ability (Abderahman 2002). Also, the finding was lower than the one reported by Koubla et al. (2008) on amarelle peels, i.e., 4-6%.

Degree of esterification

The results in Figure 7 showed the degree of esterification of pumpkin peels pectin extracted by nitric and citric acids were 66.53±0.058 and 66.57±0.058%, respectively.

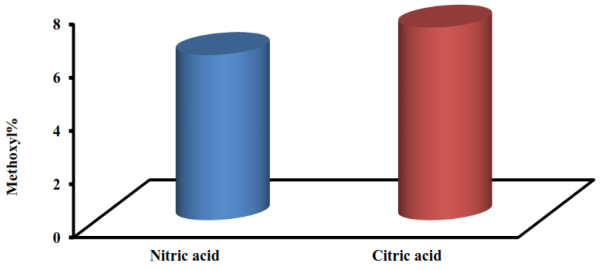

Figure 5. Methoxyl content of pectin of pumpkin peel

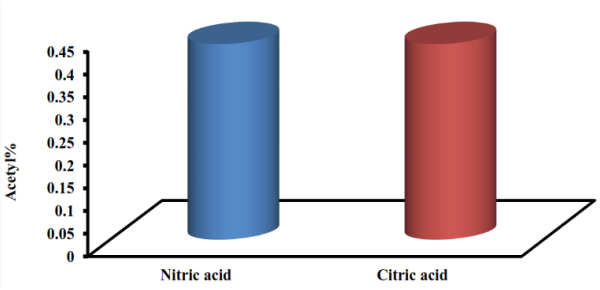

Figure 6. Acetyl content of pectin of pumpkin peel

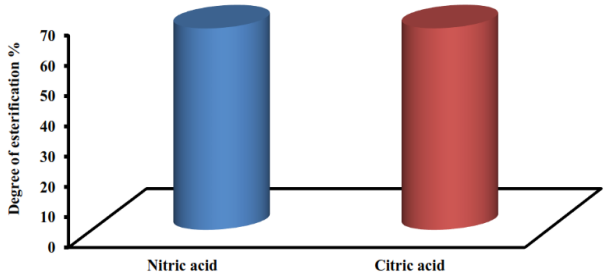

Figure 7. Degree of esterification of pectin of pumpkin peel

There was no difference of p0.05 between the two samples. The result was in agreement with the result reported by Eltinay et al. (1982) for mango peel pectin (66.9%) but higher than that obtained by Mohamed (1999) for grapefruit peels pectin, which was 51.01-51.24% and by Koubla et al. (2008) for ambarella peels which were 50-58%.

The values of degree in the esterification of this study were lower than those reported by Eltinay et al. (1982), Abderahman (2002), and Ali (2014) for pumpkin pectin (73.9-86.8%), for mango pulp pectin (87.0%) and lemon pomace pectin (79.51±0.36 and 70.39±4.20%) respectively. Pumpkin peel has a high level and good properties of pectin, and using Soxhlet, and nitric acid at 60 min for the extraction process gives the highest pectin yield.

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Modeling and synthesis of antiplasmodial chromones, chromanones and chalcones based on natural products of Kenya

MANYIM SCOLASTICA, ALBERT J. NDAKALA♥, SOLOMON DERESE
Department of Chemistry, University of Nairobi. P.O. Box 30197-00100, Chiromo Campus, Nairobi, Kenya. Tel.: +254-20-4446138, Fax.: +254-20-4446138, ♥email: andakala@uonbi.ac.ke

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Abstract. *Scolastica M, Ndakala AJ, Derese S. 2018. Modeling and synthesis of antiplasmodial chromones, chromanones and chalcones based on natural products of Kenya. Biofarmasi J Nat Prod Biochem 16: 8-21.* Despite numerous research that has been done on plants in Kenya, resulting in the isolation of thousands of natural products, data on these natural products are not systematically organized in a readily accessible form. This has urged the construction of a web-based database of natural products in Kenya. The database is named *Mitishamba* and is hosted at http://mitishamba.uonbi.ac.ke. The *Mitishamba* database was queried for chromones, chromanones, and chalcones and subjected to structure-based drug design using *Fred's* (OpenEye) docking utility program with the 1TV5 PDB structure, the *Pf*DHODH receptor, to identify complex ligands that bind with the active site. Ligand-based drug design (Shape and electrostatics comparison) was also done on the ligands against query **A77 1726** (**38**) (the ligand that is co-crystallized with *Pf*DHODH receptor) using *ROCS* and *EON* programs, respectively, of OpenEye suite. There was a substantial similarity among the top-performing ligands in the docking studies with shape and electrostatic comparison that identified compounds of interest targeted for synthesis and antiplasmodial assay. In this study, a chromanone (7-hydroxy-2-(4-methoxyphenyl) chroman-4-one (**48**)) and two intermediate chalcones (2',4'-dihydroxy-4-methoxychalcone (**45)** and 2',4'-dihydroxy-4-chlorochalcone (**47))**, were synthesized and subjected to antiplasmodial assay. Among these substances, **45** showed vigorous activity, whereas **47** and **48** had moderate activity against the chloroquine-resistant K1 strain of *P. falciparum* with IC_{50} values of 4.56±1.66, 17.62 ± 5.94, and 18.01 ±1.66 µg/ml, respectively. Since the synthesized compounds showed antiplasmodial potential, there is a need for further computational refinement of these compounds to optimize their antiplasmodial activity.

Keywords: Antiplasmodial activity, chalcones, chromones, chromanones, web-based database

INTRODUCTION

Natural products have been the center of focus for many years as the primary source of new, more effective, and safer bioactive metabolites with therapeutic properties against various infectious diseases (Dike et al., 2012). The majority of the synthetic drugs used today are either inspired or derived from bioactive compounds in nature (Zhu et al., 2012). Thus, nature continues to be an essential source of new medicines.

The significance of bioactive compounds of natural origin is that they provide novel lead compounds or pharmacological agents for drug discovery. Medicinal plants have played a vital role in the treatment of malaria, as well as the development of antimalarial drugs. The best-known potent antimalarial compound quinine was isolated from the bark of the *Cinchona* tree (Schlitzer 2007). Over the past decades, the discovery of artemisinin from *Artemisia annua* has boosted research on plants searching for new antimalarial lead compounds (Biamonte et al., 2013). Based on the historically high success rate of natural products, the diversity of chemical compounds found in nature continues to be an essential source of molecular templates in the search for novel antimalarial drugs (Nogueira and Lopes, 2011).

The African flora comprises a variety of medicinal plants, which the indigenous people have extensively utilized to treat various ailments. For example, in East Africa, there are close to 1,200 plant species with medicinal value (Kokwaro 2009). These plant species have been used to treat various diseases and ailments like malaria, typhoid, ulcers, skin diseases, diabetes, reproductive problems, aches, and pains. Multiple studies on medicinal plants of Kenya have led to the isolation of several bioactive compounds (Endale et al. 2012; Yenesew et al. 2003, 2004, 2005, 2009) with diverse structural scaffolds (Derese et al. 2003; Gumula et al. 2012; Omosa et al. 2010). Despite the enormous body of research existing on the natural products of Kenya, this information is not systematically organized in a readily accessible form. This is why a web-based searchable database of natural products in Kenya is developed in this study.

A searchable database of natural products of Kenya would be a source of templates, when combined with modeling, for the design and synthesis of drugs for treating various diseases. For example, chromone (1-benzopyran-4-one), a core scaffold in flavonoids, has been identified to bind well with diverse receptors (Gaspar et al., 2011). This is due to the vast pharmacologically active compounds with this scaffold that have exhibited antimalarial, antibacterial, anticancer, anti-HIV, and anti-inflammatory activity. Chromone derivatives have also been found to act as kinase inhibitors by binding to benzodiazepine receptors (Keri et al., 2014). These attractive binding properties and biological

activities make the chromone scaffold a template of interest for *in silico* design and synthesis of antiplasmodial compounds.

The objectives of this research were to (i) Develop a web-based *in silico* database of natural products of Kenya. (ii) Design antiplasmodial compounds based on chromone, chromanone, and chalcone scaffolds through virtual screening of the database against *Plasmodium falciparum* dihydroorotate dehydrogenase enzyme. (iii) Synthesize and evaluate the antiplasmodial activity of the promising chromones, chromanones, and chalcones from the virtual screening.

MATERIALS AND METHODS

Database construction

This study constructed a web-based in silico database of natural products in Kenya. Information on natural products was collected from different sources and processed to generate this web-based database.

Data collection

Information about compounds isolated from the natural products of Kenya was collected from various journal articles, review papers, theses, books of abstracts, and conference proceedings. The captured information was the structure of the compounds, common names, classes of the compounds, and biological activities (if available). It also included the plant species, family, parts of the plant where the compounds were extracted, places of the collection of the plant, and the reference of the source material.

Data processing

The data collected from the various sources was then captured in a Microsoft Excel spreadsheet. Accelrys Draw 4.1 SP1 software-academic version was used to draw the 2D chemical structures of the natural products collected from the literature. The *mol2nam*, a command-line utility of the *Lexichem* program (OpenEye Scientific Software), generated the compounds' IUPAC names and converted the 2D structures into SMILES files (1D format). *Omegsa2*, a command-line utility of the *Omega* program (OpenEye Scientific Software), was used to convert the 2D to 3D structures and generate the physicochemical properties of the compounds: polar surface area (PSA), molecular weight (MW), Merck molecular force field energy (MMFF), partition coefficient (log P), rotatable bonds, heavy atoms, hydrogen acceptors and hydrogen donors of the compounds. The data was visualized with the VIDA program (OpenEye Scientific Software) and exported into an initial MS excel spreadsheet. The data in the MS Excel spreadsheet was then transferred into the Discovery Studio 4.1visualizer, a product of Accelrys, where it was organized, and an SDF file was generated.

Generation of the web-based database of Kenya natural products

The Linux computer operating system was used to produce the web-based database of natural products from Kenya. The SDF file was exported into the local host database and then transferred to the web-based MySQL relational database using the command line. The information was stored in the MySQL database in normalized tables, where each compound was given a unique identification code. The creation of the database search engine was adapted from Nobert Heider's MolDBR6 (Haider 2010) software package based on the framework summarized in Figure 1. The software utilizes the *checkmol* and *matchmo*l search engines (Haider 2010). Javascript editor JSME and Java molecule editor (JME) by Peter Ertl of Novartis (Bienfait and Ertl 2013) were used to enable users to search for compounds using structures. The search engine utilizes hypertext preprocessor (PHP) scripts and structured query language (SQL) to achieve its basic utility. The web-based database generated is hosted at http://mitishamba.uonbi.ac.ke.

Preparation of the ligands and the receptor

The ligands and the receptor were prepared for the virtual screening process.

Preparation of the ligands

The *Mitishamba* database was queried separately for the chromanone, chromone, and chalcone ligands, and the SDF file of the three hits was downloaded from http://mitishamba.uonbi.ac.ke. The three sdf files were combined and filtered using a *filter* program (OpenEye Scientific Software). The filter parameters used were based on the Lipinski rule of five (molecular weight<500, hydrogen bond donors<5, hydrogen bond acceptors<10, and Log P<5) (Lipinski et al. 2012). The output was then saved as a zipped file (.oeb.gz).

Omega2 (OpenEye Scientific Software) was then used to generate 3D conformers of the filtered compounds using default parameters except for the number of conformers (maxconfs=10) and stereochemistry (strict stereo=false). The output was saved as a zipped file (.oeb.gz) and visualized using *Vida* (OpenEye Scientific Software).

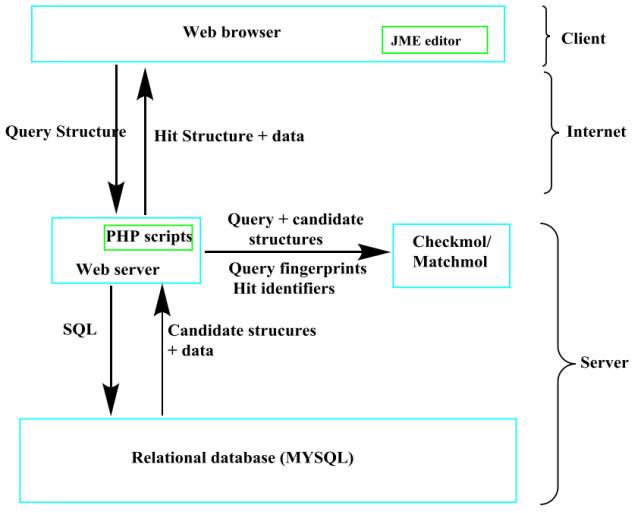


Figure 1. The architecture of the *Mitishamba* database search engine adapted from MOLDBR6

Receptor preparation

The 1TV5 PDB structure of the *Plasmodium falciparum* dihydroorotate dehydrogenase (*Pf*DHODH) co-crystallized with **A77 1726 (38)** ligand, Figure **2**, was obtained from the RCSB protein data bank at http://www.rcsb.org/pdb/explore.do?structureId=1tv5. The *Make Receptor* program (OpenEye Scientific Software) was used to define the active binding pocket of the target by selecting the ligand of interest **A77 1726** (**38)** bound to the 1TV5 PDB structure. This was followed by creating a box that encloses the active site. Then the active site shape potential was created to define the outer and inner contours.

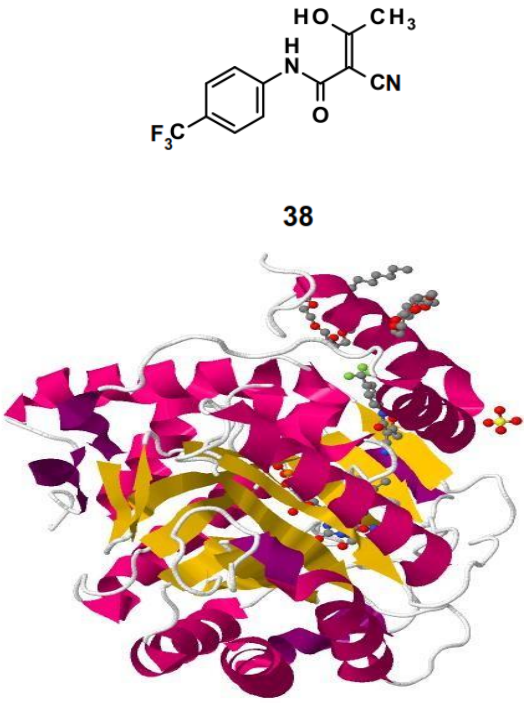


Figure 2. 3D Structure of *Pf*DHODH with bound ligands

Virtual screening

The prepared ligands were then subjected to structure-based virtual screening against the prepared receptor and ligand-based virtual screening to identify promising antiplasmodial lead compounds.

Structure-based virtual screening

Fred (OEDocking, OpenEye Scientific Software) was used to carry out docking studies against the prepared *Pf*DHODH receptor where the specified binding site contained the inhibitor (**38**). A docking study was done using the command line utility where the inputs were the zipped files of the ligands and the prepared *Pf*DHODH receptor. At the same time, the output was a zipped file of the docked poses, which were then viewed using the *Vida* (OpenEye Scientific Software) visualizing program. The zipped file of the docked poses and the receptor were then used to generate a pdf document summarizing ligand-receptor interactions.

Ligand-based virtual screening

A shape and electrostatics similarity search was conducted using the validated query for ligand-based virtual screening.

Query validation. The inhibitor (**38**) bound to the 1TV5 *Pf*DHODH receptor was chosen as the query molecule for use in shape and electrostatics similarity. A query validation run was done using the vROCS program (OpenEye Scientific Software) to determine whether the selected query was suitable for similarity search. The decoys and actives were obtained from the Database of Useful Decoys-Enhanced (DUD-E) http://dude.docking.org/target/pyrd. The databases of decoys and actives in the validation help establish whether the query is chosen to differentiate between the already known active and inactive (decoys) molecules against the target protein.

The receiver operating characteristic curve (ROC curve), together with its area under the curve (AUC) and the early enrichment values, are the statistical metrics generated by the *vROCS* program (OpenEye Scientific Software) that were used to validate the query (**38**). The validated question (**38**) was then used in ligand-based virtual screening of the chromones, chromanones, and chalcones to conduct shape matching and electrostatic similarity searches.

Shape similarity search. Using the validated query (**38)**, vROCS (OpenEye Scientific Software) performed a shape similarity search. As generated by the Othe mega program, the 3D conformers of the ligands were overlaid on the query using the vROCS program, which employs the Gaussian shape overlap to score the ligands. The ligands are scored based on shape (Shape Tanimoto score) and color (Color Tanimoto score) and ranked based on the Tanimoto Combo score (shape and color).

Electrostatics similarity search. Using the validated query (**38**), electrostatics similarity studies on 3D conformers of the ligands were done using *EON* (OpenEye Scientific Software). *EON* aligns molecules on the query and calculates the electrostatic potential using the Poisson-Boltzmann and Coulombic electrostatics tools. The molecules are scored regarding Poisson-Boltzmann electrostatics Tanimoto (ET pb), Coulombic electrostatics Tanimoto (ET_pb), and *EON* shape Tanimoto (*EON*_shape_tani). The ligands were then ranked using electrostatics Tanimoto combo (ET_combo), a combination of *EON* shape Tanimoto and Poisson-Boltzmann electrostatics Tanimoto (ET_pb).

The ranking of ligands based on structure and ligand-based virtual screening guided the identification of synthetically accessible chromones and chromanones targeted for synthesis.

Synthesis of chromanones and chromones

The approach for synthesizing chromanone and chromone-based lead compounds was based on retrosynthetic Figure 3.

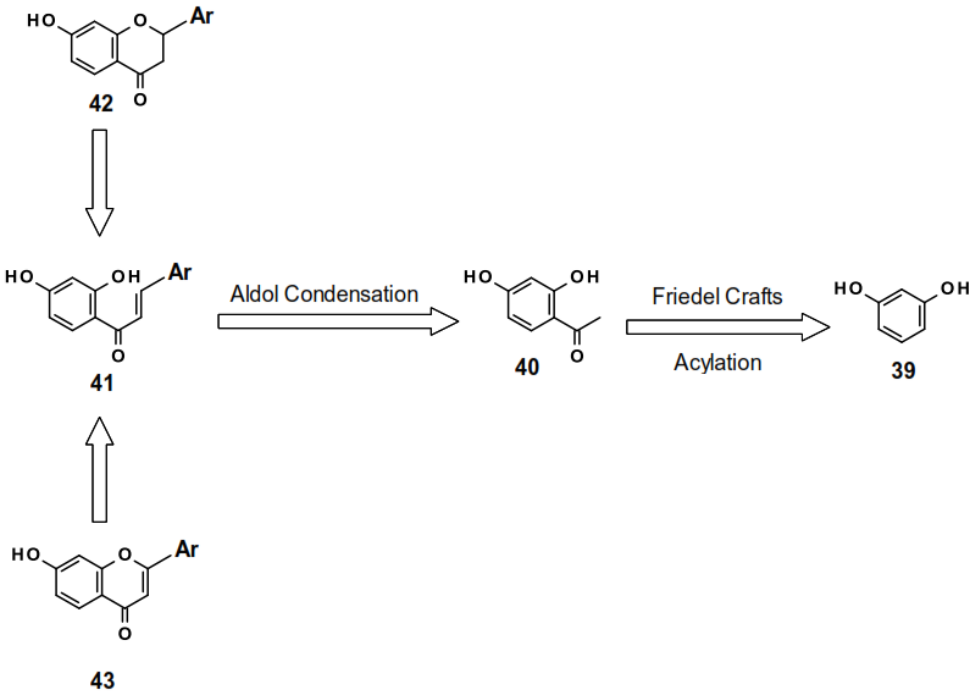
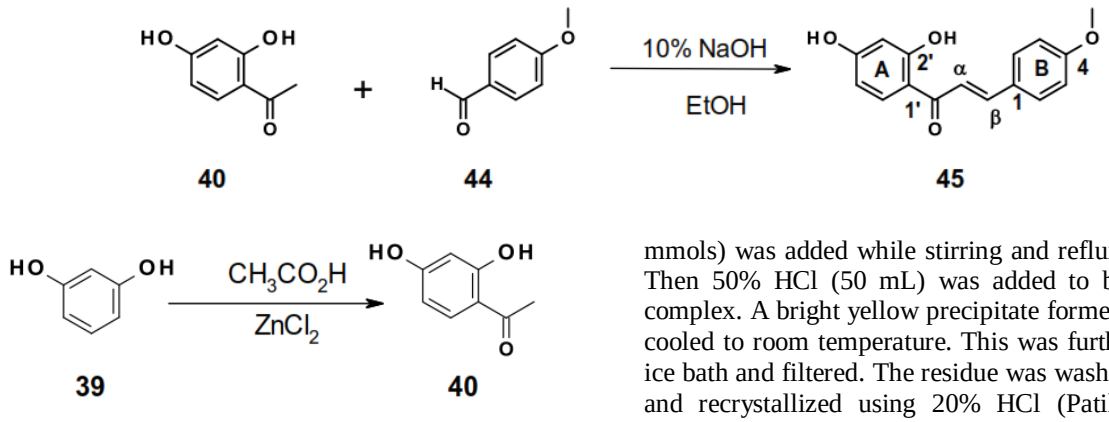


Figure 3. Retrosynthetic pathway to chromanones and chromones



General procedure

Chemicals used in the synthesis were purchased from LOBA Chemie Laboratory reagents and fine chemicals. The purchased chemicals were of synthetic grade and did not need any purification. The solvents used in column and thin layer chromatography were distilled before use. The ^{1}H NMR spectra were obtained at 600 and 500 MHz with TMS as an internal standard and deuterated dichloromethane and acetone as the solvents. Reactions were monitored on analytical TLC silica gel plates with a fluorescent indicator of 254 nm. Purification was done using column chromatography on silica gel 60-120. Purification through recrystallization was done using methanol.

Procedure for synthesis of 2,4-dihydroxyacetophenone (40)

Anhydrous $ZnCl_2$ (15 g, 110 mmols) was added to acetic acid (30 mL) and refluxed at 150 °C. When all the $ZnCl_2$ was almost dissolved, resorcinol (**39**) (10 g, 90 mmols) was added while stirring and refluxed for 3 hours. Then 50% HCl (50 mL) was added to break the $ZnCl_2$ complex. A bright yellow precipitate formed as the mixture cooled to room temperature. This was further cooled in an ice bath and filtered. The residue was washed with 5% HCl and recrystallized using 20% HCl (Patil et al., 2012). Compound **40** was obtained as a yellow crystalline solid (4.6 g, 50%), mp 146 °C: ^{1}H NMR (600 MHz, Acetone-d_6) δ 12.77 (*s*, 1H), 9.48 (*s*, 1H), 7.79 (*d*, *J* = 8.40 Hz, 1H, C-6), 6.45 (*dd*, *J* = 2.40Hz, 8.40Hz, 1H, C-5), 6.34 (*d*, *J* = 2.40 Hz, 1H, C-3), 2.57 (*s*, 3H, OCH_3). ^{13}C NMR (151 MHz, Acetone-d_6) δ 202.8 (C=O), 164.9 (C-4), 164.5 (C-2), 133.5 (C-1), 113.4 (C-6), 107.7 (C-5), 102.4 (C-3), 25.5 (CH_3).

Procedure for synthesis of 2',4'-dihydroxy-4-methoxychalcone (45)

10% NaOH (20 mL) and 99% ethanol (30 mL) were mixed in a round-bottomed flask and cooled in an ice bath for 10 minutes. 4-Methoxybenzaldehyde (6 g, 44 mmols) was added while stirring, then 2, 4-dihydroxyacetophenone (3 g, 20 mmols) was added dropwise. The reaction mixture was left stirring and monitored using TLC until all the 2,4-dihydroxyacetophenone was consumed; this took approximately 48 hours. The reaction mixture was poured over cold water and acidified using 20% HCl. Sodium bicarbonate was added while shaking until no bubbles were

observed to remove the 4-methoxy benzoic acid. It was then transferred into a separatory funnel and extracted three times using ethyl acetate (20 mL). The organic layer was dried up using anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to provide a yellow precipitate which was purified using column chromatography (5% ethyl acetate in hexane) and recrystallization (methanol). Compound **45** was obtained as a yellow solid (1.6 g, 30 %), mp 186 °C: ^{1}H NMR (600 MHz, Acetone-d_6) δ 13.64 (*s*, 1H, chelated hydroxyl), 8.16 (*d*, *J* = 8.8 Hz, 1H, C-6'), 7.86 (*m*, 4H, C-2, C-3, β-C

and (α-C), 7.05 (*d*, 2H, C-3 and C-5), 6.50 (*dd*, *J* = 8.8, 2.2 Hz, 1H, C-5'), 6.40 (*d*, *J* = 2.2 Hz, 1H, C-3'), 3.89 (*s*, 3H, OCH_3), ^{13}C NMR (150 MHz, Acetone-d_6) δ 191.9 (C=O), 166.7 (C-4'), 164.7 (C-2'), 162.0 (C-4), 143.9 (β-C), 132.5 (C-1'), 130.7 (C-2), 130.7 (C-6), 128.0 (C-1), 118.2 (α-C), 114.4 (C-3), 114.4 (C-5), 113.6 (C-6'), 107.8 (C-5'), 102.8 (C-3'), 54.9 (CH_3).

Procedure for synthesis of 2', 4'-dihydroxy-4-chlorochalcone (47)

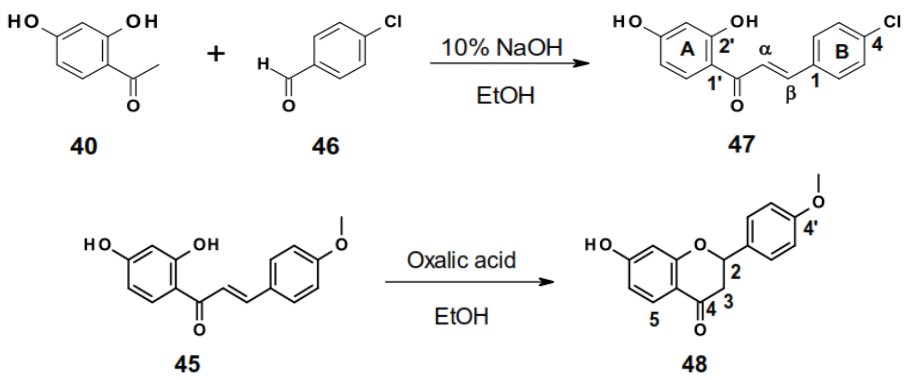
10% NaOH (10 mL) and 95% EtOH (10 mL) were mixed in a flask and cooled in an ice bath for 10 minutes. 4-Chlorobenzaldehyde (6 g, 43 mmols) was added into the flask while stirring, and then 2'4-dihydroxyacetophenone (20 mmols) was added dropwise and stirred for 30 minutes. The reaction mixture was heated under reflux at 70ºC and monitored using TLC until all the 2'4'-dihydroxyacetophenone was consumed; this took 48 hours. The reaction mixture was poured over cold water and acidified using 20% HCl. Sodium bicarbonate was added while shaking until no bubbles were observed to remove 4-chlorobenzoic acid. It was then transferred into a separatory funnel and extracted three times using ethyl acetate (20mL). The organic layer was dried using anhydrous Na2SO4, filtered, and concentrated under reduced pressure to purify a yellow precipitate using column chromatography (5% ethyl acetate in hexane) and recrystallization (methanol). Light yellow crystals (0.84 g, 16%) were obtained: ^{1}H NMR (500 MHz, Acetone-d_6) δ 13.44 (*s*, 1H, chelated hydroxyl), 8.18 (*d*, *J* = 8.9 Hz, 1H, C-6'), 8.00 (*d*, *J* = 15.5 Hz, 1H, H_β), 7.91 (*d*, *J* = 8.4 Hz, 2H, C-2 and C-6), 7.86 (*d*, *J* = 15.5 Hz, 1H, H_α), 7.52 (*d*, *J* = 8.5 Hz, 2H, C-3 and C-5), 6.50 (*dd*, *J* = 8.9, 2.4 Hz, 1H, C-5'), 6.40 (*d*, *J* = 2.4 Hz, 1H, C-3'). ^{13}C NMR (126 MHz, Acetone-d_6) δ 191.7 (C=O), 166.8 (C-4'), 165.1 (C-2'), 142.3 (C-β), 135.8 (C-4), 133.9 (C-1), 132.8 (C-6'), 130.3 (C-2), 130.3 (C-6), 129.0 (C-3), 129.0 (C-5), 121.6 (C-α), 113.6 (C-1'), 108.1 (C-5'), 102.9 (C-3').

Procedure for synthesis of 7-hydroxy-2-(4-methoxyphenyl) chroman-4-one (48)

Oxalic acid (1 g, 7.9 mmols) and 2',4'-dihydroxy-4-methoxychalcone (0.1 g, 0.37 mmols) were dissolved in ethanol (10 mL). The reaction mixture was heated at 80 °C for 2 days. It was cooled to room temperature and poured over cold water and extracted three times with ethyl acetate (20 mL). The organic extract was dried using anhydrous Na_2SO_4 and concentrated under reduced pressure then purification was done using column chromatography (5% ethyl acetate in hexane) and recrystallization (methanol). A yellow crystalline solid (29 mg, 29%) was obtained: ^{1}H NMR (600 MHz, CD_2Cl_2) δ 7.85 (*d*, *J* = 8.8 Hz, 2H, C-2' and C-6'), 7.44 (*d*, *J* = 8.6 Hz, 1H, C-5), 6.99 (*d*, *J* = 8.8 Hz, 2H, C-3' and C-5'), 6.59 (*dd*, *J* = 8.6, 2.3 Hz, 1H, C-6), 6.49 (*d*, *J* = 2.3 Hz, 1H, C-8), 5.46 (dd, J=2.4, 13.2Hz, 1H, H-2), 3.86 (*s*, 3H, OCH_3), 3.09 (*dd*, *J* = 13.2 , 16.8 Hz, 1H, H-3_{eq}), 2.81 (*dd*, *J*= 2.4, 16.8 Hz, 1H, H-$_{3ax}$). ^{13}C NMR (151 MHz, CD_2Cl_2) δ 190.5 (C=O), 163.6 (C-7), 162.7 (C-4'), 160.0 (C-9), 130.9 (C-5), 129.1 (C-1'), 127.8 (C-2'), 127.8 (C-6'), 115.1 (C-10), 114.0 (C-3'), 114.0 (C-5'), 110.3 (C-6), 103.2 (C-8), 79.7 (C-2), 55.3 (OCH_3), 44.0 (C-3).

In vitro antiplasmodial bioassay

In vitro antiplasmodial bioassay of compounds **45**, **47,** and **48** against chloroquine-resistant K1 and chloroquine-sensitive 3D7 strains of *Plasmodium falciparum* was done using a non-radioactive assay technique (Heydenreich et al. 2011). This technique uses the fluorochrome referred to as SYBR Green I, which is a non-radioactive DNA dye that accurately depicts *in vitro* parasite replication. The parasites were cultured to attain 3-8% parasitemia. At the same time two-fold serial dilution of chloroquine (1.953-1000 ng/mL), mefloquine (0.488-250 ng/mL) and test samples (97.7-50,000 ng/mL) were prepared on a 96 well plate. The culture-adapted *P. falciparum* was reconstituted to 1% parasitemia and added onto the plate containing the dose range of the reference drugs and test samples and incubated in the gas mixture (5% CO_2, 5% O_2, and 90% N_2) at 37 °C. The assay was stopped after 72 hours by freezing at-80 °C for 24 hours.



Thawing was done, followed by the direct addition of the lysis buffer containing SYBR Green I (1×final concentration) into the plates and gently mixing the final solution with the Beckman Coulter Biomek 2000 automated laboratory workstation. Incubation of the plates was done for 5-15 minutes at room temperature in the dark. Parasite growth inhibition was quantified by measuring the per-well relative fluorescence units (RFU) of SYBR Green 1 dye using the Tecan Genios Plus (Tecan US, Inc., Durham, NC) with excitation and emission wavelengths of 485 nm and 535 nm, respectively, and with the gain set at 60. Differential counts of relative fluorescence units (RFUs) were used in calculating 50% inhibition concentrations (IC_{50}'s) for each drug using Prism 4.0 windows software (GraphPad Software, San Diego, CA). At least three separate determinations were carried out for each sample. Replicates had narrow data ranges hence presented as mean ±SD.

RESULTS AND DISCUSSION

In this study, a web-based database of natural products of Kenya was developed, which was subjected to virtual screening to identify synthetically accessible chromones and chromanones for antiplasmodial assay.

Database construction

A web-based *in silico* database containing 1112 natural products of Kenya was constructed from data on natural products collected from theses, journal articles, books of abstracts, and conference proceedings. The information captured was the structure of the compounds (2D sketch files), common names, classes of the compounds and biological activities (if available). It also included the plant species, family, parts of the plant where the compounds were extracted, places of plant collection, and the reference of the source material.

Besides, the database contains physicochemical properties (polar surface area (PSA), molecular weight (MW), Merck molecular force field energy (MMFF), partition coefficient (log P), rotatable bonds, heavy atoms, hydrogen acceptors, and hydrogen donors) of the compounds. The database is named *Mitishamba* (a Kiswahili word meaning medicinal herbs) and is hosted at http:/mitishamba.uonbi.ac.ke.

Features of the Mitishamba web-based database

The *Mitishamba* web-based database has features for search (text, structure, and advanced search), browsing and downloading the entire database, and submitting structures, as shown in Figure 4.

Search options. The database has three search options: Text, structure, and advanced search. In-text search, users can search for structures using either trivial names or IUPAC names. For structure search, the database uses Peter Etrl Novartis (Haider 2010), Javascript molecule editor (JSME), or Java molecule editor (JME) for 2D structure input. The options are detected automatically on the users' browser, and one is used as a fallback in case either Javascript or Java feature is turned off. Optional parameters available for structure search include an exact match, substructure similarity search, and ratio similarity on a zero to hundred percent scale. Once an output is obtained, structures can be downloaded. The advanced search features allow users to search for compounds based on plant family and species.

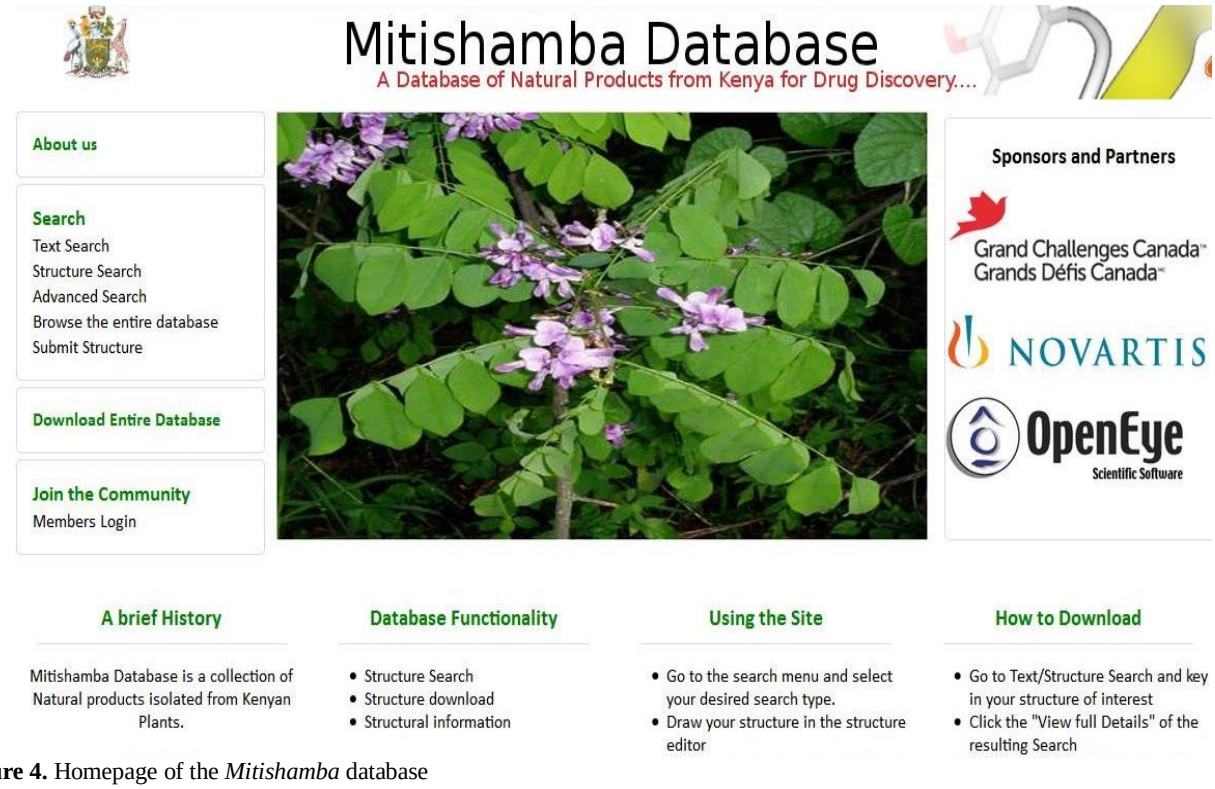


Figure 4. Homepage of the *Mitishamba* database

Browse and download options. Users can also browse the structures in the entire database. Each structure can be downloaded individually as a mol file, or users can download the entire database in sdf, mol, smiles, or oeb formats.

Submitting structures. Users can submit their work on Kenya's natural products that have not been captured in the database. To submit structure, users upload a PDF document that contains the names of the natural products, family and botanical name of the source plant, place of collection of the plant, biological activities of the compounds, and appropriately referenced source of the publication.

Virtual screening studies

A search in the *Mitishamba* database identified 157 chromone, chromanone, and chalcone ligands. The ligands were then subjected to structure and ligand-based virtual screening.

Ligand and receptor preparation

The 157 ligands were filtered using a *filter* program based on Lipinski's rule of five (molecular weight<500, hydrogen bond donors<5, hydrogen bond acceptors<10, and Log P<5) (Lipinski et al. 2012), and this yielded 103 ligands. The 3D conformers of the 103 ligands were then generated using the *omega* program for use in both structure and ligand-based virtual screening.

The PDB file of the 1TV5 PfDHODH receptor with the bound ligand (38) was obtained from the RCSB protein data bank at http://www.rcsb.org/pdb/explore.do?structureId=1tv5 and prepared using *Make Receptor* program, Figure 5.

Structure-based virtual screening studies

Docking studies of the filtered ligands against the prepared *Pf*DHODH receptor using the *Fred* program ranked the molecules using chemgausse4 docking scores (Kcal/mol). To ease the interpretation of the docking scores (raw data), z-scores were generated (Swann et al., 2011). Among the 103 ligands, the ligands with a z-score of one and above were 25, Table 1. The best scoring ligands were chromones, followed by chromanones and chalcones.

The interaction of the ligands with the *Pf*DHODH receptor was studied and compared to the binding interaction of the inhibitor (**38**). The inhibitor (**38**) bound to the 1TV5 *Pf*DHODH receptor shows hydrogen bonding interactions with three amino acid residues, ARG 265A, HIS 185A, and TRY 528A (Figure 6) (Hurt et al. 2006a,b).

Comparing the interaction of inhibitor (**38**) with those of the ligands, as expected, most of the ligands had at least one interaction with the three amino acid residues, Figure 7. Some ligands had other interactions; for example, Figure 8 shows interactions with TRY 168A and MET 536A, while Figure 9 shows interactions with GLY 181A. Other ligands showed further interactions with LEU 531, VAL 532A, LEU 172A, and CYS 184.

Ligand-based virtual screening studies

The shape and electrostatics of the 103 ligands were compared against a validated query of the inhibitor (**38**).

Query validation. The 3D structure of the 1TV5 *Pf*DHODH inhibitor (**38**), Figure 10, was used as the query molecule for shape and electrostatic similarity. To determine whether query **38** is valid for shape and electrostatic similarity studies, *vROCs* Tanimoto combo scores (combination of shape and color scores) for the actives and decoys were used to plot the ROC curve (Figure 11). In the ROCs curve, Y-axis is the fraction of actives found, and X-axis is the fraction of decoys found.

The statistical metrics derived from the ROC curve for query validation run are summarized in Table 2

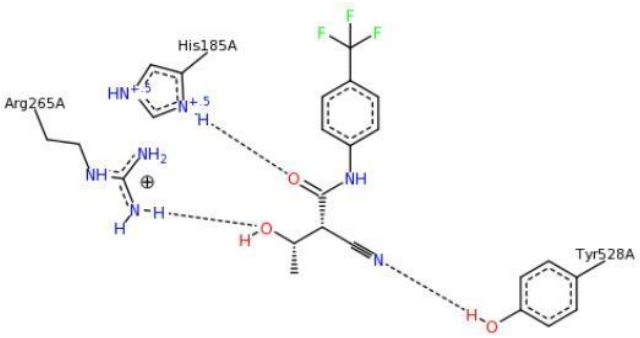


Figure 6. Hydrogen bonding interactions of the bound inhibitor (**38**) with amino acid residues of *Pf*DHODH

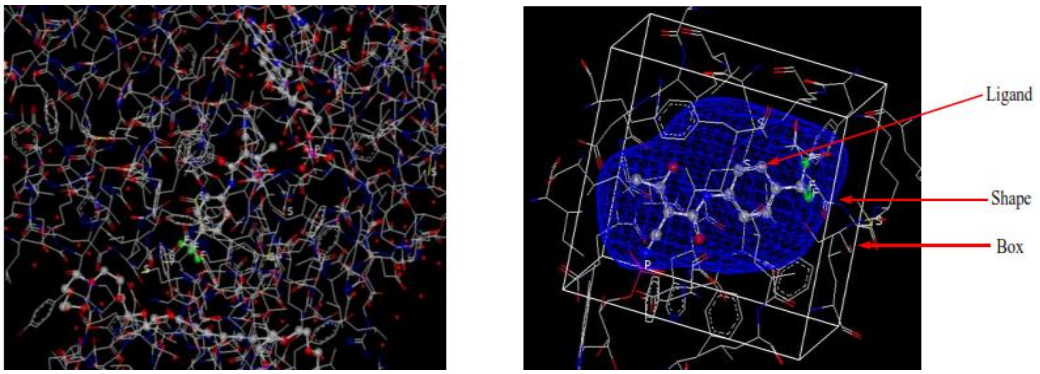


Figure 5. (A) 1TV5 PDB file (B) prepared 1TV5 Receptor showing the box enclosing the active site, the defined shape of the active site, and the bound ligand.

Table 1. Docking and z-scores of ligands

Smiles of Compounds	Class of ligand	Docking score (Kcal/mol)	Z-score
COc1c (=O)c2c (cc (cc2oc1c3ccc (c (c3)O)O)O)[O-]	chromone	-14.8608	2
COc1cc (c2c (c1)OC (CC2=O)c3ccccc3)[O-]	chromanone	-14.5211	2
COc1cc (c2c (c1)OC (CC2=O)c3ccccc3)[O-]	chromone	-14.5211	2
c1cc (ccc1c2cc (=O)c3c (cc (cc3o2)O)[O-])O	chromone	-14.5133	2
COc1cc (c2c (=O)cc (oc2c1)c3ccc (cc3)O)[O-]	chromone	-14.3959	2
COc1c (=O)c2c (cc (cc2oc1c3ccc (cc3)O)O)[O-]	chromone	-14.1036	1
c1ccc (cc1)c2cc (=O)c3c (cc (cc3o2)O)[O-]	chromanone	-13.9018	1
COc1cc (c2c (c1)OC (CC2=O)c3ccc (cc3)O)[O-]	chromone	-13.8635	1
COc1cc (c2c (c1)OC (CC2=O)c3ccc (cc3)O)[O-]	chromone	-13.8635	1
c1cc (c (cc1c2c (c (=O)c3c (cc (cc3o2)O)[O-])[O-])O)O	chromone	-13.8531	1
c1cc (c (cc1c2c (c (=O)c3c (cc (cc3o2)O)[O-])[O-])O)O	chromone	-13.8531	1
c1cc (c (cc1c2cc (=O)c3c (cc (cc3o2)O)[O-])O)O	chromone	-13.8232	1
c1cc (ccc1c2c (c (=O)c3c (cc (cc3o2)O)[O-])[O-])O	chromone	-13.7642	1
c1c (cc (c (c1O)O)O)c2c (c (=O)c3c (cc (cc3o2)O)[O-])[O-]	chromone	-13.7514	1
COc1ccc (c (c1)O)C (=O)C=Cc2ccccc2	chalcone	-13.6169	1
c1ccc (cc1)C2CC (=O)c3c (cc (cc3O2)O)[O-]	chromanone	-13.5869	1
COc1cc (cc (c1C (=O)C=Cc2ccccc2)O)O	chalcone	-13.55	1
COc1cc (c2c (c1)oc (c (c2=O)OC)c3ccc (cc3)O)[O-]	chromone	-13.5402	1
COc1cc (c (c2c1c (=O)cc (o2)c3ccccc3)OC)O	chromone	-13.4887	1
COc1cc2c (c (c1OC)[O-])C (=O)CC (O2)c3ccc (cc3)O	chromanone	-13.4786	1
COc1cc (c (c (c1)O)C (=O)C=Cc2ccccc2)O	chalcone	-13.4599	1
COc1ccc (cc1)c2c (c (=O)c3c (cc (cc3o2)O)[O-])OC	chromone	-13.4357	1
COc1c (cc2c (c1[O-])c (=O)c (c (o2)c3ccc (cc3)O)[O-])O	chromone	-13.3051	1
c1ccc (cc1)c2cc (=O)c3ccc4c (c3o2)cco4	chromone	-13.2924	1
COc1ccc (cc1)c2cc (=O)c3c (cc (cc3o2)O)[O-]	chromone	-13.2169	1

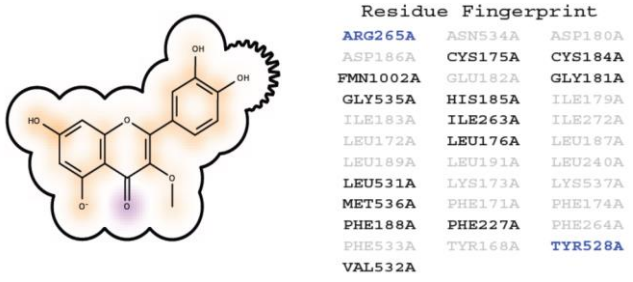


Figure 7. (A) ligand (B) amino acid residues that interacted (blue are donors)

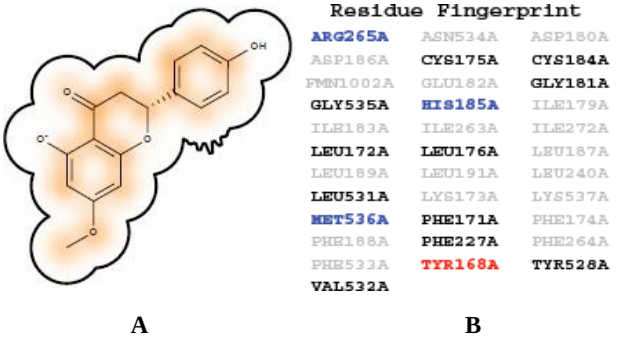


Figure 8. (A) ligand (B) amino acid residues that interacted (blue are donors, red are acceptors)

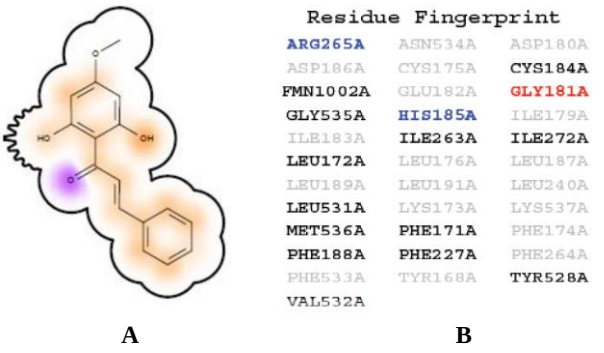


Figure 9. (A) ligand (B) amino acid residues that interacted (blue are donors, red are acceptors, and black is by contact)

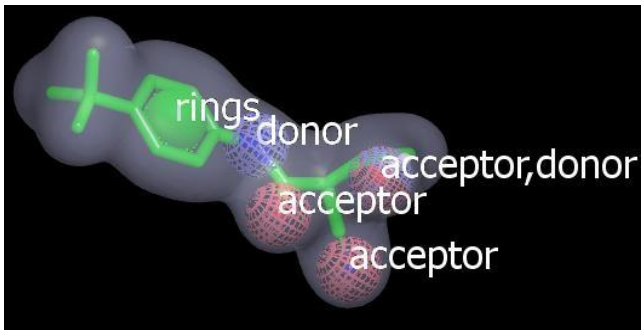


Figure 10. 3D structure of the query molecule (38) showing shape contour and color and shape atoms

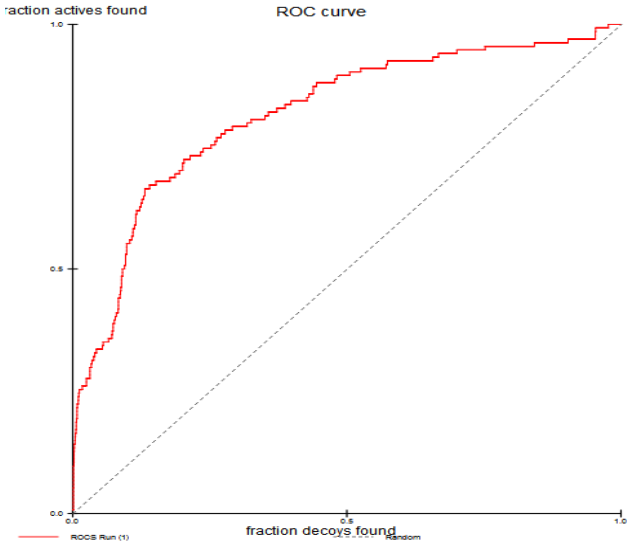


Figure 11. ROC curve for the query

Table 2. Query validation run statistical metrics

Statistical metrics	Values
AUC	0.813
0.5% enrichment	31.399
1.0% enrichment	22.320
2.0% enrichment	12.589

The area below the curve (AUC) is the probability that a randomly chosen active has a higher score than a randomly selected inactive. A highly selective query has 0.8<AUC<1.0 (Jain and Nicholls 2008). Since the AUC for query **38** is 0.813, the query is highly particular. Table 2 indicates that the early enrichment values decrease with an increase in the percentage of the decoys. Initial enrichment values refer to the fractions of the actives at the particular portion of the decoys (Senger et al., 2003). Therefore, the AUC and early enrichment values indicate that query **38** is selective and hence suitable and valid for use in both shape and electrostatics similarity studies.

The shape and electrostatics similarity studies. A shape similarity search was done on the 3D conformers of the 103 ligands to compare the shape of these ligands to the shape of the validated query. The ligands were ranked based on Tanimoto combo scores, and the results are summarized in Table 3. Similarly, an electrostatic similarity comparison between the validated query and the 3D conformers of the 103 ligands was done and ranked using the electrostatic Tanimoto score (ET score), Table 3.

The *ROCS* Tanimoto combo scores and the *EON* electrostatic tanimoto scores were combined into the total tanimoto score. The total tanimoto score was then used to calculate z scores which were used to identify the top 29 ligands, Table 3. Like structure-based virtual screening studies (Table 1), ligand-based virtual screening identified chromones as the most virtually active, followed by chromanones and chalcones.

Selection of compounds for synthesis

Generally, both structure and ligand-based virtual screening studies identified chromones (flavones) as the best inhibitors of *Pf*DHODH, followed by chromanones (flavanones) and chalcones. Although the specific ranking order varies, the general trend remains the same. The ranking of ligands based on structure and ligand-based virtual screening guided the identification of synthetically accessible chromones and chromanones targeted for synthesis.

Synthesis of chromanones and chromones

The approach for synthesizing chromanone and chromone-based lead compounds was based on retrosynthetic Figure 12. The starting material for the synthesis was resorcinol (**39**), which was acylated and then subjected to Aldol condensation and cyclization to access the target chromanones (**42**) and chromones (**43**).

Synthesis of 2,4-dihydroxyacetophenone (40). 2,4-Dihydroxyacetophenone (**40**) was synthesized through Friedel Craft acylation of resorcinol (**39**) using acetic acid in the presence of $ZnCl_2$ as the Lewis acid (Figure 13) (Patil et al. 2012).

The product was obtained as a yellow crystalline solid with a 50% yield. The structure of the product was determined using ^{1}H and ^{13}C NMR. The ^{1}H NMR signals of an acetoxymethyl at δ 2.57 (*s*, 3H), a chelated hydroxyl (δ 12.77 (*s*, 1H)), a hydroxyl (δ 9.48 (*s*, 1H)) and three mutually coupled aromatic protons at δ 7.79 (*d*, *J* = 8.40 Hz, 1H), 6.45 (*dd*, *J* = 2.40, 8.80Hz, 1H), 6.34 (*d*, *J* = 2.40 Hz, 1H) are consistent with literature (Patil et al. 2012). The ^{13}C NMR signals at δ 202.8 (C=O), 164.9 (C-4), 164.5 (C-2) 133.5 (C-1), 113.4 (C-6), 107.7 (C-5), 102.4 (C-3), 25.5 (CH_3) are also consistent with literature (Patil et al. 2012).

Synthesis of 2',4'-dihydroxy-4-methoxychalcone (45). 2',4'-Dihydroxy-4-methoxychalcone (**45**) was synthesized through base-catalyzed Aldol condensation of 2,4-dihydroxyacetophenone (**40**) and 4-methoxybenzaldeyde (**44**). The product was obtained as yellow crystals in 30% yield. The formation of 2',4'-dihydroxy-4-methoxychalcone (**45**) was found to be slow (48 hrs), which could be attributed to the presence of two hydroxyl groups on the acetophenone (**40**). Under necessary conditions, the phenolic protons are picked, thus increasing the electron density in the system and reducing the electrophilicity of the carbonyl group, thereby lowering the acidity of the methyl hydrogens (Figure 14).

The ^{1}H NMR signals for compound **45** were similar to those of compound **40** except for incorporating a methoxy δ 3.89 (*s*, 3H, OCH_3). In the ^{13}C NMR, the signal for the carbonyl is more shielded (δ 191.9) compared to the starting material (δ 202.8), consistent with an α β-unsaturated carbonyl system (118.2 (α-C) and 143.9 (β-C)) as would be expected from an Aldol condensation product.

Table 3. Ligand-based virtual screening results

Smiles of compounds	Tanimoto combo	ET combo	Total	Z-score
c1cc (c (cc1c2c (c (=O)c3ccc (cc3o2)O)[O-])O)O	0.955	0.754	1.709	2
c1cc (c (cc1c2c (c (=O)c3c (cc (cc3o2)O)[O-])[O-])O)O	0.941	0.754	1.695	2
c1cc (c (cc1c2cc (=O)c3c (cc (cc3o2)O)[O-])O)O	0.942	0.741	1.683	2
COc1ccc (cc1O)[C@H]2[C@@H] (C (=O)c3c (cc (cc3O2)OC)[O-])O	0.946	0.706	1.652	2
COc1ccc (cc1)c2cc (=O)c3c (cc (cc3o2)O)[O-]	0.888	0.755	1.643	2
COc1ccc (cc1O)c2c (c (=O)c3c (o2)cc (c (c3[O-])O)O)[O-]	0.922	0.706	1.628	2
COc1cc (c2c (=O)cc (oc2c1)c3ccc (c (c3)OC)O)[O-]	0.903	0.711	1.614	1
COc1ccc (cc1)c2cc (=O)c3c (cc (cc3o2)OC)[O-]	0.875	0.732	1.607	1
COc1c (=O)c2c (cc (cc2oc1c3ccc (c (c3)O)O)O)[O-]	0.897	0.707	1.604	1
COc1cc (c2c (c1)oc (c (c2=O)[O-])c3ccc (c (c3)OC)O)[O-]	0.889	0.71	1.599	1
COc1ccc (cc1O)c2c (c (=O)c3c (cc (cc3o2)OC)[O-])OC	0.92	0.674	1.594	1
c1c (cc (c (c1O)O)O)c2c (c (=O)c3c (cc (cc3o2)O)[O-])[O-]	0.893	0.698	1.591	1
c1cc (ccc1c2cc (=O)c3c (cc (cc3o2)O)[O-])O	0.854	0.73	1.584	1
CC1 (C=Cc2cc (cc (c2O1)O)[C@@H]3CC (=O)c4c (cc (cc4O3)O)[O-])C	0.836	0.732	1.568	1
COc1ccc (cc1OC)c2c (c (=O)c3c (cc (cc3o2)OC)[O-])OC	0.873	0.681	1.554	1
COc1cc (c2c (=O)cc (oc2c1)c3ccc (cc3)O)[O-]	0.839	0.707	1.546	1
COc1ccc (cc1)[C@H]2CC (=O)c3c (ccc (c3O2)OC)[O-]	0.844	0.698	1.542	1
COc1cc (ccc1O)c2c (c (=O)c3c (cc (c (c3o2)OC)O)[O-])[O-]	0.842	0.685	1.527	1
c1ccc (cc1)c2cc (=O)c3c (cc (cc3o2)O)[O-]	0.83	0.686	1.516	1
COc1ccc (cc1)c2c (c (=O)c3c (cc (cc3o2)O)[O-])OC	0.819	0.696	1.515	1
COc1cc2c (c (c1OC)[O-])C (=O)C[C@@H] (O2)c3ccc (cc3)O	0.79	0.723	1.513	1
COc1cc (c2c (c1)O[C@H] (CC2=O)c3ccc (cc3)O)[	0.839	0.666	1.505	1
c1cc (c (cc1c2c (c (=O)c3ccc (cc3o2)O)[O-])O)O	0.955	0.754	1.709	2
c1cc (c (cc1c2c (c (=O)c3c (cc (cc3o2)O)[O-])[O-])O)O	0.941	0.754	1.695	2
c1cc (c (cc1c2cc (=O)c3c (cc (cc3o2)O)[O-])O)O	0.942	0.741	1.683	2
COc1ccc (cc1O)[C@H]2[C@@H] (C (=O)c3c (cc (cc3O2)OC)[O-])O	0.946	0.706	1.652	2
COc1ccc (cc1)c2cc (=O)c3c (cc (cc3o2)O)[O-]	0.888	0.755	1.643	2
COc1ccc (cc1O)c2c (c (=O)c3c (o2)cc (c (c3[O-])O)O)[O-]	0.922	0.706	1.628	2
COc1cc (c2c (=O)cc (oc2c1)c3ccc (c (c3)OC)O)[O-]	0.903	0.711	1.614	1
COc1ccc (cc1)c2cc (=O)c3c (cc (cc3o2)OC)[O-]	0.875	0.732	1.607	1
COc1c (=O)c2c (cc (cc2oc1c3ccc (c (c3)O)O)O)[O-]	0.897	0.707	1.604	1
COc1cc (c2c (c1)oc (c (c2=O)[O-])c3ccc (c (c3)OC)O)[O-]	0.889	0.71	1.599	1
COc1ccc (cc1O)c2c (c (=O)c3c (cc (cc3o2)OC)[O-])OC O-]	0.92	0.674	1.594	1
COc1cc2c (c (c1OC)[O-])c (=O)c (c (o2)c3ccc (c (c3)O)O)OC	0.849	0.656	1.505	1
CC (=CCc1cc (ccc1O)[C@@H]2CC (=O)c3ccc (cc3O2)O)C	0.849	0.653	1.502	1
COc1ccc (cc1)c2c (c (=O)c3c (cc (cc3o2)OC)[O-])OC	0.816	0.684	1.5	1
c1cc (ccc1c2c (c (=O)c3c (cc (cc3o2)O)[O-])[O-])O	0.812	0.68	1.492	1
COc1cc (c (c (c1O)OC)OC)c2cc (=O)c3c (cc (cc3o2)O)[O-]	0.819	0.663	1.482	1
CC (=CCc1cc (ccc1O)/C=C/C (=O)c2ccc (cc2O)O)C	0.857	0.619	1.476	1
COc1ccc (cc1)c2c (c (=O)c3c (cc (cc3o2)OC)[O-])[O-]	0.81	0.658	1.468	1

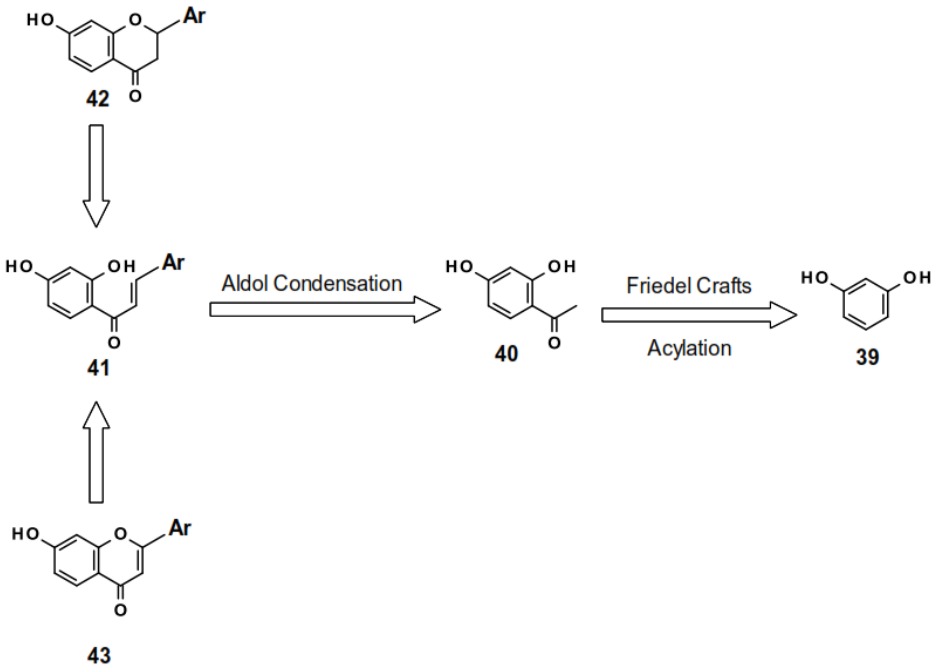


Figure 12. Retrosynthetic pathway for chromanones and chromones

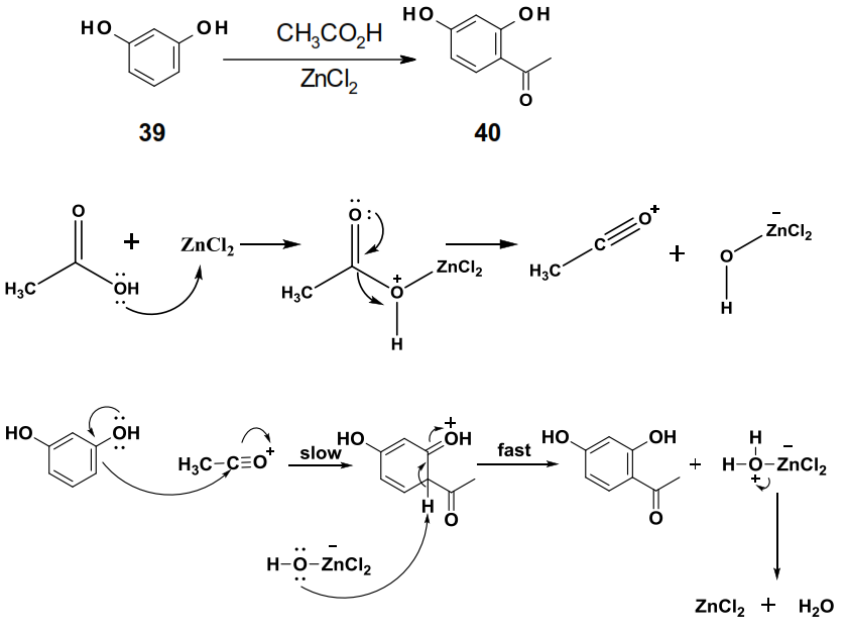
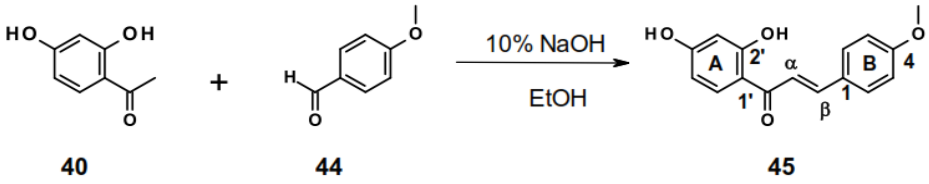


Figure 13. Mechanism of acylation



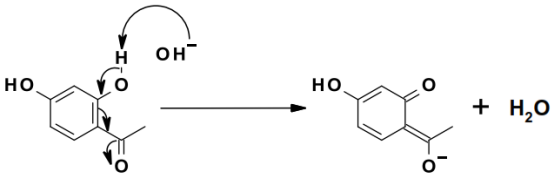


Figure 14. Mechanism of de-protonation of phenolic protons

Synthesis of 2',4'-dihydroxy-4-chlorochalcone (47). 2',4'-Dihydroxy-4-chlorochalcone (**47**) was done through base-catalyzed Aldol condensation of 2,4-dihydroxyacetophenone (**40**) and 4-chlorobenzaldeyde (**46**). Compound **47** was obtained as yellow crystals in 16% yield. Unlike the previous Aldol condensation, which took place at room temperature, this reaction required refluxing at 70 °C for 48 hrs.

The ^{1}H NMR signals for compound **47** were similar to those of compound **40** except for the incorporation of two *trans* olefinic protons at δ 8.00 (*d*, *J* = 15.5 Hz, 1H, H_{β}) and 7.86 (*d*, *J* = 15.5 Hz, 1H, H_{α}) and an AA'BB' aromatic spin system resonating at δ 7.91 (*d*, *J* = 8.4 Hz, 2H, C-2 and C-6) and 7.52 (*d*, *J* = 8.5 Hz, 2H, C-3 and C-5). In the ^{13}C NMR, the signal for the carbonyl is more shielded (δ 191.7) compared to the starting material (δ 202 .8), consistent with an α-unsaturated carbonyl system (121.6 (α-C) and 142.3 (β-C)) as would be expected from an Aldol condensation product.

The low yield of chalcones 2', 4'-dihydroxy-4-methoxychalcone (**45**) and 2', 4'-dihydroxy-4-chlorochalcone (**47**) could be due to side reaction (Cannizaro reaction of the aldehydes) which led to the formation of a mixture of more than two products, which necessitated purification through column chromatography. Cannizaro reaction involves the conversion of non-enolizable aldehydes into alcohol and carboxylic acid and takes place in the presence of a strong base. The side products formed, including p-chlorobenzoic acid, were produced in large quantities. Therefore, it prompted the use of an excess of the benzaldehyde to ensure that the 2,4-dihydroxyacetophenone was completely consumed in the reaction.

Synthesis of 7-hydroxy-2-(4-methoxyphenyl) chroman-4-one (48). The synthesis of 7-hydroxy-2-(4-methoxyphenyl) chroman-4-one (7-hydroxy-4'-methoxyflavanone) (**48**) was achieved through the acid-catalyzed cyclization of 2',4'-dihydroxy-4-methoxychalcone (**45**). Compound **48** was isolated as a yellow crystalline solid with a 29% yield. The low yield of compound **48** is due to the decomposition of the chalcone hence forming mixtures that needed to be purified by column chromatography. Compound **48** was characterized as a flavanone based on its characteristic ^{1}H NMR δ at (5.46 (*dd*, 1H, *J*=2.4, 13.2 Hz, H-2), 3.09 (*dd*, *J* = 13.2, 16.8 Hz, 1H, H-3_{eq}), 2.81 (*dd*, *J*=16.8, 2.4 Hz, 1H, H-3_{ax})) and ^{13}C NMR (δ 190.5 (C=O), 79.7 (C-2), 44.0 (C-3)) peaks.

An attempt was made to synthesize chromones (flavones) through oxidative cyclization under two different reaction conditions (Ghodile et al. 2012, Venkatachalam et al. 2012), Figure 15, was unsuccessful. Under the circumstances employed in the attempted cyclization, the chalcones decomposed.

***In vitro* antiplasmodial activity**

In vitro antiplasmodial bioassay of 2',4'-dihydroxy-4-methoxychalcone (**45**), 2',4'-dihydroxy-4-chlorochalcone (**47**), and 7-hydroxy-2-(4-methoxyphenyl)chroman-4-one (**48**) against chloroquine-resistant K1 and chloroquine-sensitive 3D7 strains of *Plasmodium falciparum* were conducted using a non-radioactive assay technique, and the results are as shown in Table 4.

Among the compounds tested, 2',4'-dihydroxy-4-methoxychalcone (**45**) showed the highest antiplasmodial activity against both strains. Moderate antiplasmodial activity was observed for compounds **47** and **48** against the chloroquine-resistant K1 strain of *P. falciparum.* 2',4'-Dihydroxy-4-methoxychalcone (**45**), and 7-hydroxy-2-(4-methoxyphenyl) chroman-4-one (**48**) showed strong antiplasmodial activity against the chloroquine-sensitive 3D7 strain of *P. falciparum.* It is worth noting that, from the docking reports, 2',4'-dihydroxy-4-methoxychalcone (**45**) ranks the best among the three synthesized and assayed compounds which is consistent with the assay results, Table 4 and Figures 16-18.

In conclusion, the first web-based *in silico* database of natural products from Kenya was successfully generated and named the *Mitishamba* database. The 157 chromone, chromanone, and chalcone ligands obtained from the *Mitishamba* database were subjected to structure and ligand-based virtual screening, leading to the identification of three synthetically accessible ligands for synthesis and antiplasmodial assay. The three ligands synthesized and assayed were a chromanone (flavanone), 7-hydroxy-2-(4-methoxyphenyl) chroman-4-one (**48**), and two chalcones, 2',4'-dihydroxy-4-methoxychalcone (**45**) and 2',4'-dihydroxy-4-chlorochalcone (**47**). Among the compounds synthesized, 2',4'-dihydroxy-4-methoxychalcone (**45**) showed the highest antiplasmodial activity against chloroquine-sensitive 3D7 and chloroquine-resistant K1 strains of *P. falciparum* with IC_{50} values of 5.14 ± 0.70 and 4.56±1.66 µg/ml, respectively.

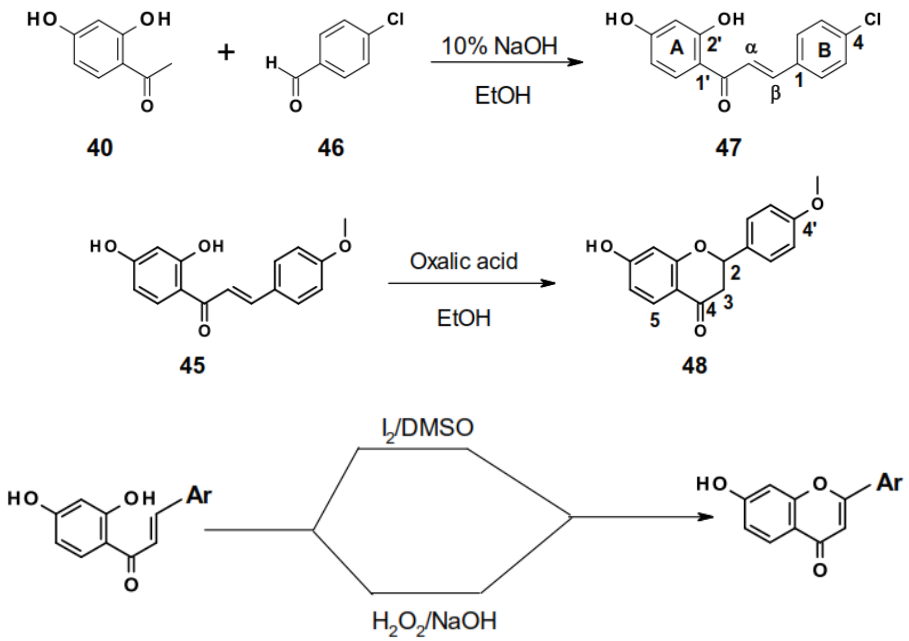


Figure 15. Oxidative cyclization of chalcones to chromones

Table 4. *In vitro* IC_{50} values against K1 and 3D7 strains of *P. falciparum*

Sample	IC_{50} (µg/ml)		Docking score (Kcal/mol)
	K1	3D7	
2',4'-Dihydroxy-4-methoxychalcone (**45**)	4.56 ± 1.66	5.14 ± 0.70	-14.60
2',4'-Dihydroxy-4-chlorochalcone **(47)**	20.36 ± 2.77		-14.14
7-Hydroxy-2-(4-methoxyphenyl) chroman-4-one (**48**)	18.01 ± 2.28	4.57 ± 2.17	-13.34
Chloroquine*	0.471 ± 0.037	0.006 ± 0.0022	
Mefloquine*	0.004 ± 0.0025	0.0008± 0.0004	

Note: *standard

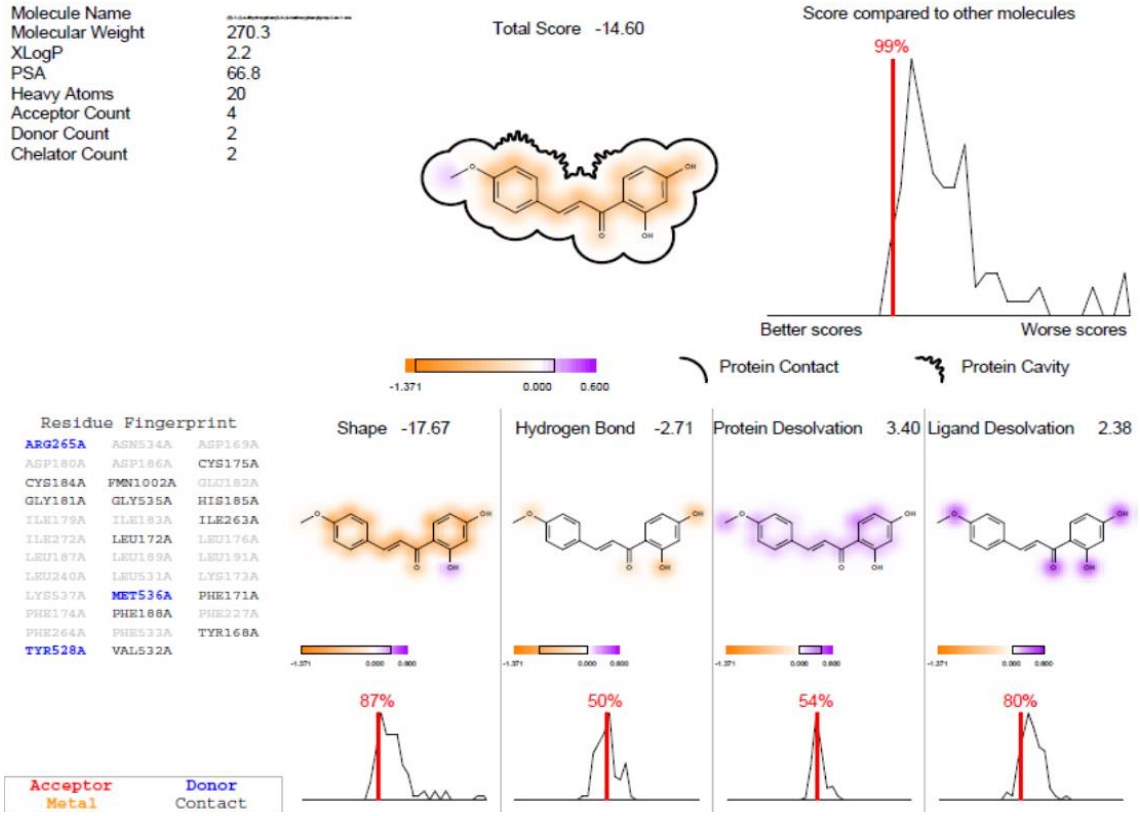


Figure 16. Docking report for 2', 4'-dihydroxy-4-methoxychalcone **(45)**

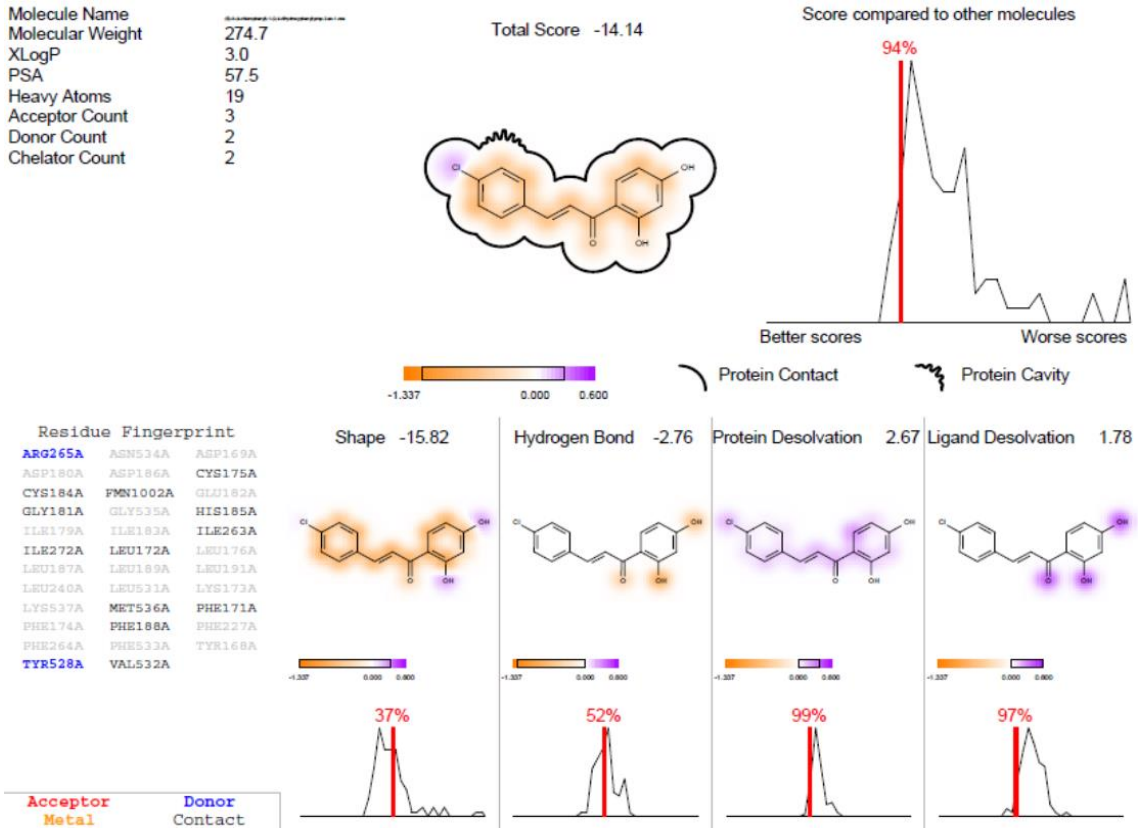


Figure 17. Docking report for 2', 4'-dihydroxy-4-chlorochalcone (**47**)

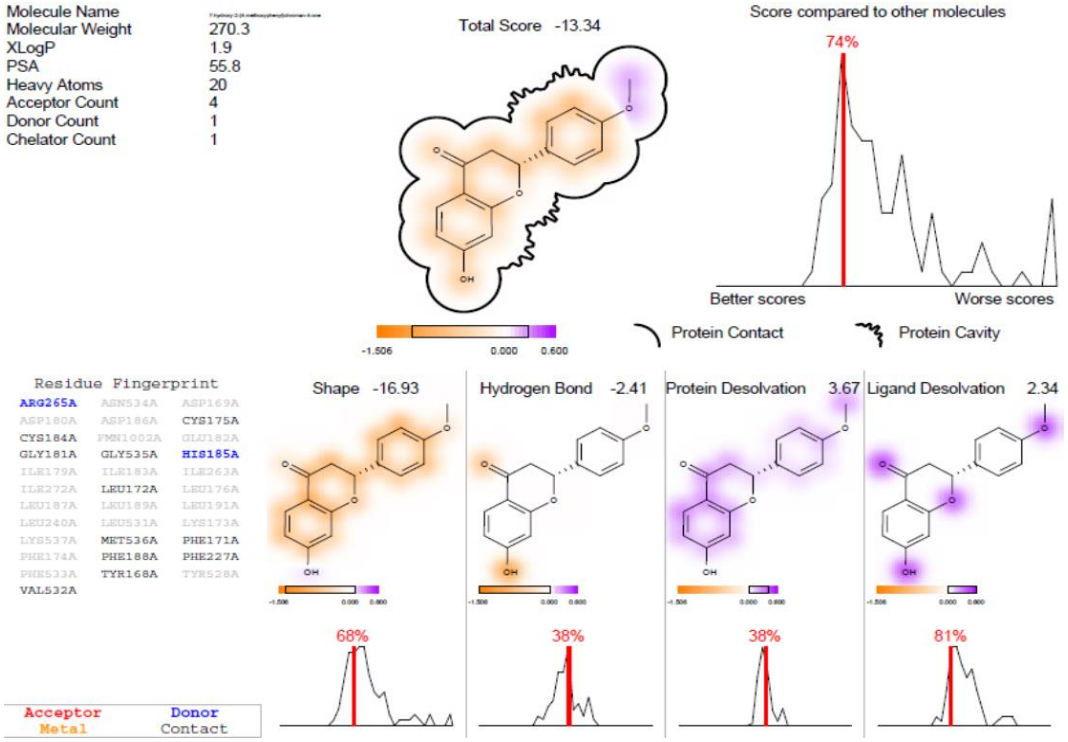


Figure 18. Docking report for 7-hydroxy-2-(4-methoxyphenyl) chroman-4-one (**48**)

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Quantification of phenolics, flavonoids and antioxidant activity of *Tamarindus indica* from selected areas in Tanzania

MOURICE MBUNDE, ROBINSON H. MDEGELA♥, H.S. LASWAI, F.P. MABIKI
Department Veterinary Medicine and Public Health, Faculty of Veterinary Medicine, Sokoine University of Agriculture. P. O. Box 3021, Morogoro, Tanzania. Tel.: +255 23 260 45 42; Fax.: +255 23 260 46 47, ♥email: mdegela@suanet.ac.tz, rmdegela@yahoo.com

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Abstract. *Mbunde M, Mdegela RH, Laswai HS, Mabiki FP. 2018. Quantification of phenolics, flavonoids and antioxidant activity of* Tamarindus indica *from selected areas in Tanzania. Biofarmasi J Nat Prod Biochem 16: 22-28.* The objective of this study was to establish the quantities and antioxidant activity in fruits and leaves of Tamarindus indica L. collected from three agroecological zones of Tanzania represented by the Morogoro, Tanga, and Dodoma regions. Samples were examined for their total phenolic and flavonoid contents as well as their antioxidant activity. The total phenolic content showed a significant difference in all fruit and leaves extracts and ranged from 1994.4±530.77 to 17874.67±5234 mg GAE/100 g. Similarly, the total flavonoid content in tamarind leaf and fruit extracts ranged from 880±609.45 to 11483.11±2559.67 mg CE /100 g dry weight. There was a significant difference between the antioxidant activity in the leaf (54.39±0.13%) and fruit extracts (40.11±0.03%). Tamarind leaf extracts exhibited significantly higher radical scavenging activity than fruit extracts. The antioxidant activity in fruit extracts expressed in percentage ranged between 29.27±0.06% and 40.11±0.03%, while in leaf extracts, the activity ranged from 22.33±0.08% to 54.39±0.13%. The radical scavenging activity from Coastal leaf extracts had the highest activity, followed by Eastern leaf extracts and lastly, Central leaf extracts. The highest activity was shown in the fruit samples by Coastal fruit extracts, followed by Central fruit extracts, and Eastern fruit extracts were the least active. The values in the Ferric reducing power (FRAP) assay ranged between 6968±3655.91 µM Fe (II)/g and 76822.67±23259.9 µM Fe (II)/g for leaves and fruits dry mass, respectively. These values correspond to the antioxidant activity, positively correlated with the total phenolic and flavonoid contents. Geographical location and climatic conditions have been reported to have enormous effects on the amount and activity of antioxidants available in both tamarind leaves and fruits. Findings from the study indicated that tamarind could be utilized as a cheap source of antioxidants. However, more agronomic studies should be considered to confirm the effects of agroecological differences on antioxidant activity.

Keywords: Antioxidant activity, flavonoids, phenolics, *Tamarindus indica*

INTRODUCTION

Oxidation reactions that occur mainly in the human body are likely to produce free radicals, bringing about various disorders, including atherosclerosis, ischemia, arthritis, reperfusion injury of many tissues, gastritis, and cancer (Seal 2011). To protect the cells, organs, and systems of the body against the deleterious effects of free radicals, humans have a highly sophisticated and complex antioxidant protection system that functions interactively and synergistically to neutralize free radicals (Percival, 1996). Antioxidants prevent the oxidative damage caused by free radicals in the body, as they can react with free radicals, chelate catalytic metals, and act as oxygen scavengers. The antioxidant compounds in the body are primarily obtained from external sources, mainly through the consumption of fruits and vegetables. The need for a supply of antioxidants becomes even more critical with increased exposure to free radicals originating from external sources, such as exposure to x-rays, cigarette smoking, ozone, air pollutants, and industrial chemicals (Dimitrios 2006; Kumar 2011).

Since ancient times, humans have depended on natural sources, especially plants, to protect against the effects of various diseases and improve their lifestyles. With technological advancement and recent research findings, it has been revealed that certain non-nutritive chemicals produced by plants, such as terpenoids, flavonoids, and other phenolic compounds, which were initially thought to be of no importance to human health, possess antioxidant properties (Seal 2011).

Antioxidant compounds have been searched in several types of plant materials, such as vegetables, fruits, leaves, barks, and roots, in the form of crude plant drugs. Polyphenolic compounds, which are dominant in antioxidant activity, are then found to be common in leaves, fruits, stems, and barks. In plants, these compounds are essential for normal growth development and defense against infection and injury (Seal 2012; Aires et al. 2013). Epidemiological reports proposed that dietary intake of natural products has proved to have a strong inverse correlation with the risk of developing cancers and coronary heart disease (Lako et al. 2007; Zidenberg-Cherr and Heneman 2008). Antioxidants in natural sources, especially fruits and vegetables, have created a high demand for natural products to control and treat various infections and diseases. Some chemically synthesized drugs claim to have undesirable side effects (Mayunzu et al., 2011).

Tamarindus indica L., commonly known as tamarind, has a long history in traditional medicine throughout Africa and Asia (El-Siddig et al., 2006; Lourith et al., 2009). In Tanzania, this species is increasingly being used by society for juice making or as a vegetable. Modern medical science has also confirmed its laxative and diuretic properties. All morphological parts of *T. indica* can be used, from the fruit pulp and seed to the leaves, bark, and flowers. Ailments such as diarrhea, ulcers, jaundice, eye infections, and digestive problems can be treated with infusions, pastes, and powders from *T. indica* (Khairunnuur et al., 2009; De Caluwé et al., 2010). Herbal practices are still widely used wherever *T. indica* is accessible (Rudrappa 2009).

Numerous studies have reported tamarind as having high levels of vitamins; A, B, and C and organic acids like citric, ascorbic acids, tartaric and malic, and polyphenols flavonoids. These compounds are primarily responsible for potent antioxidant, hepatoprotective, and antimicrobial activity (Lamien-Meda et al., 2008; Lourith et al., 2009; Rodríguez-Amado et al., 2012).

Many wild fruits and leaves contain significant amounts of antioxidant compounds, essential in preventing various diseases (Javanmardi et al., 2003). The properties of antioxidants are mainly brought about by polyphenolic compounds such as flavonoids, anthocyanins, phenolic acids, and phenolic diterpenes. *Tamarindus indica* L. is reported to contain many polyphenolic compounds with the potential for antioxidant activity (Pieta 1998). Nevertheless, the quantities of antioxidants may vary with geographical location (Aires et al., 2011; Mahmood et al., 2012). Despite the extensive utilization and availability of *T. indica* in most parts of Tanzania, little is known about the amount and activity of antioxidants from this plant.

Furthermore, there is limited information on comparative analysis of antioxidant compounds available in the wild tamarind from different agroecological zones of Tanzania. Thereby, this study was designed to fill the existing knowledge gap. Findings from the study would be useful in providing baseline information about the antioxidant and antioxidant capacity of *T. indica*.

The objectives of this research were: (i) To quantify the number of phenolics and flavonoid contents in leaves and fruit extracts of *T. indica* from the Coastal, Eastern, and Central zones of Tanzania. (ii) To determine the antioxidant activities of extracts from leaves and fruits of *T. indica* from the zones above Tanzania.

MATERIALS AND METHODS

Description of areas where samples were collected

This study involved sample collection from three different locations that fall in agroecological zones. Coastal zone (Tanga), Eastern Plateau and Mountain Blocks (Morogoro), and Central Plateau (Dodoma). The Coastal zone (Tanga) lies 500-1200 meters above sea level and has been developed over gneissic rocks. The region has poorly drained, flat, broad topographical depressions developed on young alluvium and strongly dissected areas of pronounced slopes, often rocky and severely eroded. Two main types of soils are available: sandy clay loams and sandy clays, and sands and loamy sands. The region is mostly infertile and lacks moisture acceptance properties due to a tendency for surface sealing. It experiences bimodal rainfall ranging from 700-1200 mm per annum (USDA 2005; Handeni 2008).

The Eastern Plateau and Mountain block, which encompasses the Morogoro region (Mvomero district), exhibits undulating plains to dissected hills and mountains and moderately fertile clay soil. The area experiences unimodal rainfall ranging from 800 to 1400 mm (USDA 2005; Mbogoni and Ley 2008).

The Central Plateau (Dodoma region) has undulating plains with rocky hills and low scarps. Its soil is drained with low fertility. The rainfall is unimodal and unreliable, ranging from 500 to 800 mm (USDA 2005).

Study design

This observational study design was adopted whereby samples were collected and taken to the laboratory to extract and analyze antioxidants. Samples were collected from three villages, namely Misima (Tanga), Doma (Morogoro), and Ntyuka (Dodoma), purposively selected from the three zones. The collection of the samples was done purposively based on the availability of tamarind species with mature fruits in certain areas. In each region, five samples (leaves and fruits from five tamarind trees) were taken from one village. The basis for the selection of each area was the climatic condition, i.e., semi-arid, woodland, and coastal climatic conditions.

Materials

Equipment and apparatus

Whatman no. 1 filter paper, UV-visible spectrophotometer (UNICO VIS1200 Version SS-1.24, United Products and Instruments, Inc.), bench centrifuge, Buchner funnel, separating funnel (250 mL), beakers (250 mL), volumetric flask (5, 10, 25, 50 mL), measuring cylinder (5, 10, 25, 50 mL), conical flasks (25 mL), cuvettes, Eppendorf tips, micropipettes.

Chemicals and reagents

Methanol (CH_3OH), ethanol (C_2H_5OH)), hydrochloric acid (HCl), Folin Ciocalteu Reagent (FCR), TPTZ (2, 4,6-tripyridyl-s-triazine), iron sulphate heptahydrate ($FeSO_4.7H_2O$), iron chloride ($FeCl_3$), sodium carbonate (Na_2CO_3), sodium hydroxide (NaOH), sodium acetate (CH_3COONa), sodium acetate buffer ($C_2H_3NaO_2.3H_2O$), sodium nitrite ($NaNO_2$), aluminium trichloride ($AlCl_3$), standard Gallic acid, butylated hydroxytoluene (BHT), 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), vitamin C (ascorbic acid) and catechin. All the chemicals used, including the solvents, were analytical grade and purchased from the University Suppliers.

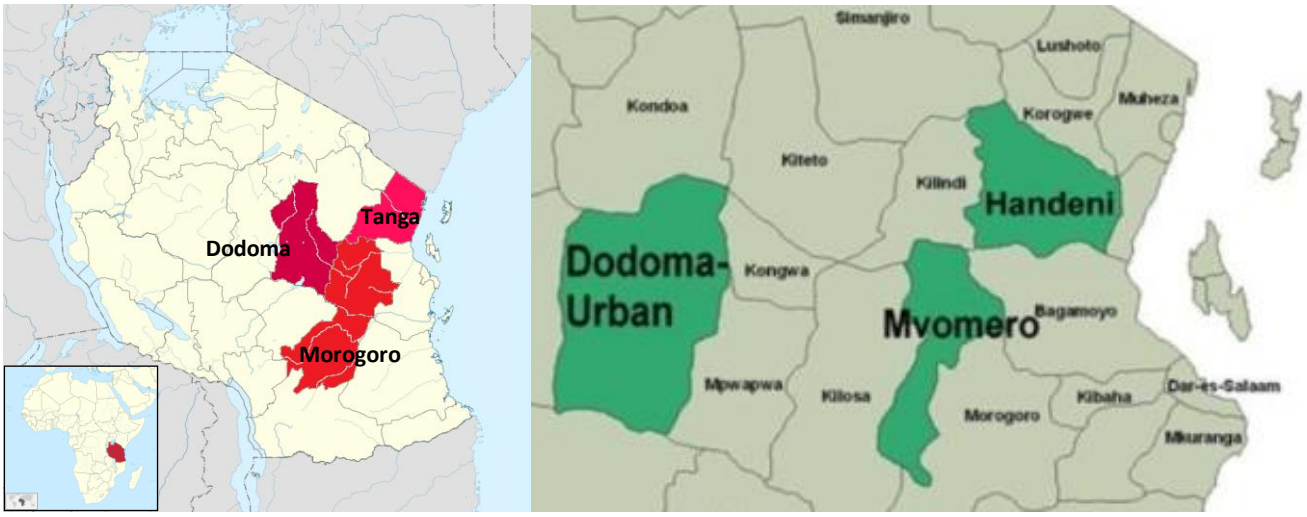

Figure 1. Map of the study area where samples were collected

Methods

Collection of plant materials

Fresh tamarind leaves and ripened fruits were collected from several populations of tamarind species in the selected agroecological zones (Plate 1). The Global Positioning System (GPS) was used to mark the coordinates and photographs of the plant taken at each location.

Extract preparations

The separated leaves were air-dried under the shade at room temperature (30ºC) and ground to a powder using a grinding machine. Ten grams of leaf powder (made from 2 g of individual sample) was extracted in 99.9% methanol for 48 hrs at 30-33ºC. The fruits were peeled, and the pulps were separated from the seed (Ashafa et al., 2010). Ten percent pulp extract was prepared by soaking 10 g (made from 2 g of individual sample) of the fresh pulp in 100 mL of 99.9% methanol, then mixed thoroughly (Khairunnuur et al. 2009). The mixture was homogenized and allowed to stand for 48 hrs. Both fruit and leaf extracts were filtered through Whatman filter paper No.1. The filtrate was evaporated under reduced pressure at 40ºC using a rotary evaporator. The crude extract obtained was stored at -20ºC until further analysis (Ashafa et al. 2010).

Coding of samples

The samples collected from three different zones were coded, as shown in Table 1.

Table 1. Coding of samples collected from three zones

Code	Agro-ecological zone	Source of tamarind extract
LVMR	Eastern zone	Leaves
FRMR	Eastern zone	Fruits
LVDM	Central zone	Leaves
FRDM	Central zone	Fruits
LVTA	Coastal zone	Leaves
FRTA	Coastal zone	Fruits

Note: LVMR = Morogoro leaf extract; FRMR = Morogoro fruit extract; LVDM = Dodoma leaf extract; FRDM = Dodoma leaf extract; LVTA = Tanga leaf extract; FRTA = Tanga fruit extract

Determination of total phenolics content

The concentration of total phenolics was measured according to the previously described method (Velioglu et al. 1998) with some modification whereby the diluted aqueous solution of each extract (0.5 mL) was mixed with Folin Ciocalteu reagent (0.2 N, 2.5 mL). The mixture was incubated at room temperature for 5 minutes and then added with sodium carbonate solution (75 g/L in water, two mL). After incubation for 2 hours, the absorbance was read at 760 nm. A standard calibration curve was plotted using Gallic acid (0, 25, 50, 75, 100, 125, 150, 175, 200, 225, 250 mg/L). The measurements were expressed as mg of Gallic Acid Equivalents (GAE)/100 g of fruit weight.

Determination of total flavonoid contents

Total flavonoid content was measured using a colorimetric assay (Zhishen et al. 1999). One mL aliquot of catechin standard solutions (5, 10, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200 mg/L) was placed in a test tube, then added with 4 mL of ddH_2O and 0.3 mL (5%) $NaNO_2$. After 5 min, 1.5 mL (2%) $AlCl_3$ was added to the test tube and shaken to homogenize. Five minutes later, 2mL of 1 M NaOH was added to the mixture and shaken well. The absorbance of the mixture, pink in color, was read by spectrophotometer at 510 nm versus the prepared standard. Total flavonoid content in the fruit extract was expressed as mg/100 g catechin equivalents (CE) (fresh weight basis). All samples were analyzed in triplicate.

Determination of 1, 1-diphenyl-1-picrylhydrazyl scavenging activity

Antioxidant activity of tamarind leaves and fruit extracts was determined using the stable DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging as described *el*sewhere with some modification (Mensor et al. 2001). A four mL of 0.1 mM DPPH solution was added into 2 mL of the solutions of Butylated hydroxyl toluene (BHT) in methanol at different concentrations (25, 50, 75, 100, 125, 150 mg/L). The mixtures were shaken vigorously and incubated at room temperature for 30 min. Next, 4 mL

of DPPH was added to 1 mL of sample diluted with 2 mL of methanol, and the mixture was shaken vigorously and allowed to stand for 30 min at room temperature. The absorbance was read at 517 nm wavelength using a UV-VIS spectrophotometer (UNICO VIS1200 Version SS-1.24) with Butylated hydroxyl toluene (BHT)was used as the reference. Lower absorbance values of the reaction mixture indicated a higher free radical scavenging activity. The solution was measured spectrophotometrically at 518 nm. The antioxidant activity (AA) was calculated as below: AA% = 100-[((Absorbance of the sample-Absorbance of the blank)/ (Absorbance of the control)] x100 (Mensor et al. 2001).

Determination of ferric reducing antioxidant power (FRAP)

Ferric reducing antioxidant power (FRAP assay) was modified from the earlier study by Benzie and Strain (1996). The stock solutions included 300 mM acetate buffer (3.1 g CH_3COONa and 16 mL CH_3OOH), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s- triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The fresh working solution was used in a ratio of (10:1:1) by mixing acetate buffer, TPTZ, and $FeCl_3 \cdot 6H_2O$. The temperature of the solution was increased to 37°C before use. A volume of 100 µL extracts/standard was placed in a test tube and diluted with 300 µL of distilled H_2O, then 2.85 mL of the FRAP solution was added and incubated for 30 min. Readings of the colored product (ferrous tripyridyltriazine complex) were taken at 593 nm. The standard curve lies between 100 and 700 µL $FeSO_4.7H_2O$. Results were expressed in µM Fe (II)/g dried mass and compared with that of catechin.

Data analysis

Results were obtained from three replicate experiments. For each variable, treatment means were subjected to Analysis of Variance (ANOVA) and Duncan's Multiple Range Test (DMRT) of the CoHort CoStat software version 6.33. Significant differences were reported at $p< 0.05$.

RESULTS AND DISCUSSION

The antioxidant capacity of the different extracts derived from fruit and leaf and the ranking order for each assay are presented in Tables 2, 3, and 4 and Figures 2 and 3. The variation observed in the number of polyphenolic contents in fruits harvested from three different agroecological zones might be attributed to the series of complex biochemical reactions during fruit ripening. The complex reactions affected the formation of phenolics, anthocyanins, flavonoids, carotenoids, and other volatile compounds leading to the development of final characteristics and distinct flavors of mature fruit (Gull et al. 2012).

Total phenolics content

The underlying mechanism of the method used in the phenolic determination (Folin-Ciocalteau assay) is an oxidation/reduction reaction according to the redox properties of antioxidant compounds that can react with the Folin-Ciocalteau Reagent (FCR), enhancing the measurement of phenolic concentration (Norshazila et al. 2010). Table 2 shows the phenolic content of tamarind extract and the ranking order for each extract.

This study demonstrated that phenolic compounds level in tamarind extracts from the three different agro-ecological zones varied significantly in both leaves and fruit extracts. In general, among the six sample extracts, the tamarind leaf and fruit extracts from the Coastal zone demonstrated the highest levels of phenolic content compared to the other zones. There was a high variation in phenolic content between tamarind morphological parts (i.e., fruits and leaves) in all cases. For instance, a tamarind leaf extract from the Coastal zone had a significantly higher concentration of phenolics (Table 2) than fruits from the same zone. The higher level of phenolics in tamarind fruits and leaves has not been reported as in the case of the present study except in seed extracts, as reported by Lourith et al. (2009). Depending on the solvent used, they found the contents to be approximately 713.24 mg GAE/100 g to 63,691 mg GAE/100 g. Compared to the present study, other groups also reported a low phenolics content in tamarind pulp (957.33 ± 13.20 g of GAE/100 g of fruit) (Lamien-Meda et al. 2008). Meanwhile, a study on the tamarind fruit done by Khairunnuur et al. (2009) reported lower levels of phenolic contents compared to the findings of this study, ranging from 19.21±0.29 g GAE/100 g in seed and 2.14±0.05 g GAE/100 g in fruit.

This study further showed a significant variation in the total phenolic content in the tamarind leaf extract from the Coastal and Central zones (Table 2). No significant difference was observed in the total phenolic content of the tamarind leaf extract from the Eastern (Morogoro) and Coastal (Tanga) zone, likewise for fruit extracts from samples collected in the Central (Dodoma) and Eastern zones. These findings suggest that tamarind leaves and fruits growing in Tanzania contain more phenolic contents than those reported elsewhere, suggesting that Tanzania *T. indica* could have potent antioxidant activity.

The presence of higher concentrations of phenolic compounds in leaves could be explained by the change in the biochemical composition in the later stages of fruit ripening, i.e., different phenolic acids condense to form complex phenolic compounds such as tannins and lignin (Gull et al., 2012). Tamarind trees sampled in this study mostly had only younger leaves, thus supporting the argument by Rodríguez-Amado et al. (2012). They reported that younger leaves of tamarind bear higher phenolic compounds since the plant needs to protect itself from predators' attacks. Plant extract containing high levels of phenolic compounds may scavenge free radicals such as superoxide anion radicals and peroxy radicals in the human body and protect human cells or tissues against oxidative stress (Norshazila et al. 2010).

Total flavonoid content

The distribution of total flavonoid content (TFC) in tamarind leaves and fruit extracts concerning geographical

regions is presented in Table 3. The level of flavonoids in tamarind leaf extracts except those samples collected from the Central zone were significantly higher than those of fruit extracts from all zones. In all extracts, fruits harvested from the Coastal zone had the lowest flavonoid content (Tables 3). Tamarind fruits extract from samples collected from the Central and Coastal zone did not show any significant differences in concentration of flavonoids among them. Likewise, the leaf extracts from samples collected from the Coastal and Eastern zones showed no significant difference in their flavonoid contents. However, a substantial difference in flavonoid content was found between the leaf and fruit extracts from the Coastal zone region, as demonstrated in Table 3.

The flavonoid content in the present study was higher than the values reported in other reports. Lamien-Meda et al. (2008) reported lower levels of flavonoids (2.18 ± 0.21 mg QE/100 g) in fruit methanolic extracts and (5.68 ± 0.10 mg QE/100 g) in fruit acetone extract tamarind growing in Burkina Faso. The presence of higher concentrations of flavonoid compounds in younger leaves compared to those in fully ripe fruits could be attributed to the fact that the later stages of fruit ripening exhibit different phenolic acids that may condense to form complex phenolic compounds such as tannins and lignin. Hence, due to changes in phenolic compounds with maturity, fully-ripe fruit possessed relatively lower amounts of total flavonoid contents (Gull et al. 2012). Concentrations of flavonoid compounds in younger leaves compared to the fruits may be attributed to the fact that flavonoids are responsible for color formation; therefore, most samples were found to have yellow and red colors (Plate 6), suggesting a higher level of flavonoids. The growing conditions could also influence differences in flavonoid contents of different samples of tamarind from different locations, the genetic make-up of the species, amount of precipitation, altitude, temperature, soil conditions, and availability of nutrients (Jaffery et al. 2003; Mahmood et al. 2012; Rodríguez-Amado et al. 2012). The above factors might have affected the concentration of flavonoids in fruit and leaf extracts tested by altering the composition of their phytochemicals.

On the other hand, it was detected that tamarind has a higher concentration of phenolics than flavonoids in both leaf and fruit extracts tested. Tamarind leaf extracts had significantly higher polyphenolic compounds (phenolics) and flavonoids than fruit extracts.

Radical scavenging activity

The DPPH test determines the antioxidant activity, which is based on the ability of the stable free radical 2,2-diphenyl-1-picrylhydrazyl to react with hydrogen donors, including phenols (Lamien-Meda et al. 2008). The bleaching of 2,2-diphenyl-1-picrylhydrazyl by a test compound represents its capacity to scavenge free radicals generated independently from any enzymatic or transition metal-based system. Antioxidant compounds available in sample extracts react with DPPH, a stable free radical, to convert it to 1,1-diphenyl-2- (2,4,6-trinitrophenyl) hydrazine (Ali et al. 2010).

The bleaching of DPPH solution regularly increases with increasing sample fruit and leaf extracts in a given volume, as shown in Figure 3. The bleaching action of antioxidant compounds like the solution (Lamien-Meda et al. 2008). The antioxidant activity of the samples tested showed variation over the tested samples (Figure 3). The results showed that scavenging activity decreased in the following order: LVTA<LVMR<LVDM<FRTA <FRDM <FRMR. This trend implied that tamarind growing in the Coastal zone had the highest reduction potential than in other zones. This observation reflected the concentration of phenolics and flavonoids observed in the sample extracts from the Coastal Zone.

There was a significant difference in the antioxidant activity between the extracts from the leaf (54.39±0.13%) and that from the fruit (40.11±0.03%). Radical scavenging activity observed in leaf and fruit extracts was correlated with the concentration of phenolics and flavonoids found in all the extracts, with a strong positive correlation between total phenolic content and radical scavenging activity (R^2 = 0.923). This correlation suggested that polyphenols are responsible for antioxidant activity. Lamien-Meda et al. (2008) underscored that variation in radical scavenging ability among the tamarind extracts over the regions can be brought by the difference in climate and solvent used.

Table 2. Total phenolics content (mg GAE/100 g) of tamarind leaf and fruit extracts collected from different agro-ecological zones in Tanzania (n = 6)

[2]Code	Agro-ecological zone	[1]Total phenolics content (mg GAE/100 g)
LVMR	Eastern zone	17874.67±5234[a]
LVTA	Coastal zone	17799.25±4825.05[a]
LVDM	Central zone	6144.6±2205.23[b]
FRTA	Coastal zone	4755±1699.25[bc]
FRMR	Eastern zone	2073.33±287.39[c]
FRDM	Central zone	1994.4±530.77[c]

Note: [1]Values are mean ± SD of 6 samples analyzed individually in triplicates. Values with superscripts significantly different at ($p<0.05$) in leaf and fruits extracts. [2]LVMR = Morogoro leaf extract; FRMR = Morogoro fruit extract; LVDM = Dodoma leaf extract; FRDM = Dodoma leaf extract; LVTA = Tanga leaf extract; FRTA = Tanga fruit extract

Table 3. Total flavonoid contents of tamarind leaves and fruit extracts from different agro-ecological zones in Tanzania (n = 6)

[2]Code	Agro-ecological zones	[1]Total flavonoid content (mg Ce 100/G Dry Wt)
LVTA	Coastal zone	11483.11±2559.67[a]
LVMR	Eastern zone	9853.33±6588.47[a]
LVDM	Central zone	3957.33±390.82[b]
FRMR	Eastern zone	2146.67±107.7[bc]
FRDM	Central zone	1088±294.23[c]
FRTA	Coastal zone	880±609.45[c]

Note: [1]Values are mean ± SD of 6 samples analyzed individually in triplicates. Values with superscripts significantly different at ($p<0.05$) in leaf and fruits extracts. [2]LVMR = Morogoro leaf extract; FRMR = Morogoro fruit extract; LVDM = Dodoma leaf extract; FRDM = Dodoma leaf extract; LVTA = Tanga leaf extract; FRTA = Tanga fruit extract

Ferric reducing activity

The ferric reducing antioxidant potential (FRAP) assay was applied to examine the free radical scavenging capacities and the lowering possibilities of the antioxidant constituents of the tamarind extracts. This assay is usually based on the reducing power of a compound (antioxidant) and measures the reduction of Fe^{3+} (ferric iron) to Fe^{2+} (ferrous iron). As the ferric ion is reduced to a ferrous ion, the values in the FRAP assay express the corresponding concentration of electron-donating antioxidants (Ali et al., 2010).

Table 4 displays the FRAP values of tamarind leaf and fruit extracts. The antioxidant activity was found to vary among the extracts from different agroecological zones of Tanzania. The trend for the decrease in FRAP values or reduction potential among the extracts was LVTA< LVMR< LVDM<FRTA<FRMR<FRDM. When comparing the FRAP values among the extracts, it was found that tamarind leaf extracts collected from the Coastal zone had the highest FRAP values ($p<0.05$), followed by leaf extracts from the Eastern zone (Table 4).

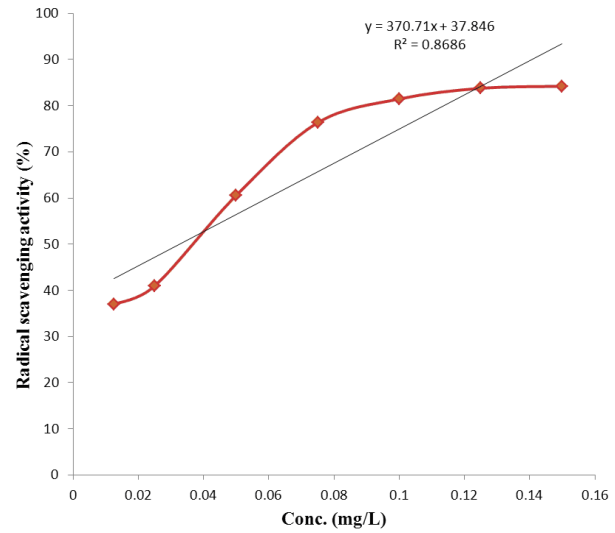

Figure 2. DPPH-free radical scavenging activity (RS %) of butylated hydroxytoluene (BHT) used as standard

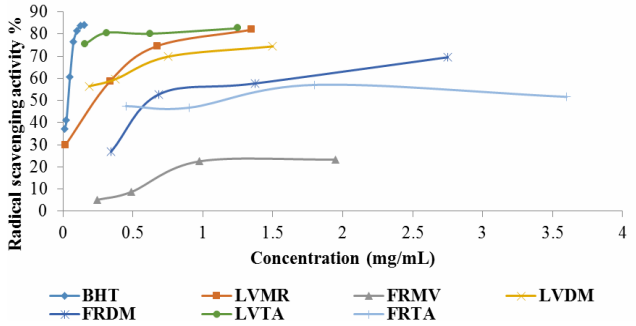

Figure 3. Effect of concentration of tamarind extracts in free radical scavenging activity. BHT = Positive Control; LVMR = Morogoro leaf extract; FRMR = Morogoro fruit extract; LVDM = Dodoma leaf extract; FRDM = Dodoma leaf extract; LVTA = Tanga leaf extract; FRTA = Tanga fruit extract

Table 4. Ferric Reducing Antioxidant Power (FRAP) values of tamarind leaf and fruit extracts from different Agro-ecological zones in Tanzania (n = 6)

[2]Code	Agro-ecological zones	[1]µM Fe (II)/g dry mass
LVTA	Coastal zone	76822.67±23259.9[a]
LVMR	Eastern zone	32776±24506.66[b]
LVDM	Central zone	8199.33±2929.49[c]
FRTA	Coastal zone	6968±3655.91[c]
FRMR	Eastern zone	5328±2945.96[c]
FRDM	Central zone	3860±2377.57[c]

[1]Values are mean ± SD of six samples analyzed individually in triplicates. Values with the superscripts significantly different at ($p<0.05$) in leaf and fruits extracts. [2]LVMR = Morogoro leaf extract; FRMR = Morogoro fruit extract; LVDM = Dodoma leaf extract; FRDM = Dodoma leaf extract; LVTA = Tanga leaf extract; FRTA = Tanga fruit extract

Leaf and fruit extracts obtained from the Central zone did not show any difference between them but showed a significant difference ($p<0.05$) with the leaves from the Coastal and Eastern zone. There was a considerable difference in FRAP values (76822.67±23259.9 µM Fe (II)/g dry mass) in leaf extracts from the Coastal zone compared with FRAP values of fruit extracts from the same location. Total phenolic and flavonoid content correlates with ferric reducing antioxidant activity. These results agreed with Khairunnuur et al. (2009), who found a significant difference between FRAP values of tamarind fruit and seed extracts. The impact of geographical factors on the antioxidant property has been shown by variations in FRAP values observed among tamarind extracts obtained from the three agroecological zones.

This study showed that the amount and activity of antioxidants were higher for samples from the Coastal zone (moderate temperature) than for samples from the Central zone (extreme temperature). This indicated the effect of the agroecological zone on the amount and activity of antioxidants available in both tamarind fruits and leaves. The Coastal zone (Tanga) and Eastern zone (Morogoro) were observed to possess favorable factors that promote the production of phytochemicals in tamarind as compared to the Central zone (Dodoma). These two zones (Tanga and Morogoro region) share the same geographical factors: temperature, soil type, and rainfall (USDA 2005). According to Gull et al. (2012), moderate temperature conditions (25/30°C) are suitable for increasing antioxidant content. The authors further argued that plants growing in extreme cold (18/12°C) or hot (above 35°C) temperatures produce fruits and leaves with lower antioxidant content (Gull et al. 2012).

In conclusion, from the results, the amount of phenolics and flavonoid content of leaf extracts is higher than that of fruit extracts. Furthermore, there is variation in phenolics and flavonoid content amongst the three agroecological zones in which sample extracts obtained from the Coastal zone contained the highest amount of these polyphenols. Leaf extract from the Eastern and Coastal zone exhibited significantly higher antioxidant activity levels than extracts from the Central zone. Also, the leaf extracts showed higher radical scavenging activity than fruit extracts in the

following orders LVTA<LVMR<LVDM <FRTA<FRDM< FRMR, from high to low amounts, respectively. Antioxidant activity was positively correlated with the total phenolics and flavonoid contents. The polyphenols content and the mean DPPH and FRAP in all sample extracts differed significantly. This behavior is frequent in natural products due to variation that relates to climate, soil characteristics, and phenological stages of the plant, at the fructification stage. Phenolics and flavonoid compounds in leaves of the plants are higher in earlier growth stages, probably as a strategy of the plant to protect itself from the insects and predators' attacks.

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Effect of crude extract of *Acalypha hispida* and *A. indica* leaves on the growth of *Staphylococcus aureus* bacteria in vitro

RIMA PUTRI TUNAS KARTIKA, TJAHJADI PURWOKO, SUNARTO♥
Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Ir. Sutami 36A Surakarta 57126, Central Java, Indonesia. Tel./fax.: +62-271-663375, ♥email: rm.sunarto@yahoo.com

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Abstract. *Kartika RPT, Purwoko T, Sunarto. 2018. Effect of crude extract of* Acalypha hispida *and* A. indica *leaves on the growth of* Staphylococcus aureus *bacteria in vitro. Biofarmasi J Nat Prod Biochem 16: 29-35. Staphylococcus aureus* is one of the bacteria causing infectious diseases. One alternative to dealing with the infectious diseases caused by *S. aureus* is using natural plant materials, often called traditional medicine. *Ekor kucing* (*Acalypha hispida* Brum.f.) and *anting-anting* (*Acalypha indica* L.) plants are typical herbs producing useful chemical substances in medicine, such as saponin, tannin, flavonoid, acalyphin, and volatile oil functioning as an antibacterial agent, among others. The aims of this research are to (i) to examine the inhibitory potential of crude extracts of *A. hispida*, and *A. indica* leaves against the growth of *S. aureus* bacteria, and (ii) to find out the comparison of potential bacterial inhibitory activity between crude extracts of *A. hispida* and *A. indica* leaves. The bacterial inhibition potential testing was conducted using Poisoned Food Techniques (PFT) method. Crude extracting with 70% ethanol solvent was done on *A. hispida* and *A. indica* leaf and the extract was dissolved serially using aquadest solvent to obtain the various extract concentrations: 0,5%, 0.4%, 0.3%, 0.2%, 0.1% and 0% (control). Then, the colony width was measured using the gravimetry method. The Completely Random Design was used to analyze the data result of this experiment. The crude extract of *A. hispida* and *A. indica* leaves at all concentrations can inhibit the *S. aureus* bacteria growth. The crude extract of *A. hispida* is more able to pursue the bacterium of *S. aureus* than the crude extract of *A. indica*.

Keywords: *Acalypha hispida, Acalypha indica*, antibacterial, *Staphylococcus aureus*

INTRODUCTION

Infectious diseases are a high cause of morbidity and mortality worldwide, especially in developing countries such as Indonesia (Guntur 2007). Infectious diseases can be transmitted from one organism to another by various microorganisms, one of which is bacteria (Gibson 1996). It was reported from Guntur's research (2007) that the factors causing infection were mostly Gram-positive and negative bacteria, including the genus *Staphylococcus*.

Staphylococcus aureus is one of the bacteria that cause various infections from the genus *Staphylococcus*. These pathogenic bacteria cause skin, lower respiratory tract, digestive tract infections, bones, joints, mucous membranes, and eczema, causing acne, boils, and pneumonia. The *S. aureus* is a normal microflora on the skin, but when there is an increase in the number, this bacterium can cause infection (Taukhid et al. 2002).

One alternative to treat infectious diseases caused by *S. aureus* is to use natural plant materials or traditional medicines (Lestari et al. 2006). The soaring prices of synthetic drugs and their side effects on health have increased the community's use of traditional medicines by utilizing the natural resources around them. The advantages of using natural ingredients include being more environmentally friendly, easy to obtain, inexpensive, and having relatively smaller side effects when used correctly and appropriately, both in the right dose, time of use, method of use, the accuracy of material selection, and accuracy of selection of traditional medicines for indications certain (Nugroho et al. 1999).

Ekor kucing (*Acalypha hispida* Brum.f.) and *anting-anting* (*Acalypha indica* L.) plants are a type of herb that produces chemical compounds that are useful in medicine, including saponins, tannins, flavonoids, acalyphin, and essential oils, one of which functions as an antibacterial (Hutapea 1993; Villes and Reese 1995; Akintola and Ande 2006). In traditional medicine, the efficacy of this *A. hispida* plant is as a hemostatic drug, coughing up blood, treatment of white patches on the skin, burns, intestinal inflammation, intestinal worms, vomiting blood, efficacious as a wound cover, and laxative urine (Dalimarta 1991). The *A. hispida* is an ornamental plant usually found in house yards. At the same time, *A. indica* is a weed commonly found growing wild on roadsides, grass fields, or mountain slopes. In the Malay peninsula, the leaves of *A. indica* are used for laxatives and eye pain medications (Hutapea 1993). The benefit of this plant as traditional medicine is an added value to improve the function of *Acalypha* so that it is not just a weed or ornamental plant. Cahyanti's research (2004) shows that the root and shoot extract of *A. indica* can inhibit growth and reduce the chlorophyll content of purslane (*Portulaca oleracea* L.).

Besides being easy to obtain and useful for natural herbicides, it turns out that secondary metabolites of plants *A. hispida* and *A. indica* can be used as ingredients for traditional medicines, so it is suspected that they have potential as alternative materials for controlling bacterial

diseases. So far, there have been many studies to determine the antibacterial power of various species of the genus *Acalypha* against various kinds of bacteria. In addition, although *A. hispida* and *A. indica* plants are widely used as traditional medicinal plants for various diseases, it is not widely known how much antibacterial effectiveness these two plants inhibit or kill bacteria that cause disease.

Therefore, it is necessary to compare the antibacterial power of crude extracts of *A. indica,* and *A. hispida* leaves against *S. aureus* bacteria to find out which is more effective in inhibiting or killing *S. aureus* bacteria and to find alternative materials for controlling bacterial diseases, especially those caused by *S. aureus* bacteria.

The aims of this research are to (i) to examine the inhibitory potential of crude extracts of *A. hispida,* and *A. indica* leaves against the growth of *S. aureus* bacteria, and (ii) to find out the comparison of potential bacterial inhibitory activity between crude extracts of *A. hispida* and *A. indica* leaves.

MATERIALS AND METHODS

Materials

The ingredients were leaves of *A. hispida,* and *A. indica* obtained from B2P2TO2T (*Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional*) Tawangmangu, Karanganyar, Central Java, Indonesia, while pure culture of *S. aureus* bacteria obtained from the Faculty of Pharmacy, Universitas Setia Budi, Surakarta, Central Java, Indonesia.

Research procedure

Sterilization of tools and materials

Petri dishes, test tubes, Erlenmeyer, NA media, and all tools and materials (except *A. hispida* and *A. indica* leaf powder extract) to be used were sterilized in an autoclave for 30 minutes by setting the pressure at 1 atm (15 dyne/cm) and a temperature of 121°C after previously being washed, dried and wrapped in paper.

Production of bacterial suspension stock

The bacterial suspension was made to increase the stock by inoculating 1 ose of pure culture into 5 mL of NA media, then incubating at 37°C for 24 hours in an incubator.

Leaf powder making

The plant parts used for extracting were the leaves of *A. hispida* and *A. indica*. from each plant, healthy and fresh leaves with optimum growth with uniform age and size were selected, then washed with running water to remove dust and other impurities. After that, the leaves are drained, then dried in indirect sunlight by covering them with a black cloth. The purpose of drying is to obtain an extract that is not easily damaged so that it can be stored longer. The dried leaves are made into powder with an electric blender, then stored in a closed container. The dried leaf powder will be used to make the extract.

Extract preparation

Two hundred and fifty grams of dried *A. hispida* and *A. indica* leaf powders were soaked in 500 mL of 70% ethanol and then shaken for 24 hours at 120 rpm. The extract was filtered using a Buchner funnel, and the filtrate was taken. Furthermore, the filtrate and solvent that are still mixed are dried with a rotary evaporator at a maximum temperature of 50°C to obtain a dry extract. The extract obtained was serially diluted with distilled water to obtain extract concentrations of 0.5%, 0.4%, 0.3%, 0.2%, 0.1% and 0% (control) (Modification of Sunarto et al. 1999; Ogbebor and Adekunle 2005).

Bacterial inoculum preparation

Pure cultures of *S. aureus* were rejuvenated in NA media. The liquid NA media (at 50°C), mixed with one bacterial loop in a test tube, was vortexed for the growth of bacteria to be evenly distributed. Then it was poured into a petri dish and incubated for 48 hours at 37°C. For the antibacterial test, the bacteria grown were molded with a cork drill with a diameter of 0.5 cm and a thickness of 1-2 mm, then inoculated right in the middle of the agar media.

Inhibition potential test

First, a comparative test of negative control (sterile aquades) and positive control (1% ethanol) was conducted to determine any inhibitory activity of ethanol solvent in NA media. Then *S. aureus* bacteria inoculum was inoculated on each control and incubated for 7 x 24 hours. The results of bacterial cultures were photographed to compare with the growth of bacteria tested with the extract. Next, 6 g of NA media plus 2 g of agar was dissolved in 300 mL of sterile distilled water and boiled at 100°C. After boiling, the media was put into test tubes for as much as 10 ml each and then autoclaved for 15 minutes at 121°C, with a pressure of 1 atm.

Furthermore, the media was cooled to a temperature of 50°C, mixed with the extract, and poured into a petri dish aseptically. Bacterial mold was inoculated right in the middle of the test medium aseptically. After the bacterial culture was about 7 days old, the diameter of the colony was calculated. The process of calculating the colony diameter for each concentration started after bacterial growth; the results were then compared with the control. The treatment was repeated 3 times for each concentration (Modification of Sunarto et al. 1999).

Determination of bacterial colony growth area

The colony area was measured by the gravimetric method by making a replica of the growth using paper. However, before making a replica, the paper used to make the replica is weighed, and its area is measured so that the paper's initial weight and the original paper's area are known. After that, a replica of the growth of bacterial colonies was made, and the weight of the replica was weighed. The formula can calculate the width of bacterial colonies:

$$\text{Bacterial colony width} = \frac{\text{weight of replica x initial width of paper}}{\text{initial weight}}$$

The choice of this calculation method is based on the results of the study that the area of bacterial colony growth is not completely circular, so it has different radii from each side.

Percentage determination

The percentage of growth inhibition of *S. aureus* bacteria is calculated by the following formula (Ogbebor and Adekunle 2005):

% inhibition = [(control-treatment) / control] x 100 %

Data collection technique

The qualitative research data shows the size of the bacterial culture colony diameter (expressed in mm^2) at the five levels of extract concentration (expressed in %). The experimental design used a factorial Completely Randomized Design (CRD), with the following conditions:

E: Extract 70% ethanol *A. hispida*, and extract 70% ethanol *A. indica*

K: Concentration, 6 levels, i.e.: 0%(control), 0.1%, 0.2%, 0.3%, 0.4%, 0.5%

So, there were 14 combinations, each treatment combination with 3 replications.

Data analysis

The data in this study was quantitative data showing the size of the growth area of *S. aureus* bacterial colonies (expressed in mm^2). First, the data was analyzed by General Linear Model (GLM) to determine whether there were differences in each treatment. Then, the data was tested with Tamhane at a 5% level test to compare the results.

RESULTS AND DISCUSSION

The *A. hispida* plants are a type of herb that produces chemical compounds useful in medicine, including saponins, tannins, flavonoids, acalyphin and essential oils which function as an antibacterial (Dalimartha 1991; Hutapea 1993; Akintola and Ande 2006). Besides being easy to obtain, secondary metabolites from *Acalypha* can be used as a potential source of traditional medicinal ingredients, so it is suspected that *Acalypha* also has potential as an alternative material for controlling bacterial diseases. Therefore, the benefit of this plant as traditional medicine is an added value to improve the function of *Acalypha* so that it is not just a weed or ornamental plant.

So far, although *A. hispida* and *A. indica* plants are widely used as traditional medicinal plants for bacterial diseases, the amount of antibacterial activity of these plants is not widely known, so it is necessary to do a comparative test of the antibacterial power of leaf crude extract of *A. indica* and *A. hispida* against changes in *S. aureus* bacteria to find out which is more effective in inhibiting or killing *S. aureus* bacteria.

Inhibitory potential test

The potential inhibitory test of leaves crude extracts of *A. indica* and *A. hispida* on the growth of *S. aureus* was carried out using the Poisoned Food Techniques (PFT) method, which aims to examine the inhibitory potential and compare the effect of leaves crude extracts of *A. indica* and *A. hispida* on the growth of *S. aureus*. In this test, crude leaf extracts of *A. indica* and *A. hispida* were obtained from the extraction using 70% ethanol and then dissolved and diluted with distilled water to obtain concentrations of 0.5%, 0.4%, 0.3%, 0, 2%, and 0.1%. The choice of solvent for ethanol and aquadest is because they both have similar polarities. Ethanol is a polar solvent, and this solution obtains the chemical content of *A. indica* and *A. hispida* leaves which are also polar, such as tannins, saponins, volatile oils, acalyphins, and flavonoids. So that these compounds can be mutually soluble with the principle of "like dissolved like," which means that a polar compound will dissolve in a polar solvent and vice versa (Noerono 1994).

Using distilled water as a solvent was based on preliminary research, which showed that aquadest did not affect bacterial growth, so only the leaf extracts of *A. indica* and *A. hispida* were affected.

Table 1 shows the test results of leaves crude extract of *A. indica* at various concentration levels in the form of the growth area of *S. aureus* bacteria. From Table 1, it is known that crude extract of *A. indica* leaves can inhibit the growth of *S. aureus* with an average bacterial growth colony area ranging from 42,150-54,810 mm^2. While in the control plate, the average bacterial growth area was around 557,340 mm^2. While the crude extract of *A. hispida* leaves is presented in Table 2. In the administration of *A. hispida* leaf extract, the average area was only around 1000-4170 mm^2 (Table 2).

In this study, there was a significant broad difference in the administration of the two extracts compared to the control. It is supported by the statistical analysis of the Tamhane follow-up test at a 5% level, where there is a significant difference between the control and the test concentration. However, for each test concentration for a crude extract of *A. indica* leaf, there was no significant difference, as well as for each test concentration for the crude extract of *A. hispida* leaf. Perhaps this is due to the concentration range not being too far away.

All test concentrations of the two extracts showed the potential for inhibition of bacterial growth. The similarity of the effects given by the two extracts was closely related to the chemical content of the extracted leaves in each leaf extract. This similarity is thought to be due to the similarity of the compounds with antibacterial properties, such as tannins, saponins, flavonoids, essential oils, and acalyphins (Dalimartha 1991; Hutapea 1993; Villes and Reese 1995; Akintola and Ande 2006).

From the two extracts tested, it was generally seen that the crude extract of *A. hispida* leaves was more able to inhibit the growth of *S. aureus* bacteria. This difference is thought to be due to the different concentrations of the active ingredients in the two extracts.

The histogram above shows that the area of bacterial colony growth with *A. indica* leaf extract and *A. hispida* leaf crude extract was significantly different; namely, the bacterial growth area with *A. indica* leaf extract was higher than *A. hispida* leaf extract. It indicates that the leaves of *A. hispida* were more effective in inhibiting the growth of *S. aureus* bacteria. This difference is due to the levels of chemical compounds in the two plants. The percentage of inhibition of growth of *S. aureus* bacterial colonies from Tables 1 and 2 are presented in Table 3.

Table 1. Average area (mm^2) of colony growth of *Staphylococcus aureus* bacteria by administration of leaves crude extract of *A. indica* at five concentrations (%) compared to control

Concentrations (%)	Growth area (mm^2)
0%	557,340a
0.1%	54,810b
0.2%	47,500b
0.3%	46,520b
0.4%	44,040b
0.5%	42,150b

Note: The numbers followed by the same letter are not significantly different from the 5% Tamhane test

Table 2. Average area (mm^2) of colony growth of *Staphylococcus aureus* bacteria by administration of leaves crude extract of *A.hispida* at five concentrations (%) compared to control

Concentrations (%)	Growth area (mm^2)
0%	557,340a
0.1%	4,170b
0.2%	2,580b
0.3%	2,550b
0.4%	2,040b
0.5%	1,000b

Note: The numbers followed by the same letter are not significantly different from the 5% Tamhane test

Table 3. Percentage of inhibition of bacterial colony growth of *Staphylococcus aureus* (%) by administering a crude extract of *A. indica* and *A. hispida* leaves at various concentration levels (%)

Concentration (%)	Percentage of inhibition (%)	
	Extract of *A. indica* leaf	Extract of *A. hispida* leaf
0 (Control)	0.000	0.000
0.1	90.171	99.233
0.2	91.454	99.528
0.3	91.475	99.540
0.4	91.621	99.631
0.5	92.420	99.822

Based on Table 3, the difference between the crude extract of *A. indica* and *A. hispida* leaves can be seen. The percentage of inhibition of the leaf extract of *A. hispida* had a higher inhibitory value than that of *A. indica*. The administration of crude leaf extract of *A. indica* and leaf crude extract of *A. hispida* with a concentration of 0.5% had the highest percentage of inhibition. In comparison, a concentration of 0.1% had the lowest percentage of inhibition. From Table 3, it can be seen that the higher the concentration of the extract, the greater the inhibitory power of bacteria. Sunarto et al. (1999) stated that increasing the extract's concentration will increase the percentage of growth inhibition. It is because the concentration of chemical compounds is also getting higher.

Staphylococcus aureus isolates

The *S. aureus* is a Gram-positive, immobile, found singly, in pairs, short chains, or clusters. It is the cause of various kinds of infections such as acne, boils, pneumonia, and abscesses on any part of the body (Fardiaz 1993; Schegel 1994; Pratama 2005).

From the results of observations, colonies of *S. aureus* were obtained, as shown in Figures 1-2.

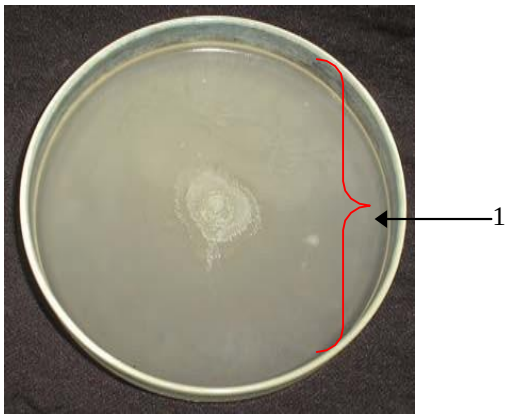

Figure 1. *Staphylococcus aureus* colonies on NA media (7 days incubation). Note: 1. Colonies of *S. aureus* bacteria

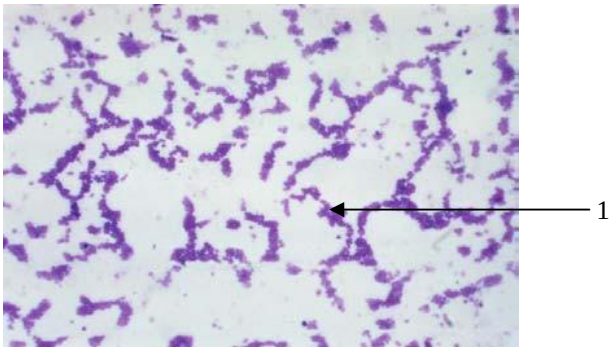

Figure 2. Colony of *Staphylococcus aureus* with gram stain, magnification 100x. Note: 1. Colonies of *S. aureus* bacteria

With gram staining, *S. aureus* appears purple because these bacteria are resistant to alcohol during staining, so they bind to the first paint and do not bind to the second paint. By observing using a visible light microscope, *S. aureus* bacteria are spherical and form clusters like grapes (Syahrurachman et al. 1993; Pratama 2005).

Growth inhibition of *Staphylococcus aureus*

The two types of extracts generally showed that all concentrations of crude extract of *A. indica* and *A. hispida* leaves could only inhibit the growth of bacterial colonies but did not kill bacteria. It can be seen from the difference in the area of bacterial colonies. In general, increasing the extract's concentration will increase the growth inhibition percentage, although the response is not always linear (Sunarto et al. 1999). The condition caused this increase in inhibitory power that the amount of extract concentration would affect the amount of chemical content, which played a role in inhibiting the growth of *S. aureus* bacteria.

The two extracts tested found that the crude extract of *A. hispida* leaf was more able to inhibit the growth of *S. aureus* bacteria than the crude extract of the *A. indica* leaf. This difference is thought to be due to the different concentrations of the active ingredients in the two extracts. In addition, it is influenced by environmental factors such as temperature, the altitude where it grows, humidity of air and soil, light intensity, and availability of water. A good growing environment for a plant will also improve its growth so that the metabolism of secondary metabolites in the plant is also more optimal. For the environment where plants live, *A. hispida* plants are usually found living in the highlands, while *A. indica* plants mostly live in the lowlands (Dalimartha 1991; Hutapea 1993).

Each chemical compound agent has its mechanism to inhibit or kill bacteria. However, one of the weaknesses of natural extracts for antimicrobial substances is the inconsistent effect because the type and content of the active ingredients obtained from each extraction are not always the same, depending on the extraction method, age, the part of the plant organ extracted and the environment in which the plant grows. In addition, the process of inhibiting bacterial growth can be carried out by all types of active ingredients in the extract, not depending on only one type (Sunarto et al. 1999).

The type and content of the active ingredients of the leaf extract are also determined by the source of the extract and the age of the source of the extract. The part of the plant used for extracting is the leaves because it contains tannins, saponins, flavonoids, essential oils, and acalyphin. The leaves of older plants have relatively higher metabolites than younger ones (Hutapea 1993).

Tannins have properties as a chelating agent with a spasmolytic effect, which can shrink cell walls or cell membranes so that they interfere with the permeability of the cell itself. Due to the disruption of permeability, cells cannot carry out living activities, so their growth is inhibited or even dies (Harborne 1996). According to Masduki (1996), tannins also have antibacterial power by precipitation of protein because it is suspected that tannins have the same effect as phenolic compounds. Saponins are secondary metabolites belonging to the glycoside group (Robinson 1991). Saponin compounds damage the cytoplasmic membrane and kill cells (Assani 1994). Flavonoid compounds have a mechanism of action: denaturing bacterial cell proteases and damaging cell membranes beyond repair (Naim 2003; Subroto and Saputro 2006). Essential oils can inhibit the growth or kill bacteria by interfering with the process of forming cell walls, or cell walls are not formed or formed imperfectly (Ajizah 2004).

Acalyphin is an active ingredient found in the leaves of *A. hispida* which has a cyanide chain (HCN) toxic, so it is suspected that this compound is the most active in killing bacteria. The content of this compound in *A. indica* leaf is 0.03%. While in the leaf of *A. hispida*, it is not yet known, if seen in Table 2, it is known that the leaf extract of *A. hispida* is more inhibiting than the leaf of *A. indica*, so it is suspected that the acalyphin content in the leaf of *A. hispida* is greater than that of the leaf of *A. indica* (Lenny 2006).

The solvent used to make the extract also affects the levels of chemical compounds. This test uses ethanol as a solvent because it is a universal polar solvent that can dissolve polar compounds. According to Cowan (1999), ethanol can dissolve active compounds such as tannins, polyphenols, polyacytelin, terpenoids, sterols, alkaloids, essential oils, volatiles, curcumin, anthraquinones, flavonoids, steroids, resins, and chlorophyll, and improve the stability of soluble substances, can inhibit enzyme activity, and is effective in producing an optimal amount of active ingredient, where the free material slightly enters the extracting fluid. Still, it does not cause swelling of the cell membrane (Voight 1995).

Meanwhile, water is a dilution solvent because the ethanol extract is easily soluble in water due to its polarity. Water can dissolve alkaloids, saponins, terpenoids, volatile oils, glycosides, tannins, sugars, gums, starch, proteins, mucus, enzymes, waxes, fats, pectin, dyes, and organic acids (Cowan 1999).

Inhibition mechanism

The compounds thought to be antibacterial in the crude extract of the leaves of *A. indica* and *A. hispida* were tannins, saponins, essential oils, flavonoids, and acalyphin. According to Assani (1994), tannins have antibacterial power by precipitation of bacterial cell proteins so that bacterial protein synthesis will be disrupted or lead to enzyme inactivation reactions and destruction or inactivation of genetic material functions. Saponins are surface-active compounds that are antibacterial by lowering the surface tension that can bind lipids so that antibacterial compounds can enter through the membrane and will damage the cytoplasmic membrane and kill cells. Finally, essential oils can inhibit the growth or kill bacteria by interfering with forming a membrane or cell wall so that the membrane or cell wall is not formed or is formed imperfectly (Ajizah 2004).

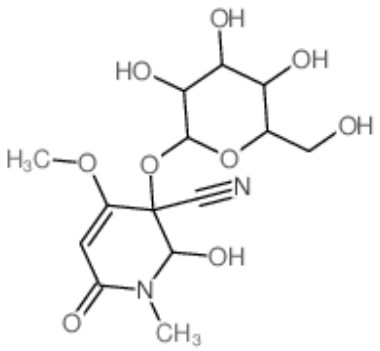

Figure 3. Chemical structure of acalyphin

According to Masduki (1996), flavonoid compounds are antibacterial with their mechanism of action is to damage cell membranes beyond repair and degrade bacterial cell proteins. In addition, flavonoids are thought to be able to inhibit the growth of *S. aureus* bacteria because of the phenolic effect of flavonoids. While the most active compound from the leaves of *A. indica* and *A. hispida* is acalyphin, this compound presented in Figure 3.

Although only 0.03% in *A. indica* leaves, acalyphin has a cyanide chain (HCN) which is toxic, so it is suspected that cyanide enters the cell structure of *S. aureus* and poisons it so that it interferes with metabolic processes in cells and even kills cells (Lenny 2006). According to Jawetz et al. (2001), inhibited bacterial growth or death due to an antibacterial substance can be caused by inhibition of the cell wall, cell membrane function, protein synthesis, or nucleic acid synthesis.

Antibacterial compounds that diffuse into the agar media can cause inhibition of cell wall formation so that cells are only limited by a thin membrane and can be lysed (Madigan and Martinko 1997). Antimicrobial compounds will damage the cell wall's structure by inhibiting the cell wall's growth. The mechanism of the destruction of the cell wall is by lysing the cell membrane, which is the structure of the cell wall. Fessenden and Fessenden (1999) say that the cell membrane is formed from embedded proteins and fused with a double layer (bilayer) of phosphoglyceride molecules, with the hydrophobic ends facing in and the hydrophilic ends facing out. The function of these proteins is to allow the entry of water, ions, and compounds. Compounds with high concentrations will diffuse and be captured by hydrophilic sensors. Hydrophilic components will bind to compound molecules which eventually causes the lysis of the entire lipoprotein membrane to inhibit the growth of the cell wall. If the cell wall, which is a protective barrier for cells, is damaged, it will cause the death of microbial cells.

Antimicrobial compounds, according to Pelczar and Chan (1988), will work to affect the permeability of the cell cytoplasmic membrane, where the cytoplasm functions to regulate the entry and exit of substances between cells and the outside environment. The cytoplasmic membrane is also the site of enzyme reactions. The *S. aureus* bacteria are Gram-positive bacteria with a structure with lots of peptidoglycans and relatively little lipid, so the bacterial cell wall becomes hydrated during treatment with ethanol. Peptidoglycan plays a role in hardness and gives cell shape. The ethanolic extracts of the leaves of *A. hispida* and *A. indica* will easily dissolve in water because they are polar. Therefore, the compounds in the leaves of the two plants are generally polar. At the same time, S. aureus has teichoic and trichuronic acids, which are polymers that are soluble in water (in peptidoglycan) so that polar compounds easily penetrate the walls.

Ethanol is relatively polar, so the compound extracted is relatively polar. The polarity of these compounds causes the compounds to more easily penetrate the cell walls of Gram-positive bacteria (Hugo and Russell 1998; Gopalakrishnan et al. 2000). Therefore, the destruction of cytoplasmic membrane permeability and protein will inhibit the growth of *S. aureus* bacteria.

Inhibition also occurs in the process of protein synthesis or the synthesis of nucleic acids. Therefore, it is suspected that tannin compounds have an inactivation mechanism of genetic material. According to Pelczar and Chan (1982), the process of inhibition of protein synthesis occurs in the transcription process and the transition of genetic material, where there is a translation error, so that the amino acids produced are misplaced in the peptide chain and produce non-functioning proteins.

Inhibition by antimicrobial compounds can also occur against enzymes that work in cells. According to Pelczar and Chan (1988), enzymes are potential targets for the work of an antimicrobial agent. Furthermore, it is stated that the inhibition of antimicrobial substances is generally irreversible; that is, a change occurs, so the enzyme is not active. The inhibition or cessation of enzyme activity can cause the mechanism of enzyme work to be disrupted. For example, *S. aureus* has a coagulase enzyme that can clot plasma supplemented with oxalate or in the presence of a factor or serum. It also has the enzyme catalase, which can convert hydrogen peroxide into water and oxygen. With these enzymes, *S. aureus* will produce an enterotoxin, leukocidin, exfoliatin, and lysostaphin toxins which can cause red blood cell lysis and cause infection. Disruption of the working enzyme mechanism will affect the formation of bacterial cells and bacterial growth.

The combined activity of several antibacterial compounds can be more effective than the work of each compound (Jawetz et al. 2001). But it is also possible that antibacterial compounds with the largest percentage can affect the effectiveness of their work. On the other hand, the combined working activity of several antibacterial compounds can also be less effective than the work of each compound (Kusumaningrum 2002).

Judging from the antibacterial activity of each chemical compound in the leaves of *A. indica* and *A. hispida*, the inhibition of the growth of *S. aureus* bacteria may be carried out by all chemical compounds or only one chemical compound. However, this can not be ascertained because it is unclear how many levels of each chemical compound are contained in the leaves of *A. indica* and *A. hispida.*

In conclusion, the crude extract of the leaves of *A. hispida* at a concentration of 0.1%, 0.2%, 0.3%, 0.4%, and 0.5% were able to inhibit the growth of *S. aureus* bacteria. Crude extract of the leaves of *A. hispida* at a concentration of 0.1%, 0.2%, 0.3%, 0.4%, and 0.5% were able to inhibit the growth of *S. aureus* bacteria. Crude extract of *A. hispida* leaf was more effective in inhibiting *S. aureus* bacteria than crude extract of *A. indica* leaf.

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Anti-bacterial properties and GC-MS analysis of extracts and essential oils of selected plant product

OMBUNA DINAH NYAITONDI, RUTH NDUTA WANJAU♥, HUDSON NYAMBAKA, AHMED HASSANALI
Department of Chemistry, School of Pure and Applied Sciences, Kenyatta University. Nairobi, Kenya. ♥email: ruthwanjau@yahoo.com, wanjau.ruth@ku.ac.ke

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Abstract. *Nyaitondi OD, Wanjau R, Nyambaka H, Hassanali A. 2018. Anti-bacterial properties and GC-MS analysis of extracts and essential oils of selected plant product. Biofarmasi J Nat Prod Biochem 16: 36-50.* Plants are traditionally used to treat bacterial infections though not clinically regulated due to a lack of awareness and sufficient data to support the reported therapeutic claims. Some plants used as food and vegetables are hardly considered in such studies. This study aimed to investigate the antibacterial properties associated with garlic, ginger, turmeric, lemon, and onion in the form of juices, methanol extracts, and essential oils. These materials were tested against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Salmonella typhi*. Identification of suspected antibacterial compounds was made by comparing retention indices and the mass spectra with those in National Institute of Standards and Technology (NIST) libraries using GC-MS analyses. Garlic juice was bactericidal against all tested strains. Lemon/garlic juice exhibited significantly higher activity against *E. coli* and *S. typhi*. Turmeric/lemon/garlic methanol extracts blend was most active against *S. aureus*. Preliminary screening of the essential oils indicated significant antibacterial activity of lemon/garlic essential oil blend against *P. aeruginosa*. GC-MS analysis of the active samples confirmed the presence of compounds containing OOH,-OH,-N,-Cl,-F,-NH_2, and-S groups associated with bacterial inhibition in conventional antibiotics. The 10 major constituents obtained from samples suspected to contain antibacterial activity, include limonene; 3-vinyl-1,2-dithiacyclohex-4-ene; α-zingiberene; diallyl disulphide; 2-butanone,4-(-hydroxy-3-methoxyphenyl); 3-chlorothiophene; methanehydrazonic acid, N-[3-(methylthio)-1,-2,4-thiadiazol-5-yl]-,ethyl ester; n-hexadecanoic acid; γ-sitosterol and propanamide,2-amino-3-phenyl. Juices of garlic, lemon, and lemon/garlic blend were active against one or more bacteria tested, unlike methanol extracts and essential oils. They should be used in raw form as heating and drying are likely to render them inactive. Further studies on methanol extract and fresh juice of lemon/garlic blend need to be undertaken to elucidate the active principles in these extracts and may lead to the discovery of novel antimicrobial agents and models for the new generation of synthetic antibiotics.

Keywords: anti-bacterial, garlic, ginger, turmeric, lemon, onion, GC-MS analysis

INTRODUCTION

The continuous spread of multidrug-resistant pathogens has become a threat to public health and a significant concern for infection control practitioners worldwide (Borowitz and Naser 2011). Not only increasing the cost of drug regimens, but this scenario has also paved the way for the re-emergence of previously controlled diseases and has contributed substantially to the high frequency of opportunistic and chronic infection cases in developing countries (Collins et al. 1999). Among some pathogens, bacteria cause a wide range of infections, resulting in mild to life-threatening illnesses that require immediate interventions (Martin and Edzard 2003). Common bacterial infections include respiratory infections, gastrointestinal infections, ear infections, and skin disorders (Mandal et al. 2005; Arthur 2006).

In developing countries, outbreaks of bacterial infections occur most often in densely populated areas such as refugee camps and slums. Food vendors, slum dwellers, riparian communities, fishers, and school children are among the risk groups (Brooks et al. 2005; Change 2009). Studies conducted by the Center for Microbiology Research in KEMRI, Nairobi, Kenya, show that 41% of people contracted typhoid fever in 2008 (Kariuki 2008). The study further demonstrated that 52% of the cases affected children under 10 years old and 40% of people aged 15 and 45 years. More than half of these cases were from the informal settlements (slum areas) surrounding the capital city (Kariuki 2008). There have been studies of sporadic outbreaks of bacterial infections in many regions, including three districts in Central Kenya, Malindi, and Kwale in the Coast Province and some parts of Nyanza Province (Onyango 2005). However, not all outbreaks were confirmed, leading to a lack of reliable data on the prevalence of diseases caused by bacteria (WHO 2009).

Conventional antibiotics usually provide effective therapy for bacterial infections (Martin and Edzard 2003). Nevertheless, these bacteria have become resistant to one or more antibiotics, and the population of Multidrug-Resistant (MDR) bacteria is increasing (Stewart and Costerton 2001). Mechanisms that microorganisms have developed to resist antibiotics include inactivation of antibiotics by enzymes, alteration of drug target sites, blockage of drugs from entering into the cell membrane, and chromosomal and plasmid-mediated resistance.

Closely related to bacterial infections is malaria, caused by the *Plasmodium* parasite (Ndyomugyenyi et al., 2007;

Charles, 2010*)*. The symptoms include fever, shaking, chills, headache, muscle aches, tiredness, nausea, vomiting, and diarrhea (Ali et al. 2007; Mohanna et al. 200, similar to some bacterial infections (especially typhoid). Therefore, patients are focused on treating malaria instead of bacterial infections (Balentine 2009). The symptoms are more dramatic in children, and if untreated, they may kill fast (Onyango 2009).

Antibiotics such as ampicillin, chloramphenicol, Trimethoprim/Sulfamethoxazole (TMP-SMX), amoxicillin and ciprofloxacin have been commonly used to treat bacterial infections (Wain and Kidqell 2004). The bioactive parts against bacteria in these conventional antibiotics include structural moieties that include Cl,-F,-N,-NH_2,-S,-COOH, and-OH, which are also found in many herbs used traditionally against bacterial infections. Studies have shown that sulfur-containing compounds have strong inhibitory antibacterial activities (Julia and Ann 1947; Kyung and Fleming 1996; Yanyali et al. 2001). Nitrite exhibits toxic properties while nitrous acid is bactericidal; chlorine-releasing compounds such as chlorine dioxide (ClO_2) and acidic and alcoholic compounds act as antibacterial agents (Gerald and Russell 1999).

Some vegetable trials have been comparable to conventional treatments and provide therapy for bacterial infections (Martin and Edzard 2003). The compounds in drugs vary in different species. Even within a single species, the phytochemical composition may be affected by the plant‟s growing conditions, and various parts of a herb can have distinct chemical structures (Linda et al. 2008). Most herbs, foods, and spices contain antibacterial properties; for instance, allicin, a compound produced in garlic, was proven to be active against bacteria and fungi (Serge 2001; Onyeagba et al. 2007; Lian-fang et al. 2009; Pandey et al. 2011).

The objectives of this research were (i) To determine *in vitro* antibacterial activities of juices, methanol extracts, and essential oils of garlic (*A. sativum*), ginger *(Z. officinale*), onion (*A. cepa*), turmeric (*C. longa*), and lemon (*C. lemon*) individually and as blends. (ii) To determine the time-course antibacterial activities of garlic (*A. sativum*), ginger *(Z. officinale*), onion (*A. cepa*), turmeric (*C. longa*), and lemon (*C. lemon*) juices individually and as blends. (iii) To identify suspected antibacterial constituents of the active samples and blended essential oils by GC-MS.

MATERIALS AND METHODS

Experimental procedures

Over time, this study involved bioassay of garlic, ginger, turmeric onion, lemon juices, methanol extracts, and essential oils. Identification of active compounds was performed using GC-MS.

Sample collection and pretreatment

The vegetable materials (garlic, ginger, onion, and turmeric) and lemon were purchased from Githurai market in Nairobi. The ginger, turmeric, lemon fruit, and onion rhizomes were washed using tap water to remove dirt. The materials were dried up at room temperature for six hours, then stored in a dry cabin at room temperature awaiting extraction.

Instrumentation

The HP 5890 series II Gas Chromatograph was interfaced to a 5973 Mass Selective Detector (MSD) and controlled by HP Chemstation software (version b.02.05, 1989-1997). The chromatographic separation was achieved using an HP5-MS capillary column (30.0 m x 250 m x 0.25 m). The stationary column phase comprises a 5: 95% diphenyl: dimethylpolysiloxane blend. The operating GC condition was an initial oven temperature of 35 °C for 3 min, then programmed to 280^0 C at the rate of 10^0 C/min, and then kept constant at 280^0 C (23 min). The injector and detector temperatures were set at 270^0 C, and the carrier gas was nitrogen-flowing at a rate of 1.2 ml/min. The mass spectrometer was operated in the electron impact mode at 70 eV. Ion source and transfer line temperature were kept at 280^0 C. The mass spectra were obtained by centroid scan of the range from 40 to 800 amu. Retention index made identification of the constituents, library mass search database (NIST & WILEY, and compared with the mass spectral data.

Isolation and extractions

Methanol extractions

The vegetable materials and lemon were cut into small pieces and dried at room temperature for three weeks. The materials were ground into powder using a blender and soaked in methanol for 72 hours with occasional stirring. The extracts were filtered using Whatman's No. 1 filter paper (9 cm). The filtered extracts were then concentrated using a rotatory evaporator and dried to a paste in a hood. The crude extract was then used for bioassay.

Steam distillation

The essential oils were isolated from all the materials except onion since they did not produce a significant amount of hydrodistillate. The materials were chopped into small pieces. Using a round-bottomed flask, 1 kg of each material was mixed with 1 liter of water and then steamed distilled using Clevenger-type apparatus (Figure 3.2). A flask containing the homogenate was heated for three to four hours, and the oil was separated from water using a Pasteur pipette. The essential oils were put in amber-colored vials, labeled, and stored at −4° C before bioassay (Tassou et al. 1995)

The oil isolated from garlic was extracted using DCM. The mixture of oil and DCM was treated with anhydrous sodium sulfate to remove any dissolved water and evaporated using a rotatory evaporator. The oil was labeled, put in an amber-colored vial then stored at −4° C before bioassay.

Juice extractions

The bulbs of garlic and onion, rhizomes of ginger and turmeric, and lemon were cut into small pieces and crushed using a juice extractor. The juice was sieved, put in amber-colored vials, and concentrated by freeze-drying. The

extractions were done two hours before the commencement of the sensitivity test. The sensitivity test was done within five days of preparation.

Bioassays

Preparation of McFarland standard

McFarland equivalent turbidity standard (0.5 McFarland) was made by adding 0.6 ml of 1 % $BaCl_2$. $2H_2O$ to 99.4 ml of 1 % H_2SO_4 and mixed. About 5 ml of the turbid solution was transferred to a stopped test tube of the same type that was used to prepare the test and control inoculums, then stored in the darkroom at a temperature of 25^0 C. Exactly 0.5 McFarland gives an equivalent approximate density of bacteria $1x10^{-8}$ Colony Forming Units (CFU) (Baron and Yolken 1999).

Preparation of inoculums by direct colony suspension method

Microorganisms obtained from KEMRI included one gram-positive bacteria, *S. aureus* (ATCC 25923), and three gram-negative bacteria, *E. coli* (ATCC 25922), *S. typhi* (ATCC 20613), and *P. aeruginosa* (ATCC 27853). Before use, the test strains were tested biochemically for viability and purity (Elgayyer et al., 2000). Sterile water (small volume) was poured inside a test tube to which general colonies of the test organisms, and the suspension was adjusted to match the 0.5 McFarland's standard (10^8 CFU/ ml), which resembles the appearance of an overnight broth culture by adding distilled water (Azu et al. 2007).

Screening for antibacterial activity

Disc diffusion test. Antibacterial efficacy was tested using the filter paper disc diffusion method (Elgayyar et al., 2000). Each extract (3 g) was dissolved in DMSO and 10 µL (100 mg/mL) loaded onto 6 mm (Whatman's No. 3) filter paper discs and air-dried. The vegetable and lemon blends were made in a ratio of 1: 1. The nutrient agar (NA) was used in culturing the bacteria. The media (NA) was prepared using the manufacturer's instructions, while plates were prepared by adding Mueller-Hinton (MH) agar.

Each plate was inoculated with 0.1 ml of bacteria culture directly from the 24-hour broth culture and diluted to match the 0.5 McFarland standard. The discs loaded with the extracts were placed onto the seeded plates. The bacterial cultures were incubated at 37^0 C for 24 hours, after which zones of inhibition were measured and recorded in mm. Negative control plates had discs with DMSO and water; positive control had standard antibiotic discs of chloramphenicol, ciprofloxacin, and ampicillin. An inhibition zone of 9.0 mm was taken as the base, and any sample that recorded less value was treated as inactive against the test microorganism.

Minimum inhibitory concentration (MIC) Test. The active samples (with an inhibition zone of ≥9) from the antibacterial screening were tested for minimum inhibitory concentration (MIC). Different concentrations of essential oils, juices, and methanol extracts were prepared by dissolving 3.0 g of the crude samples in 2.0 ml of DMSO to determine the MIC. The blends were prepared by mixing the resultant mixtures in the ratio of 1: 1, and 100 µL of the samples were drawn into a 96-well microtiter plate. Concentrations of 750 mg/mL, 375 mg/mL, 188.5 mg/mL, 93.8 mg/mL, 46.9 mg/mL, 23.4 mg/mL, 11.7 mg/mL, 5.9 mg/mL, 2.9 mg/mL and 1.5 mg/mL were made using serial dilution method (Elgayyar et al. 2000; Kariba 2001).

The test strains adjusted to 0.5 McFarland standard were drawn into wells. Blends of active essential oils and methanol extracts were made at 1: 1. The MIC for bacteria was measured using a broth dilution method of the active extracts. Tubes containing only nutrient broth were seeded with the test organism, as described above, to serve as the control. The cultures were incubated at 37 °C for 24 hours and were examined for bacterial growth by observing turbidity. The MIC was the first tube showing no growth (the lowest concentration inhibited growth) (Kariba 2001; Michael et al. 2003).

Minimum bactericidal concentration (MBC). The minimum bactericidal concentration (MBC) of the active extracts was done by subculturing 0.1 ml (100µl) of all the tubes showing no growth on nutrient agar. After 24 hours of incubation at 37 °C, the first plate showing no growth was the MBC (Michael et al. 2003).

GC-MS analyses

Samples of 3.0 g garlic, ginger, lemon, and turmeric were crushed and dissolved separately in 5 ml of DCM. They were shaken and mixed using the ultrasound path for 3 min, then filtered using glass wool. The sample was drawn into small vials, and then 1 µL was injected into the GC-MS. China garlic was also prepared similarly and analyzed for comparison with garlic (used for bioassays). The active methanol extracts were blended in 1: 1, and 2 ml of pentane was added to each blend. The mixture was left overnight, filtered using glass wool, and 5 µL of the filtrate was dissolved in 1 ml of pentane. The sample (1 µL) was injected into the GC-MS for analysis. The active essential oil blends (in the ratio of 1: 1) were also drawn into small vials, and then 1 µL was analyzed.

Data analyses

The inhibition zone data obtained from juice and methanol extracts were subjected to analysis of variance (ANOVA). Individual essential oils recorded less than 9 mm activities, and their results were not subjected to ANOVA. The mean inhibition zones of their active juices and methanol extracts against *S. typhi*, *P. aeruginosa*, *S. aureus,* and *E. coli* were compared to their blends. Treatment means showing a significant difference ($p \leq 0.05$) were separated using Student-Newman-Keuls (SNK) at a 5% significance level. The GC-MS chromatograms acquired from each active sample were subjected to HP Chemstation software; each peak was analyzed for the most abundant compound that contains active constituents-OH,-COOH,-Cl,-S, N,-F, and-NH_2. The compounds were identified by directly comparing their mass spectra to the Wiley NBS and MIST database library of mass spectra.

RESULTS AND DISCUSSION

Antibacterial activities

Vegetable and lemon juices

The inhibition zones of juices on gram-positive and gram harmful bacteria were determined using the filter paper disc diffusion method (Elgayyar et al., 2000). The results are indicated in Table 1.

Garlic juice (Figure 1, sample 40) inhibited the growth of all bacteria tested to variable levels (10.0 mm for *P. aeruginosa*, Figure 1.A; 11.7 mm for *E. coli*, Figure 1.B; 14.7 mm for *S. aureus* Figure 1.C*;* and 17.7 mm for *S. typhi*, Figure 1.D.). Lemon juice inhibited only the growth of *S. typhi* with a zone of 11.0 mm. Turmeric, lemon, and ginger juices had no activity against *P. aeruginosa*, *E. coli*, and *S. aureus*; this can be attributed to the low concentration (10 µL) of samples used for bioassay. Earlier studies on their activity show that they had antifungal and antibacterial agents at concentrations of 50 µL and 100 µL (Gopalan et al. 2000; Jayaprakasha et al. 2002; Fisher and Phillips 2006).

The high antibacterial activity exhibited by garlic compared to lemon may be attributed to sulfur-based compounds such as alliin, which possess strong antibacterial activities (Larkcom 1976: Bocchini et al. 2001). These compounds are found in the intact bulbs, flavorants formed on cutting or crushing the bulbs, substances derived from further reactions of these flavorants, or metabolic degradation of these three types of compounds (John and Timothy 1997). The results agree with earlier reports where garlic was effective against a plethora of gram-positive and gram harmful bacteria such as *S. aureus*, *Proteus*, *Pseudomonas*, *E. coli*, *Salmonella*, and *Klebsiella* (O'Gara and Hill 2000). On the other hand, the activity of lemon can be attributed to the presence of-COOH and-OH group, which act against the bacteria (Angel 2006). However, *E. coli* was not susceptible to lemon juice, due to its unusual acid-resistant properties. The microorganism can survive and grow in acidified media (Greg and Ann 2007).

Research has found the aqueous extract of garlic to be more potent than organic extracts (Roy et al. 2006; Jaber and Al-Mossawi 2007). This could be a result of the fact that some phenolases and hydrolases are released when plant materials are ground in water . These enzymes might modulate the active compounds' activity in the extract (De and Ifeoma 2002). Since the herbalist usually uses water to prepare infusions and decoctions, and since most constituents of garlic are soluble in water, there is a likelihood that the herbalist can extract all the bioactive drug components in garlic, making it a proper home remedy against some infections.

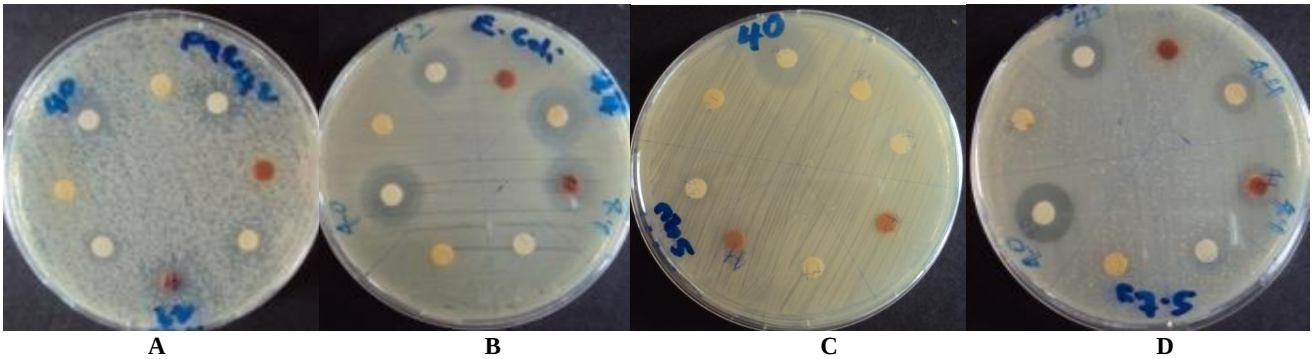
Positive controls had diverse activities depending on the type of sample used. The activity of ampicillin on *E. coli* and *S. typhi* was 11.7 mm and 18.7 mm, respectively (Table 1). Ciprofloxacin had an activity of 41.7 mm against *P. aeruginosa*, 30.7 mm against *E. coli*, and 17.3 mm against *S. aureus*, and 34.67 mm against *S. typhi*. Chloramphenicol had the highest activity against *E. coli* (35.0 mm) and *S. aureus* (34.0 mm) (Figure 2).

The activity of standards (+ve controls) is considerably high compared to the samples used. This can be accredited to the pure form of the standards and, therefore, no interferences from other compounds. The natural juices contain mixtures of compoun , including non-active constituents, which may dilute of the active constituents (Narayana et al. 2000).

Table 1. Antibacterial activity exhibited by various juices against *P. aeruginosa, E .coli, S. aureus*, and *S. typhi*

Juice/ antibiotic	Inhibition zone in mm[a]			
	P. aeruginosa	***E.coli***	***S.aureus***	***S.typhi***
Turmeric	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
Lemon	6.0±0.0	6.0±0.0	6.0±0.0	11.0±1.0
Ginger	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
Garlic	10.0±0.0	11.7±0.3	14.7±2.5	17.7±2.5
Onion	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
DMSO (-ve)	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
Water (-ve)	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
Ampicillin (+ve)	6.0±0.0	11.7±0.3	6.0±0.0	18.7±0.6
Ciprofloxacin (+ve)	41.7±0.6	30.7±0.3	17.3±2.1	34.7±0.6
Chloramphenicol (+ve)	20.0±0.0	35.0±0.0	34.0±0.0	30.7±0.3

Note: [a] includes the diameter (6 mm) of the disk used; +ve: positive control; -ve: negative control



A **B** **C** **D**

Figure 1. Plates showing inhibition zones of garlic against *P. Aeruginosa* (A), *E. coli* (B), *S. aureus* (C), and *S. typhi* (D)

Figure 2. Plate showing inhibition zones of chloramphenicol and ciprofloxacin on *S. aureus.* Note: C-Chloramphenicol; CF-Ciprofloxacin

Juices of turmeric, ginger, and onion did not significantly inhibit the growth of any microorganism tested; their inhibition zones were 6.0±0.0 mm each. The sulfur-based compounds which are accredited to bacterial activities might have been destroyed during the cutting and crushing of onion, the bacteria may have developed resistance to the onion, ginger, and turmeric or the relative percentage of the active compounds in the samples was low (Griffiths et al. 2002). Onion has also proven ineffective against gram-negative bacteria such as *S. aureus*, *E. coli*, and *S. typhi* due to fewer amounts of allicin (Farbman et al. 1983).

Although ginger, turmeric, and onion juices tested individually showed no significant inhibition, some blends of these vegetables (1: 1, v/v) were active (Table 2). The highest activity was exhibited by lemon/garlic (15.0 mm) and lemon/garlic/turmeric (14.7 mm) against *E. coli.* The blends of lemon/garlic, ginger/garlic, lemon/garlic/ginger, turmeric/ginger/garlic, and lemon/garlic/turmeric had appreciable activities against *E. coli* and *S. typhi*. Ginger/ lemon, lemon/turmeric, ginger/turmeric, and lemon/ginger/ turmeric blends had no significant activity against all the four bacteria tested. *S. typhi* was susceptible to turmeric/ garlic and lemon/garlic/turmeric/ginger at a zone of 12.0 mm and 9.7 mm, respectively. The test bacteria, *P. aeruginosa* and *S. aureus*, did not record any activity when the juice blends were used.

Table 3 gives the results of the Student-Newman-Keuls (SNK) test on the mean inhibition zones of individual juices and their blends against *S. typhi*, *P. aeruginosa*, *S. aureus*, and *E. coli* bacteria. The mean inhibition zone of garlic juice against *S. typhi* was significantly different ($p < 0.05$) compared to other tested materials and not significantly different from ampicillin. Lemon/garlic/ turmeric blend gave inhibition zones against *E. coli* and *S. typhi* that are substantially different from pure garlic ($p < 0.05$).

From the mean inhibition zones, it can be noted that ginger and turmeric lower the activity of blends and the lemon/garlic blend has lower activity on *S. typhi* (12.0±0.0) compared to pure garlic (17.7±2.5). This may be due to the deactivating effect of citric acid on allinase, an enzyme that converts alliin to allicin (Bocchini et al. 2001). The transformation of alliin to allicin is exceptionally rapid, taking mere seconds. Even more intriguing is the instability of allicin (Blania and Spangenberg 1991). The allicin molecule's most crucial and reactive part is the sulfur-sulfur bond coupled to an oxygen atom (Mohammad et al., 2007). It remains active only for a short period before degrading when allicin degrades, 20 sulfur compounds are formed (Bocchini et al. 2001).

Blends that comprised garlic had antibacterial activity against one or more microorganisms tested. Studies on rats infected with Klebsiella pneumoniae using plant extracts (ginger and garlic) for seven days show that the garlic treated group recovered fully on day four. Still, all the animals in ginger managed group died. NHowever, no death wasrecorded in rats treated with the mixture of garlic and ginger (Olatunde et al. 2009). All tests performed against *P. aeruginosa* showed inactivity except for garlic. This might be a result of the bacteria developing resistance against individual juices and blends (Baliga 2005).

Methanol extracts

All individual methanol extracts except lemon showed no activity against the tested microorganism (Table 4). The lemon extract had an activity of 11.0±0.0 mm against *P. aeruginosa* and 10.0±0.0 mm against *S. aureus*, respectively.

The methanol blends, also made in the ratio of 1: 1 (v/v), had sensitivities against the bacteria, as shown in Table 5. The turmeric/lemon extract blend had the activity of 11.0±0.0mm against *S. aureus*. The increase in activity can be attributed to favorable interactions between the natural compounds present in the mixture leading to synergism (Bocchini et al. 2001). Addition of garlic to the mix of turmeric/lemon methanol extract increases the activity to 12.0±1.0 mm. The activity of turmeric/ginger/lemon extract on *S. aureus* is 10.0±0.0 mm, but on the addition of garlic, the activity reduces to 9.3±0.6 mm. The blend of turmeric/garlic/ginger/lemon/onion extracts had an activity of 9.0±0.0 mm against *S. aureus*. The methanol blends recorded an inhibition zone of less than 9.0±0.0 mm against *E. coli* and *S. typhi*, thus inactive. The factors associated with the reduced activities of the mixtures are not apparent and therefore require further studies to be undertaken on the blends.

Table 6 gives a summary of the overall mean inhibition zones of individual methanol extract and blends against *S. aureus*. The whole mean inhibition zone of turmeric/lemon/garlic methanol blend against *S. aureus* is significantly different ($p < 0.05$) to the other test materials. The activities of individual lemon and turmeric/ginger/ lemon, turmeric/garlic/ginger/lemon/onion, and turmeric/ garlic/ginger/lemon blends are not significantly different. The data acquired from the susceptibility tests on *P. aeruginosa*, *E. coli* and *S. typhi*, was not subjected to ANOVA as only lemon/ginger was active against *P. aeruginosa*.

The result of antibacterial susceptibility assay showed promising evidence for the antibacterial effects of lemon methanol extract against *S. aureus* (10.0±0.0 mm) and *P.*

aeruginosa (11.0±0.0 mm). This is in line with a study conducted by Pandey et al. (2011) which showed methanol extract of lemon to be effective against *P. aeruginosa* with an inhibition zone of 23 mm. The results of antibacterial testing revealed that methanol extract of lemon had inhibitory effect on *P. aeruginosa* (11.0 mm) and *S. aureus* (10.0 mm) due to better solubility in the organic solvent as compared to the juice (Malu 2009: Mohamma et al. 2009: Pandey et al. 2011).

Table 2. Antibacterial activity exhibited by various juice blends against *P. aeruginosa, E .coli, S. aureus* and *S. typhi*

Sample juice blends	Mean inhibition zone in mm [a]			
	P. aeruginosa	***E .coli***	***S. aureus***	***S. typhi***
Lemon/garlic	7.7±0.6	15.0±0.0	6.0±0.0	12.0±0.0
Ginger/garlic	6.0±0.0	12.0±0.0	6.0±0.0	11.0±0.0
Turmeric/garlic	6.0±0.0	6.0±0.0	6.0±0.0	12.0±0.0
Ginger/turmeric	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
Ginger/lemon,	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
Lemon/turmeric,	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
Lemon/garlic/ginger	6.0±0.0	13.7±0.6	6.0±0.0	9.7±0.6
Turmeric/ginger/garlic	6.0±0.0	11.0±0.0	6.0±0.0	10.0±0.0
Lemon/garlic/turmeric	8.7±0.6	14.7±0.6	6.0±0.0	11.0±0.0
Lemon/ginger/turmeric	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
Lemon/garlic/turmeric/ginger	6.0±0.0	6.0±0.0	6.0±0.0	9.7±0.6
DMSO (-ve)	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
Water (-ve)	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
Ampicillin (+ve)	6.0±0.0	11.7±0.3	6.0±0.0	18.7±0.6
Ciprofloxacin (+ve)	41.7±0.6	30.7±0.3	17.3±2.1	34.7±0.6
Chloramphenicol (+ve)	20.0±0.0	35.0±0.0	34.0±0.0	30.7±0.3

Note: [a] includes the diameter (6 mm) of the disk used

Table 3. The mean (±SD) inhibition zones exhibited by individual juices and their blends against *P. aeruginosa, E. coli, S. aureus* and *S. typhi*

Sample juice / antibiotic	Inhibition zone (mm) (±SD)			
	P. aeruginosa	***E. coli***	***S. aureus***	***S. typhi***
Lemon	N.A	N.A	N.A	11.0±1.0^{a}
Garlic	10.0±0.0^{a}	11.7±0.6^{ab}	14.7±2.5^{a}	17.7±2.5^{b}
Lemon/garlic	N.A	15.0±0.0^{d}	N.A	12.0±0.0^{a}
Ginger/garlic	N.A	12.0±0.0^{b}	N.A	11.0±0.0^{a}
Turmeric/garlic	N.A	N.A	N.A	12.0±0.0^{a}
Lemon/garlic/ginger	N.A	13.7±0.6^{c}	N.A	9.7±0.6^{a}
Turmeric/garlic/ginger	N.A	11.0±0.0^{a}	N.A	10.0±0.0^{a}
Lemon/garlic/turmeric	N.A	14.7±0.6^{d}	N.A	11.0±0.0^{a}
Lemon/garlic/turmeric/ginger	N.A	N.A	N.A	9.7±0.6^{a}
Ampicillin	N.A	11.7±0.3^{ab}	N.A	18.7±0.6^{b}
Ciprofloxacin	41.7±0.6^{c}	30.7±0.6^{e}	17.3±2.1^{a}	34.7±0.6^{d}
Chloramphenicol	20.0±0.0^{b}	35.0±0.0^{f}	34.0±0.0^{b}	30.7±0.6^{c}

Note: Mean (±SD) followed by the same small letters within the same column are not significantly different at $\alpha = 0.05$ (Student-Newman-Keuls test). N.A-not active

Table 4. Antibacterial activity exhibited by individual methanol extracts against *P. aeruginosa, E. coli, S. aureus* and *S. typhi*

Sample methanol extract	Inhibition zone in mm[a]			
	P. aeruginosa	***E. coli***	***S. aureus***	***S. typhi***
Turmeric	6.0±0.0	6. 0±0.0	8.7±0.6	6. 0±0.0
Onion	7.7±0.6	7.3±0.6	6. 0±0.0	7.3±0.6
Lemon	11.0±0.0	7.3±0.6	10.0±0.0	6.3±0.6
Ginger	6. 0±0.0	6.0±0.0.	7.3±0.6	6. 0±0.0
Garlic	6. 0±0.0	6. 0±0.0	6. 0±0.0	6. 0±0.0
Ampicillin	6. 0±0.0	11.7±0.3	6. 0±0.0	18.7±0.6
Ciprofloxacin	41.7±0.6	30.7±0.6	17.3±2.1	34.7±0.6
Chloramphenicol	20.0±0.0	35.0±0.0	34.0±0.0	30.7±0.6

Note: [a] includes the diameter (6mm) of the disk used

Table 5. Antibacterial activity exhibited by various methanol extract blends against *P. aeruginosa E. coli*, *S. aureus* and *S. typhi*

Sample methanol extract	Mean Inhibition Zone in mm [a]			
	P. aeruginosa	***E. coli***	***S. aureus***	***S. typhi***
Turmeric/lemon	8. 0±0.0	6.3±0.6	11.0±0.0	8.3±0.6
Lemon /ginger	9.67±0.6	6.3±0.6	8.0±0.0	8.0±0.0
Ginger/garlic	6. 0±0.0	6.0±0.0	7.0±0.0	6.0±0.0
Turmeric/ginger/lemon	6· 0±0.0	6.0±0.0	10.0±0.0	6.0±0.0
Turmeric/lemon/garlic	6. 0±0.0	6.0±0.0	12.0±1.0	6.0±0.0
Turmeric/garlic/ginger/lemon	6. 0±0.0	7.0±1.0	9.3±0.6	8.0±0.0
Turmeric/garlic/ginger/lemon/onion	6· 0±0.0	6.7±0.6	9.0±0.0	8.0±0.0
Ampicillin	6. 0±0.0	11.7±0.3	6. 0±0.0	18.7±0.6
Ciprofloxacin	41.7±0.6	30.7±0.6	17.3±2.1	34.7±0.6
Chloramphenicol	20.0±0.0	35.0±0.0	34.0±0.0	30.7±0.6

Note: [a] includes the diameter (6mm) of the disk used

Table 6. The mean (±SD) inhibition zones exhibited by individual methanol extracts and blends against *S. aureus*

Sample/antibiotic	Mean inhibition zone (mm) of *S. aureus*(±SD)
Lemon	10.0±0.0[a]
Turmeric/lemon/garlic	12.0±1.0[b]
Turmeric/ginger/lemon	10.0±0.0[a]
Turmeric/garlic/ginger/lemon	9.3±0.6[a]
Turmeric/garlic/ginger/lemon/onion	9.0±0.0[a]

Note: Mean (±SD) followed by the same small letters within the same column are not significantly different at α = 0.05 (Student-Newman-Keuls test)

Table 7. Antibacterial activity exhibited by various essential oil blends against *P. aeruginosa E. coli*, *S. aureus* and *S. typhi*

Sample	Inhibition zone in mm [a]			
	P. aeruginosa	***E. coli***	***S. aureus***	***S. typhi***
Lemon	6.3±0.6	6 .0±0.0	6.3±0.6	6.3±0.6
Garlic	6.3±0.6	6 .0±0.0	7 .0±0.0	6 .7±0.6
Turmeric	6 .0±0.0	6 .0±0.0	6 .0±0.0	6 .0±0.0
Ginger	6 .0±0.0	6 .0±0.0	7 .0±0.0	6 .0±0.0
Lemon/garlic	10.0±0.0	6.3 ±2.1	6.7±0.6	6.3±0.6
Lemon/ginger	7.7±0.6	9.0±1.0	7.0±1.0	9.7±0.6
Lemon/garlic/turmeric	7.0±1.0	6 .0±0.0	9.3±0.6	6 .0±0.0
Ampicillin	6. 0±0.0	11.7±0.3	6. 0±0.0	18.7±0.6
Ciprofloxacin	41.7±0.6	30.7±0.6	17.3±2.1	34.7±0.6
Chloramphenicol	20.0±0.0	35.0±0.0	34.0±0.0	30.7±0.6

Note: [a] includes the diameter (6 mm) of the disk used

Essential oils

Essential oils of garlic, lemon, turmeric, and ginger were obtained through steam distillation using Clevenger-type apparatus. Onion did not yield sufficient oil with steam distillation using Clevenger-type apparatus. Bioassay of all the essential oils gave an inhibition zone of less than 9.0 mm and thus inactive against the test gram positive and gram harmful bacteria (Kariba et al. 2001). The inactivity of garlic may be attributed to the relative instability of the organosulphur compounds which might have been destroyed during hydrodistillation and drying (Ewa et al. 2002). Steam-distilled garlic does not contain significant amounts of alliin or allicin, but instead contains various products of allicin transformation; none appears to have as much physiological activity as fresh garlic (Mohammad et al. 2009: Salem et al. 2010).

Bioassay results obtained from blends of essential oils are summarized in Table 7. The lemon/garlic blend gave an inhibition zone of 10.0 mm with *P. aeruginosa*. Lemon/ginger essential oil blend was active against *E. coli* and *S. typhi* with an inhibition zone of 9.0 mm and 9.7 mm, respectively. Lemon/garlic/turmeric blend had an inhibition zone of 9.3 mm against *S. aureus.*

The results indicate that lemon/garlic essential oil blend showed an increase in the antibacterial activity against *P. aeruginosa* (10.0±0.0 mm) as compared to their essential oils. The increase may be due to synergistic interaction of essential oil constituents of lemon and garlic (Esimone et al. 2006). Lemon/ginger also showed an increase in the antibacterial activity against *E. coli* (9.0±1.0)and *S. typhi*(9.7±0.6). These results are consistent with the previous study which showed that some blends of plant essential oils could have higher *in vitro* activity against bacteria (Junior et al. 2005; Betoni et al. 2006; Horiuchi et al. 2007). Interestingly, although neither lemon nor garlic essential oil exhibited activity; the blend of the two was active, suggesting that the volatile constituents of lemon interact synergistically with the transformed products of garlic (Ewa et al. 2002).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The fresh juice of garlic inhibited the growth of *S. aureus*, *E. coli*, *S. typhi* and *P. aeruginosa* at a concentration of 375 mg/mL, 187.5 mg/mL, 93.8 mg/mL and 46.9 mg/mL respectively (Table 8).

Methanol extract of lemon and lemon/ginger inhibited the growth of *P. aeruginosa* at a concentration of 2.9 mg/mL and 5.9 mg/mL respectively. Lemon, turmeric/ lemon, turmeric/lemon/ginger, turmeric/lemon/ garlic, turmeric/ginger/garlic, turmeric/lemon/ginger/garlic, and turmeric/lemon/ginger/garlic/onion methanol extracts exhibited an MIC of 187.5 mg/mL, 23.4 mg/mL, 46.9 mg/mL, 93.8 mg/mL, 187.5 mg/mL, 187.5 mg/mL and 23.4 mg/mL against *S. aureus* respectively. All the methanol extracts had no activity against S. typhi. Essential oils of lemon/garlic inhibited growth of *P. aeruginosa* at a concentration of 187.5 mg/mL, and lemon/ginger inhibited growth of *E. coli* and *S. typhi* at 750 mg/mL and 187.5

mg/mL respectively. Lemon/garlic/turmeric essential oil had an MIC of 375.0 mg/mL against *S. aureus*.

The plates showing no growth on nutrient agar were sub-cultured and incubated for 24 hours at 37 °C. The MBC results obtained are displayed in Table 4.8. Garlic juice, methanol extracts, and essential oils were bactericidal on all the bacteria tested at concentrations similar to their MIC's except essential oil blend of lemon/ginger which was bacteriostatic against *E. coli* bacteria at 750 mg/mL.

Methanol extracts prevented the growth of bacteria at lower concentrations (2.9 mg/mL, 23.4 mg/mL, and 5.9 mg/mL) as compared to juices and essential oils. The bactericidal properties of the essential oils might have been evaporated, destroyed or transformed to other forms during hydrodistillation and drying while methanol extracted most of the components from the samples (Ewa et al. 2002). Interestingly, ginger and for relief during abdominal discomforts (Jayaprakasha et al. 2002; Apariman et al. 2006), but did not show unusual bactericidal activity on the tested microorganisms.

Time-course antibacterial efficacy

Juices which had recorded activity against any one or more bacteria of ≥9.0 mm were tested for effectiveness within five days and their results summarized in Table 9.

Garlic showed inhibitory activity against all the strains used for the five days (Figure 3). Turmeric and individual ginger juices recorded an inhibition zone of <9 and thus did not show activity against any of the tested bacteria.

Table 8. MIC and MBC results for active samples

		MIC's mg/mL				MBC's mg/mL			
	Sample	***P. aeruginosa***	***E. coli***	***S. aureus***	***S. typhi***	***P. aeruginosa***	***E. coli***	***S. aureus***	***S. typhi***
Fresh extracts	Garlic	46.9	187.5	375	93.8	46.9	187.5	375	93.8
Methanol	Lemon	2.9	ND	187.5	ND	2.9	ND	187.5	ND
extracts	turmeric/lemon	ND	ND	23.4	ND	ND	ND	23.4	ND
	lemon/ginger	5.9	ND	ND	ND	5.9	ND	ND	ND
	turmeric/lemon/ginger	ND	ND	46.9	ND	ND	ND	46.9	ND
	turmeric/lemon/garlic	ND	ND	93.8	ND	ND	ND	93.8	ND
	turmeric/ginger/garlic	ND	ND	187.5	ND	ND	ND	187.5	ND
	turmeric/lemon/ginger/garlic	ND	ND	187.5	ND	ND	ND	187.5	ND
	turmeric/lemon/ginger/garlic /onion	ND	ND	23.4	ND	ND	ND	23.4	ND
Essential oils	lemon/garlic	187.5	ND	ND	ND	187.5	ND	ND	ND
	lemon/ginger	ND	750	ND	187.5	ND	750(static)	ND	187.5
	lemon/garlic/turmeric	ND	ND	375	ND	ND	ND	375	ND

Note: ND: Test not done, static: bacteriostatic

Table 9. Antibacterial activity exhibited by juices and blends against *P. aeruginosa*, *E. coli*, *S. aureus* and *S. typhi* for a period of 5 days

Antibiotic/ Sample	**Bacteria**	**Mean (±SD) inhibition zone(mm) [a]**				
		Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Lemon	*S. aureus*	6.7±1.2^{b}	7.0±1.0 ^{b}	6.7±0.6 ^{b}	6.0±0.0 ^{b}	11.0±0.0 ^{a}
	S. typhi	11.3±0.6^{a}	7.0±1.0 ^{b}	6.7±1.2 ^{b}	6.3±0.6 ^{b}	6.3±0.6 ^{b}
Turmeric	*S. aureus*	6.0±0.0 ^{a}	6.0±0.0 ^{a}	6.0±0.0 ^{a}	6.0±0.0 ^{a}	6.0±0.0 ^{a}
Ginger	*S. typhi*	6.0±0.0 ^{a}	6.0±0.0 ^{a}	6.0±0.0 ^{a}	6.0±0.0 ^{a}	6.0±0.0 ^{a}
Garlic	*P. aeruginosa*	10.0±0.0^{b}	6.0±0.0^{c}	10.3±3.8 ^{b}	9.7±3.2 ^{b}	15.0±0.5 ^{a}
	E. coli	11.7±0.6 ^{ab}	19.7±3.8 ^{a}	17.0±3.6 ^{a}	10.3±3.8 ^{b}	6.0±0.0 ^{b}
	S. aureus	14.7±2.5^{b}	26.0±2.6 ^{a}	27.0±6.1 ^{a}	11.0±2.9 ^{b}	6.0±0.0 ^{b}
	S. typhi	17.7±2.5^{a}	13.7±1.2 ^{ab}	11.7±2.8 ^{b}	10.7±0.6 ^{b}	11.0±1.0 ^{b}
Lemon /garlic	*E. coli*	15.0±0.0^{a}	10.3±0.6^{c}	6.0±0.0^{d}	12.3±0.6 ^{b}	14.3±0.6 ^{a}
	S. typhi	12.3±0.6^{b}	11.3±0.6 ^{b}	7.0±1.0^{c}	11.7±2.1 ^{b}	18.7±1.2 ^{a}
Ginger/garlic	*E. coli*	12.3±0.6^{b}	9.3±0.6 ^{a}	7.0±1.0 ^{a}	7.3±1.5^{b}	7.7±1.5 ^{a}
	S. typhi	12.0±1.0^{a}	12.3±1.2 ^{a}	7.0±1.0^{b}	7.3±1.5 ^{b}	7.3±1.5 ^{b}
Turmeric/garlic	*S. typhi*	13.0±1.0^{a}	8.0±1.0 ^{b}	6.3±0.6^{b}	6.7±1.2 ^{b}	6.7±1.2 ^{b}
Lemon/garlic/ginger	*E. coli*	13.7±0.6 ^{a}	13.3±1.5 ^{a}	6.7±1.2^{b}	9.0±1.0 ^{b}	6.7±1.2 ^{b}
	*S. typh*i	10.3±0.6^{ab}	9.3±0.6 ^{b}	6.3±0.6^{c}	6.3±0.6^{c}	12.0±2.0 ^{a}
Turmeric/garlic/ginger	*E. coli*	12.3±1.5 ^{a}	7.7±1.5 ^{b}	6.3±0.6^{b}	7.0±1.0 ^{b}	7.0±1.7 ^{b}
	S. typhi	11.3±1.5 ^{a}	8.0±1.0 ^{b}	7.0±1.0^{b}	7.0±1.7 ^{b}	7.0±1.0 ^{b}
Lemon/garlic/turmeric	*E. coli*	14.7±0.6 ^{a}	13.3±1.5 ^{ab}	6.7±0.6^{c}	11.7±2.1 ^{b}	7.0±1.0^{c}
	S. typhi	11.7±1.2 ^{a}	10.3±0.6 ^{a}	6.7±1.2^{b}	7.3±1.5 ^{b}	6.7±1.2 ^{b}
Lemon/garlic/turmeric/ Ginger	*E. coli*	6.3±0.6 ^{b}	12.0±1.0 ^{a}	7.5±1.4^{b}	6.7±1.2 ^{b}	7.3±1.5^{b}
	S. typhi	10.0±1.0 ^{a}	6.7±0.6 ^{a}	7.7±1.5 ^{a}	7.0±1.7 ^{a}	7.3±1.5 ^{a}

Note: [a] includes the diameter (6mm) of the disk used

Lemon and garlic individual juices showed decreasing activities against *S. typh*i from day 1 to day 5. The individual juice of lemon and turmeric/garlic blend did not show any changes in activity against *E. coli*. Ginger/garlic, lemon/garlic/ginger, and turmeric/garlic/ginger blends show decreasing activities against *E. coli* with time. The activity of lemon/garlic against *E. coli* dropped from day 1 to 3 then increased again up to day 5 (Figure 4.4). The activity of lemon/garlic/turmeric against E. coli dropped from day 1 to 3 then raised back up to day 4 (Figure 4). Individual lemon juice and the other blended test materials did not record any activity against *P. aeruginosa* for the 5 days.

Lemon/garlic blend showed an interesting pattern against *S. typhi*: the activity dropped from 12.3±0.6 to 7.0±1.0 by day 3 but increased to 18.7±1.2 on day 5 (Figure 5). A similar pattern of activity was shown by lemon/garlic/ginger blend against the same bacterium (Figure 5). This pattern suggests that the intermediate products formed are inactive, but that their further transformation leads to products that are inhibitory to *S. typhi* (Farbman et al. 1983). Monitoring (by GC-MS or LC-MS) of the specific changes of the constituents that take place can shed light on these exciting findings.

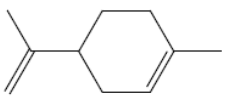
GC-MS analyses

Fresh juices

Juices of lemon, local garlic, ginger, and turmeric were analyzed by GC-MS, and each sample gave a chromatogram having several peaks. The suspected antibacterial compounds with their molecular formula and weight are listed in Table 10.

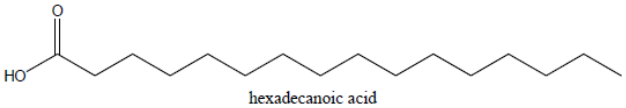
Garlic originating from China was also analyzed by GC-MS for comparison with garlic used (local garlic) in the bioassays. The candidate antibacterial compounds are listed with their molecular formula, percentage abundance and weight in Table 10.

The GC-MS analyses showed that lemon juice contained limonene (**14**) (85.08%), an antibacterial agent (Hiroyuk et al. 2006); 3-hexen-1-ol (0.16%); mentha-2,8-dien-1-ol (0.18%); hexadecanoic acid (**15**) (0.46%); 9,12-octadecadienoic acid (0.14%); 2-ethoxycarbonyl-3-methyl-7-nitro-4-azafluorenone,phenylimine (0.72%); phthalic acid, cyclohexylmethyl-3-phenylpropylester (0.40%) and α-terpineol (0.14), which may have confered bacterial inhibition property to this terpene (Angeh 2006; Fisher and Phillips 2006).



limonene

14



hexadecanoic acid

15

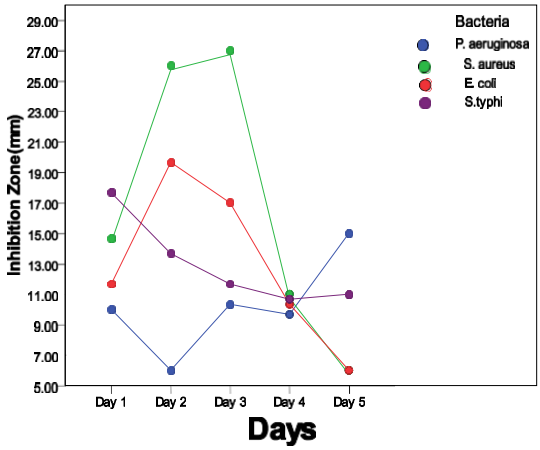


Figure 3. Comparison of mean (±SE) inhibition zones of garlic juice against *S. typhi*, *E. coli*, *P. aeruginosa* and *S. aureus*

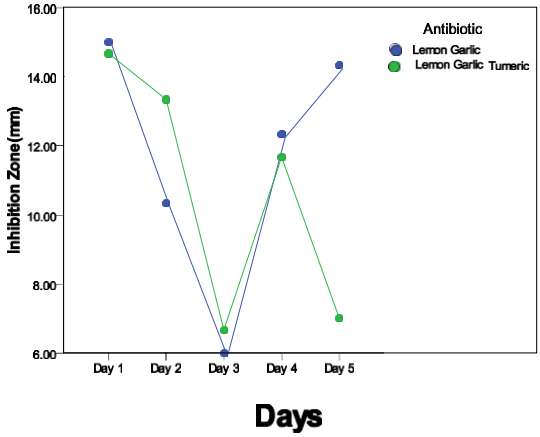


Figure 4. Comparison of mean (±SE) inhibition zones of two juice blends against *E. coli*

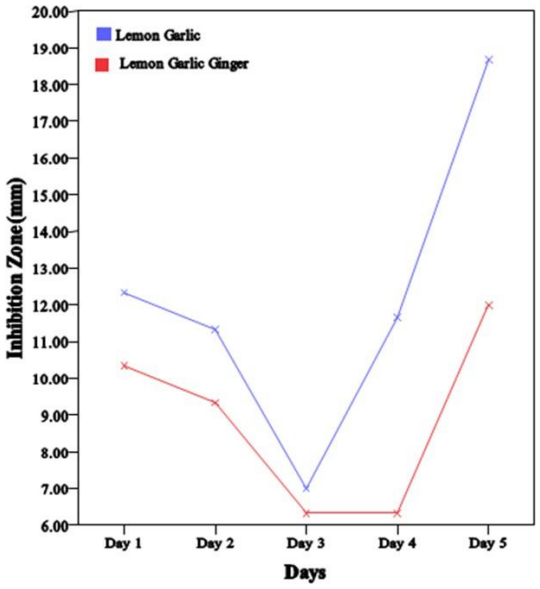
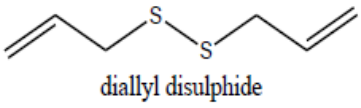


Figure 5. Comparison of mean (±SE) inhibition zones of two juice blends against *S. typhi*

Table 10. The GC-MS profile of compounds suspected to contain antibacterial properties identified in lemon, China garlic, local garlic, ginger and turmeric juices

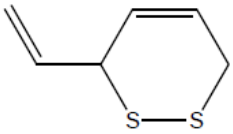
No	Compound	Molecular formula	M+ (g/mol)	Retention time (min)	Relative %				
					Lemon	China garlic	Local garlic	Ginger	Turmeric
1	α-Terpineol	$C_{10}H_{18}O$	154	14.562	0.41	-	-	0.61	-
2	Limonene	$C_{10}H_{16}$	136	11.906	85.08	-	-	-	-
3	3-Hexen-1-ol	$C_6H_{12}O$	100	8.154	0.16	-	-	-	-
4	Mentha-2,8-dien-1-ol	$C_{10}H_{16}O$	152	13.428	0.18	-	-	-	-
5	Hexadecanoic acid	$C_{16}H_{32}O_2$	256	23.749	0.46	-	-	-	-
6	9,12-Octadecadienoic acid	$C_{18}H_{32}O_2$	280	25.434	0.14	-	-	-	-
7	2-Ethoxycarbonyl-3-methyl-7-nitro-4-azafluorenone,phenylimine	$C_{22}H_{17}N_3O_4$	387	40.316	0.72	-	-	-	-
8	Pyrrolo[2,3-b] indole	$C_{14}H_{16}N_2O_4$	218	20.502	-	-	-	-	0.73
9	Methanehydrazonic acid, *N*-[3-(methylthio)-1,-2,4-thiadiazol-5-yl]-,ethylester	$C_6H_9N_4OS_2$	218	21.331	-	-	-	-	8.87
10	Selenourea, phenyl-	$C_7H_8N_2Se$	200	21.531	-	-	-	-	0.17
11	Imidazole, 4-methyl-5-[3,3,3-trifluoropropionylpropyl]-	$C_{10}H_{13}F_3N_2O$	234	22.248	-	-	-	-	0.47
12	1,6,10-Dodecatriene-3 ol,3,7,11-trimethyl-	$C_{15}H_{26}O$	222	22.358	-	-	-	-	0.48
13	2-Butenoic acid, 3-methyl-, methylester	$C_6H_{10}O_2$	114	22.43	-	-	-	-	0.60
14	2-Azabicyclo[3.2.1]octan-3-one	$C_7H_{11}NO$	125	22.58	-		-	-	0.12
15	2,4-Quinolnediol	$C_9H_7NO_2$	161	22.724	-	-	-	-	0.44
16	3-[4-Hydroxybenzoylhydrazono]-*N*-Mesitylbutyramide	$C_{20}H_{23}N_3O_3$	353	22.974	-	-	-	-	0.29
17	Phthalic acid, cyclohexylmethyl-3-phenylpropylester	$C_{24}H_{28}O$	380	23.14	0.40	-	-	-	0.53
18	Linalool	$C_{10}H_{18}O$	154	13.066	-	-	-	0.50	0.05
19	Terpinen-4-ol	$C_{10}H_{18}O_4$	154	14.563	-	-	-	-	0.05
20	Bicyclo[3.2.2]non-8-en-6-ol, (1R,5-cis,6-cis)-	$C_9H_{14}O$	138	16.105	-	-	-	-	0.03
21	Guaiacol<para-vinyl->	$C_9H_{10}O_2$	150	16.377	-	-	-	-	0.07
22	*N*-(2-Phenylethenyl)acetamide	$C_{10}H_{11}NO$	161	17.266	-	-	-	-	0.03
23	Ethanone,1-cyclopropyl-2-[3-pyridinyl]-	$C_{10}H_{11}NO$	161	19.5	-	-	-	-	0.73
24	1,5-Dimethyl-2-pyrrolecarbonitrile	$C_7H_8N_2$	120	20.104	-	-	-	-	0.61
25	6-Octen-1-yn-3-ol, 3,7-dimethyl-	$C_{10}H_{16}O$	152	20.207	-	-	-	-	1.11
26	Ethyl homovanillate	$C_{11}H_{14}O_4$	210	23.353	-	-	-	-	0.47
27	Ezlopitant , dehydro-	$C_{32}H_{24}N_2O$	452	32.758	-	-	-	-	0.14
28	Phenol, 4-pentyl-	$C_{11}H_{16}O$	164	33.341	-	-	-	-	0.76
29	[1,3,5]Triazine-2,4-diamine,6-	$C_9H_{13}N_7$	219	34.608	-	-	-	-	0.21
30	*O*-methoxy-α,-methylbenzyl alcohol	$C_9H_{12}O_2$	152	36.307	-	-	-	-	0.22
31	Methyl-4-deoxy-2-0-methyl.beta.1-threo-hex-4-enopyrid urinate	$C_8H_{12}O_4$	120	20.104	-	-	-	-	0.61
32	Benzenethiol	C_6H_6S	152	20.207	-	-	-	-	1.11
33	3,4-Dimethylthiophene	C_6H_8S	210	23.353	-	-	-	-	0.47
34	Ethylthiazole	C_5H_7NS	452	32.758	-	-	-	-	0.14
35	Thiophene, 3-methyl	C_5H_6S	164	33.341	-	-	-	-	0.76
36	Disulphide, methyl-2-propenyl	$C_4H_8S_2$	219	34.608	-	-	-	-	0.21
37	1-propene-3, 3-thiobis	$C_6H_{10}S$	152	36.307	-	-	-	-	0.22
38	Thiourea,*N*-*N*''-dimethyl	$C_3H_8N_2S$	204	5.878	-	3.77	-	-	-
39	Diallyl disulphide	$C_6H_{10}S_2$	110	8.952	-	1.18	-	-	-
40	3-Chlorothiophene	C_4H_3ClS	112	9.268	-	1.21	-	-	-
41	3-Vinyl-1,2-dithiacyclohex-4-ene	$C_6H_9S_2$	113	13.64	-	0.29	-		-
42	3-Vinyl-1,2-dithiacyclohex-5-ene	$C_6H_9S_2$	98	16.804	-	1.18	-	-	-
43	Cyclohexen-1-ol, 3-methyl	$C_7H_{12}O$	120	5.878	-	3.38	-	-	-
44	Ethyl trifluoromethyl trisulphide	$C_3H_5F_3S_3$	114	8.170	-	1.33	2.90	-	-
45	1,3-Dioxolane-2-[dichloromethyl]-	$C_4H_6Cl_2O_2$	104	9.869	-	0.48	0.84	-	-
46	Acetic acid, chloro-2-butoxyethyl ester	$C_6H_{15}ClO_3$	146	12.734	-	3.62	10.84	-	-
47	Acetamide,n-tetrahydrofurfuryl-2-methoxy	$C_8H_{15}NO_3$	173	14.250	-	1.44	1.35	-	-
48	Octadecanoic acid,3-hydroxy, methyl ester	$C_{19}H_{38}O_3$	314	20.178	-	-	0.66	-	-
49	1,2,3-Thiadiazole,5-methyl-	$C_3H_4N_2S$	100	14.727	-	2.59	-	-	-
50	1,4-benzenediol-2-chloro	$C_6H_5ClO_2$	144	16.656	-	1.61	-	-	-
51	Propanoic acid,2-chloro	$C_6H_5ClO_2$	108	17.163	-	1.86	-	-	-
52	3,4-Dimethylthiophene	C_6H_8S	112	9.268	-	1.46	-	-	-

53	Disulphide,methyl-2-propenyl	$C_4H_8S_2$	120	9.528		4.07	-	-	-
54	1,2-dithiolane	$C_3H_6S_2$	106	10.885	-	0.32	-	-	-
55	2-ethylthiacyclohexane	$C_7H_{14}S$	130	12.192	-	0.67	-	-	-
56	(methylthio)-acetonitrile	C_3H_5NS	87	13.738	-	0.88	-	-	-
57	3-Vinyl-1,3-dithiane	$C_6H_{10}S_2$	146	15.009	-	1.22	-	-	-
58	1,4-Diathiane	$C_4H_8S_2$	120	9.527	-	1.65-	3.176	-	-
59	Octadecanoic acid,3-hydroxy, methyl ester	$C_{19}H_{38}O_3$	314	20.179	-	1.24	-	-	-
60	N-Methoxy-N-methyl	$C_2H_6NF_2OP$	129	21.927	-	0.44	-	-	-
61	Amidinothiourea	$C_2H_6N_4S$	118	12.341	-	0.94	0.671	-	-
62	2-Heptanol	$C_7H_{16}O$	58	9.222	-	-	-	0.24	-
63	Borneol	$C_{10}H_{18}O$	154	14.49	-	-	-	0.81	-
64	Citronellol	$C_{10}H_{20}O$	156	15.067	-	-	-	0.50	-
65	Geraniol	$C_{10}H_{18}O$	154	15.458	-	-	-	1.05	-
66	Geranic acid	$C_{10}H_{16}O_2$	168	16.825	-	-	-	0.15	-
67	Elemol	$C_{10}H_{16}O_2$	222	19.447	-	-	-	0.73	-
68	E-Nerolidol	$C_6H_{26}O$	222	19.537	-	-	-	0.43	-
69	2-Butanone,4-(-hydroxy-3-methoxyphenyl	$C_{11}H_{14}O_3$	194	20.628	-	-	-	14.14	-
70	Ketone,1-cyclohexen-1-yl methyl,semicarbazone	$C_9H_{15}N_3O$	181	28.736	-	-	-	0.51	-
71	α-Zingiberene	$C_{15}H_{24}$	204	18.769	-	-	-	25.08	-



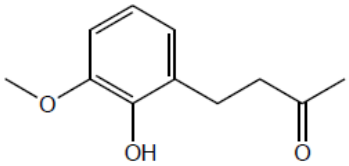
diallyl disulphide

16



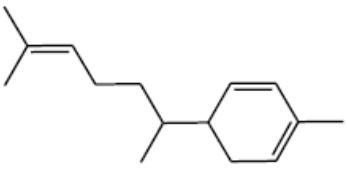
3-Vinyl-1,2-dithiacyclohex-4-ene

17



2-butanone,4-(-hydroxy-3-methoxyphenyl)-

18



19 (α-zingiberene)

GC-MS analysis of local garlic juice showed the presence of: diallyl disulphide (**16**) (10.84%); 3-chlorothiophene (6.49%); 3-vinyl-1,2-dithiacyclohex-4-ene (21.4%); 3-vinyl-1,2-dithiacyclohex-5-ene (**17**) (3.09%); acetic acid, chloro-2-butoxyethyl ester(2.73%); ethyl trifluoromethyl trisulphide (1.67%); acetamide, *n*-tetrahydrofurfuryl-2-methoxy (1.35%); 1-propene, 3,3‟thiobis (2.90%); 1,4-diathiane (3.18%); thiourea, N,N-dimethyl-(0,84%); octadecanoic acid,3-hydroxy, methyl ester (0.66%); Cyclohexen-1-ol, 3-methyl (0.62%); 1,3-Dioxolane-2-[dichloromethyl]-(0.36%) and amidinothiourea (0.67). All these compounds except acetamide n-tetrahydrofurfuryl-2-methoxy are sulphur-containing compounds, which might be responsible for antibacterial activity of garlic juice (Kathi 2000; O'Gara et al. 2000). China garlic gives additional sulphur compounds compared with local garlic. This may reflect some genetic or chemotypic differences between the two.

Ginger juice reaveled the presence of α-terpineol (0.61%); 2-heptanol (0.24%); linalool (0.50%); borneol (0.81%); citronellol (0.50%); geraniol (0.05%); geranic acid (0.15%); elemol (0.73%); *E*-nerolidol (0.43%); 2-butanone,4-(-hydroxy-3-methoxyphenyl)-(**18**) (14.14%); ketone,1-cyclohexen-1-ylmethyl,semicarbazone (0.51%) and α-zingiberene (**19**) (25.08%). These compounds are mainly terpenoids, some of which have shown strong inhibitory activity against pathogenic bacteria (Malu et al. 2009).

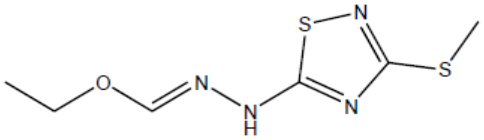
Turmeric had a wide range of suspected antibacterial components including; pyrrolo [2,3-b] indole (0.73%); Methanehydrazonic acid, *N*-[3-(methylthio)-1,-2,4-thiadiazol-5-yl]-,ethylester (**20**) (8.87%); Selenourea, phenyl-(0.17%); Imidazole, 4-methyl-5-[3,3,3-trifluoropropionyl-propyl]-(0.47%); 1,6,10-Dodecatriene-3 ol,3,7,11-trimethyl-(0.48%); 2-Butenoic acid, 3-methyl-, methylester (0.60%); 2-Azabicyclo[3.2.1]octan-3-one (0.12%); 2,4-Quinolnediol (0.44%); 3-[4-Hydroxybenzoylhydrazono]-*N*-mesityl-butyramide (0.29%); Phthalic acid, cyclohexylmethyl-3-phenylpropylester (0.53%); Linalool (0.05%); Terpinen-4-ol (0.05%); Bicyclo[3.2.2]non-8-en-6-ol, (1R,5-cis,6-cis)-(0.03%); Guaiacol<para-vinyl->(0.07%); *N*-(2-Phenylethenyl) acetamide (0.03%); Ethanone,1-cyclopropyl-2-[3-pyridinyl]-(0.73%); 1,5-Dimethyl-2-pyrrolecarbonitrile(0.61%); 6-

Octen-1-yn-3-ol, 3,7-dimethyl-(**21**) (1.11%); Ethyl homovanillate (0.47%); Ezlopitant , dehydro-(0.14%); Phenol, 4-pentyl-(0.76%); [1,3,5]Triazine-2,4-diamine,6-(0.21%) and *O*-methoxy-α,-methylbenzyl alcohol (0.22%) but they exhibited low or no anti-bacterial activity. This may be attributed to their low concentrations (Gopalan et al. 2000; Ghulam et al. 2009).

Methanol extracts

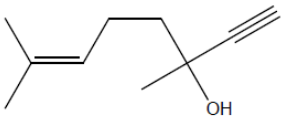
The suspected antibacterial compounds identified from active methanol extracts by GC-MS are listed in Table 11 with their relative percentage abundance, molecular formula, and weight.

The candidate antibacterial constituents obtained from methanol extracts include cyclohexanol,2-methylene-5-(1-methylene-5-[1-methylethenyl]-(4.41%); trans-carveol (1.49%); n-hexadecanoic acid (8.01%%); heptadecanoic acid (2.37%); γ-sitosterol (8.00%); borneol (0.59%); citronellol (0.64%); 2-butanone,4-[4-hydroxy-3-methoxy-phenyl]-**(22)** (5.15%); linoleic acid **(23)** (5.86%); ethyl hexadecanoate (1.28%); 2-[3-hydroxy-2-nitrocyclohexyl]-1-phenylethanone (2.53%); propanamide,2-amino-3-pheny (6.71%); 5,6,7,8-tetrahydroindolizine (1.03%); E-nerolidol (0.59%); 1,5-dimethyl-2-pyrrolecarbonitrile (1.10%); β-cadren-9-alpha-ol (0.69%); α-zingiberene (33.75%) and phenol,4-ethyl-2-methoxy-(0.94%). Methanol extracts contain aromatic hydrocarbons, ketones, phenols, organic acids and terpenes which have good inhibitory effect against gram positive and gram negative bacteria. Their varied occurrences in various blends may indicate that, their therapeutic effect(s) are not the direct effect of a single group or compound, but rather that the compounds possibly act in combination to bring about antibacterial effect (Abba et al. 2009).



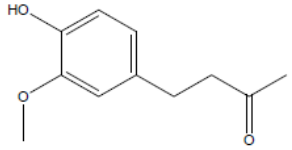
Methanehydrazonic acid, N-[3-(methylthio)-1,-2,4-thiadiazol-5-yl]-,ethylester

20



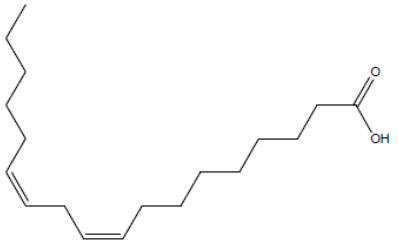
6-Octen-1-yn-3-ol, 3,7-dimethyl-

21



2-Butanone,4-[4-hydroxy-3-methoxyphenyl]-

22



Linoleic acid

23

Table 11. The GC-MS profile of compounds suspected to contain antibacterial properties identified in methanol crude extract and blends

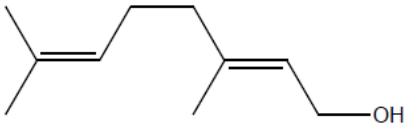
No.	Compound	Molecular formula	M^+ (g/mol)	Retention time (min)	Relative %					
					Lemon	LG	GiLT	LGT	GGiT	LGTGi
1	Cyclohexanol,2-methylene-5-(1-methylene-5-[1-methylethenyl]-	$C_{10}H_{16}O$	152	14.517	4.41	-	-	-	-	-
2	Carveol	$C_{10}H_{16}O$	152	14.987	1.49	-	-	-	-	-
3	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	23.767	8.01	0.81		0.41	0.37	0.69
4	Linoleic acid	$C_{18}H_{32}O_2$	280	25.460	5.86	-	-	-	-	-
5	Heptadecanoic acid	$C_{17}H_{34}O_2$	312	25.933	2.37	-	-	-	-	-
6	γ-Sitosterol	$C_{29}H_{50}OH$	414	39.136	8.00	-	-	-	-	-
7	Borneol	$C_{10}H_{18}O$	154	14.197	-	0.59	-	-	-	-
8	Citronellol	$C_{10}H_{20}O$	156	15.076	-	0.64	-	-	-	-
9	2-Butanone,4-[4-hydroxy-3-methoxyphenyl]-	$C_{11}H_{14}O_3$	194	20.585	-	5.15	4.27	0.63	2.54	5.50
10	Ethyl hexadecanoate	$C_{18}H_{36}O_2$	284	24.082	-	1.28	-	-	-	-
11	2-[3-Hydroxy-2-nitrocyclohexyl]-1-phenylethanone	$C_{14}H_{17}NO_4$	263	20.856	-	-	2.53	-	-	-
12	Propanamide,2-amino-3-phenyl	$C_9H_{12}N_2O$	164	21.216	-	-	6.71	-	-	-
13	5,6,7,8-Tetrahydroindolizine	$C_8H_{11}N$	121	21.089	-	-	-	1.03	-	-
14	E-Nerolidol	$C_6H_{26}O$	222	19.540	-	-	-	-	-	0.59
15	1,5-Dimethyl-2-pyrrolecarbonitrile	$C_7H_8N_2$	120	20.722	-	-	-	-	-	1.10
16	Beta-cadren-9-alpha-ol	$C_{15}H_{24}O$	220	22.407	-	-	-	-	-	0.69
17	Phenol, 4-ethyl-2-methoxy-	$C_9H_{12}O_2$	152	26.900	-	-	-	-	-	0.94
18	α-Zingiberene	$C_{15}H_{24}$	204	18.769		33.75				

Note: **LG:** Lemon/ginger, **GiLT:** Ginger/lemon/turmeric, **LGT:** Lemon/garlic/turmeric, **GGT:** Garlic/ginger/turmeric, **LGTG:** Lemon/garlic/ginger/turmeric

Table 12. The GC-MS constituents identified from three essential oil blends with antibacterial properties against *S. typhi*, *P. aeruginosa*, *E. coli* and *S. aureus*

No.	Compound	Molecular Formula	M^+ (g/mol)	Retention time (min)	Relative %		
					GL	LGi	GLT
1.	Diallyl disulphide	$C_6H_{10}S_2$	146	12.771	1.87	-	0.66
2.	Limonene	$C_{10}H_{16}$	136	11.921	84.27	49.78	36.16
3.	Linalool	$C_{10}H_{18}O$	154	14.612	0.91	1.13	-
4.	Terpinen-4-ol	$C_{10}H_{18}O$	154	13.113	4.46		1.72
5.	α-Terpineol	$C_{10}H_{18}O$	154	14.415	1.74	1.65	0.82
6.	[4-Aminophenyl]2-methylpiperidin-1-y1) methanone	$C_{13}H_{18}N_2O$	436	21.275	-	-	8.56
7.	Borneol	$C_{10}H_{18}O$	154	14.411	-	2.95	-
8.	Geraniol	$C_{10}H_{18}O$	154	15.510	-	0.09	-
9.	Elemol	$C_{10}H_{16}O_2$	222	19.490	-	0.67	-

Note: GL-Lemon/garlic, LGi-Lemon/ginger, GLT-Lemon/garlic/turmeric



geraniol

24

Essential oils

Three essential oil blends (lemon/garlic, lemon/ginger, and lemon/garlic/turmeric) that were active against *S. typhi*, *P. aeruginosa*, *E. coli*, and *S. aureus* were analyzed by GC-MS. The compounds suspected to have antibacterial properties with their molecular formula, mass and their relative proportions in the essential oils are given in Table 12 concerning the sample of origin.

The compounds suspected to have antibacterial properties are fewer in the essential oils as compared to juices and methanol extracts. The compounds which were present include: diallyl disulfide; [4-Aminophenyl]2-methylpiperidin-1-yl) methanone; limonene; terpinen-4-ol; α-terpineol; borneol; geraniol (**24**) and elemol. Limonene and α-terpineol are present in all the analyzed essential oils. Lemon/garlic essential oil does not show any sulphur derived compound in the GC-MS analysis due to the fact that during cutting and heating of garlic to obtain the oil, the compounds might have escaped (Lawson 1991; Yongabi et al. 2009; (Ahmet et al. 2006; Hérent et al. 2007; Ahmed et al. 2009; Mohamed et al. 2010).

Citrus essential oils contain significant amounts of terpenes, oxygenated derivatives and aromatic hydrocarbons (Ahmet et al. 2006; Hérent et al. 2007; Ahmed et al. 2009; Mohamed et al. 2010). Among the components (limonene and linalool) limonene was more abundant than linalool. Limonene shows the lowest effect against microorganisms. (Hérent et al. 2007; Tao et al. 2009; Palakawong et al. 2010). The inhibitory effect against microorganisms resulted from linalool rather than limonene (Fisher and Phillips 2006). Results of the previous report showed that greater antimicrobial potential could be ascribed to the oxygenated terpenes, including phenols (Maruti et al. 2011).

Conclusion

The most potent sample among the juices was garlic juice which inhibited the growth of all bacteria tested. The mean inhibition zones of Lemon/garlic juice against *E. coli* and *S. Typhi* were significantly higher among the juice blends. Among methanol extract samples, lemon had the highest activity against *P. aeruginosa* and *E. coli*. The results of antibacterial testing revealed that the juices of garlic and lemon had higher inhibitory effects as compared to methanol extracts and essential oils. The results of this study support the traditional usage of the studied vegetables and lemon and suggest that some of the extracts possess compounds suspected to have antimicrobial properties that can be used as agents in new drugs for therapy of infectious diseases caused by pathogens.

GC-MS analyses revealed that the compounds which were present in all the samples contain one or more of the following functional groups:-COOH,-OH,-N,-Cl,-F,-NH_2 and-S groups which may be associated with bacterial inhibition and found in conventional antibiotics. Individual juices and methanol extracts contained more compounds that were suspected to have antibacterial properties as compared to the blends. For example, lemon and garlic individual juices had a total of 22 compounds that were suspected to have antibacterial properties, lemon methanol extract contained 6, while lemon/garlic blend contained only 6 compounds.

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