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Full Length Research

Identification of the Antimicrobial Compounds in Garlic Grown at Laikipia County.

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This study was undertaken to identify antimicrobial compounds in garlic grown in Laikipia County. Bioactive compounds and their structures were identified in garlic extract using GC-MS, (30 m × 0.25 mm × 0.2 µm film thicknesses) cm. The compounds identified were these compounds, with sulfur group, have previously been shown to have antimicrobial activity against pathogens. It was concluded that variety of garlic grown in Laikipia County, Kenya, has potential for use as a meat decontaminant and preservative after slaughter. Diallyl-disulphide (2.89%), 6-(Methylthio) hexa-1,5-dien-3-ol (2.79%), Trisulfide di-2-propenyl (5.11%), 2-Vinyl-[4H]-1,3-dithiane(23.43%), Tetrasulfide, Hexadecanoic acid,2,3-dihydroxy propyl ester (2.58%), Oleic acid (1.34%), 5-cyano-7-methyl-6(methylthio)benzo[c] carbazole (5.83%), 5-cyano-7-methyl-6(methylthio)benzo[c] carbazole (5.44%), Tetrasulfide, di-2-propenyl (10.17 %) and Abietic acid (5.75%). Eight of the 10 compounds identified contain a sulfur group, while three (3); Hexadecanoic acid, Oleic acid and Abietic acid did not. Concentration of vinyl-dithiane(23.43%) and tetrasulfide, (10.17 %) compounds from garlic extract were higher compared to others. Garlic from Laikipia County has organosulfur and non organosulfur compounds that are effective in control of meat pathogens.

Keywords: Garlic, *Allium sativum*, Garlic extract, Antibacterial compound, GC-MS

INTRODUCTION

The use of higher plants and their extracts to treat infections is an ancient practice in traditional medicine. Humans have used natural products of animals, plants and microbial sources for thousands of years either in the pure forms or crude extracts for this purpose (Parekh and Chanda, 2007). About 80% of people in developing countries use traditional medicines of plant origin. In the last few years, a number of studies have been conducted in different countries to prove such efficacy (Parekh and Chanda, 2007). Many plants have been used because of their antimicrobial traits, which are due to secondary metabolism of the plant, and these should be investigated to better understand their properties, safety and efficacy (Prusti *et al*., 2008).

Garlic (*Allium sativum*) is a spice in the family *Liliaceae.* It has been used as flavoring agent and in folk medicine (Rivlin, 2001). It has been used for centuries to fight infections (Onyeagba *et al*., 2006): the early Egyptians used it to treat diarrhea; the ancient Greeks used it for intestinal and extra-intestinal diseases; while the ancient Japanese and Chinese used it to treat headache, flu, sore throat and fever. In Africa, particularly in Nigeria, it is used against abdominal discomfort, diarrhea, otitis media common cold, cough and respiratory tract infections (Jaber and Al-Mossawi, 2007). Garlic has been reported to contain useful biological properties like antimicrobial, anticancer, antioxidant, immunomodulatory and anti-inflammatory effects (Corzo-Martine *et al*., 2007). It has been shown to exhibit inhibitory action against both Gram-positive and Gram-negative bacteria (Whitemore and Naidu, 2000). Allicin is a key component of Garlic responsible for antimicrobial activity and its characteristic odour (Jabar and Al- Mossawi, 2007). The antimicrobial activity of garlic diminishes at high temperature, because high temperature denatures its active compound allicin (Sato and Miyata, 2000). In recent years antibiotic resistance in pathogenic microorganisms has increased due to disorganized use of antimicrobial drugs which has created serious threat to human health (Parekh and Chanda 2007). Therefore, therapeutic use of plants can be a useful alternative strategy to combat microbial resistance (Mandal *et al*., 2010) and increase shelf life of food. Many modern drugs are derived from plant sources and their extracts dominate ayurvedic medicines (Murugesan *et al*., 2011).

Identification of particular bioactive compound of plants has become easier due to the development of modern analytical tools. Gas Chromatography-Mass Spectrometry is an instrument which separates components of a mixture and identifies them quantitatively using a detector, which also provides information concerning the structure of each of the components (John *et al*., 2005) Compounds can be identified not only by comparing the retention time to a standard, but also by its mass spectrum. An unknown compound can also be identified in most cases based solely on its mass spectrum, eliminating the need to run standards for retention time data. The column used in the GC/MS is a capillary column of 30 cm with an internal diameter of 0.25 mm. The stationary phase is bonded to the interior of the glass capillary, eliminating the need for packing a solid support in the column.

After the components of a mixture are separated in the column, they reach the ion trap detector as pure compounds. The compounds are ionized by electron impact (EI) by passing the stream of gas over a beam of electrons accelerated to energy of 70 eV. This energy is used to form ions by stripping away an electron. Differing populations of the ions have differing amounts of internal energy. Some of the molecules may become ionized but may not fragment, forming a “parent ion”. A parent ion, or molecular ion, has the same mass in atomic mass units as the neutral molecule (it differs by only the mass of an electron). It is the highest mass peak in the spectrum. Many of the ions formed may have sufficient internal energy to fragment, forming a smaller mass ion and a neutral. (The neutrals formed are not detected. Only ions are detected). By using the same energy in electrons to ionize the compounds, the resulting mass spectra are highly reproducible. Fragments from different classes of compounds have some characteristics that are used to help identify unknown compounds. For example, compounds with many strong bonds, such as aromatic compounds, may be less likely to fragment. These compounds are characterized by mass spectra which are dominated by a single peak, the molecular ion. Straight chain hydrocarbons, however, fragment much more easily, and may show little or no abundance of the molecular ion in their mass spectra. Characteristic fragmentations of various classes of compounds are identified using a reference manual (Marvin and Christopher, 1988).

Ethyl acetate is an organic solvent which extracts non-polar, medium and polar natural compounds from plant materials quite efficiently. It can help to extract many biological compounds for evaluation of their activities and has low cellular toxicity (Wilhelm *et al*., 2005). Ethyl acetate extracts are used in GC-MS analysis for identification of the compounds.

The aim of this study was to identify compounds found in Garlic (*Allium sativum*) extract that are responsible for its antimicrobial activity.

MATERIALS AND METHODS

Sample collection

Laikipia County is one of the major counties in Kenya, where garlic is commercialized by small scale farmers. The soils are volcanic soils found at the slopes of Aberdares Mountains. Garlic cultivar used was Italian, a selection of Creole from Italy (Singh, 1983). Garlic samples were collected from Nganoini farm Laikipia County in Kenya. Samples were selected from garlicharvested within the four month before sampling. Garlic cultivar used was Italian, a selection of Creole from Italy (Singh, 1983). Garlic was collected from Nganoini farm Laikipia County in Kenya. Garlic used was not kept more than four months after harvesting. Laikipia County is one of the major counties in Kenya, where garlic is commercialized by small scale farmers. The soils are volcanic soils found at the slopes of Aberdares Mountains. Research experiments were conducted in the Chemistry, Public Health, Pharmacology and Toxicology Departments, Nairobi University, Kenya.

Preparation of Garlic Cloves

Garlic bulbs were washed with distilled water in a clean basin to remove any soil adhering on the surface. This also softened the outer dry skin for easier peeling. After rinsing the bulbs with clean distilled water, the washed garlic was placed in another clean basin to dry in the demonstration laboratory. The garlic cloves were then peeled and placed on an aluminum foil for weighing (Njue *et al*., 2015).

Preparation of ethyl acetate extract

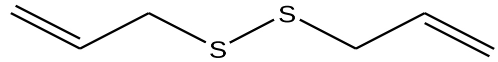
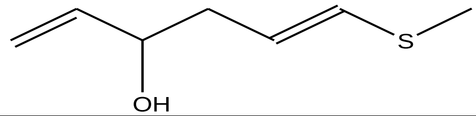
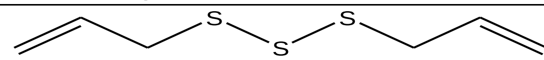
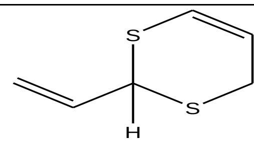
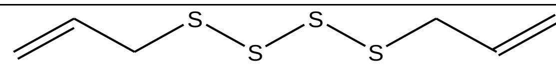
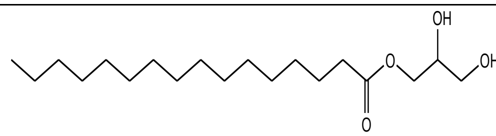
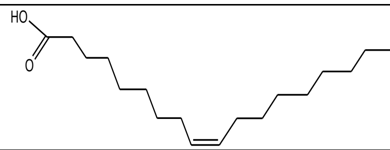
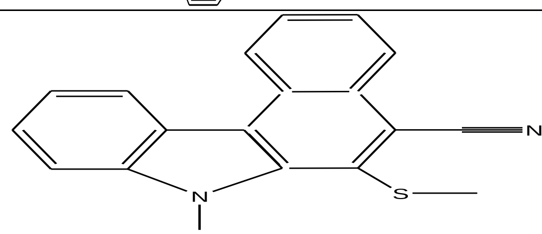
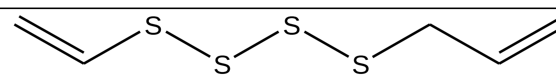
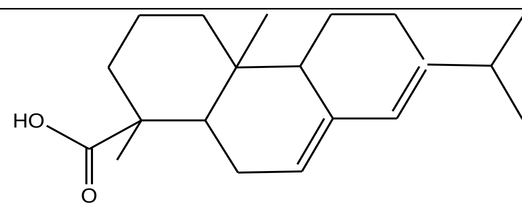
Garlic extract was prepared with 99 % ethyl acetate AR (Oxoid). One hundred grams (100g) of the peeled garlic cloves were weighed on a clean aluminium foil using a weighing balance (Mettler pm 4600, Deltarange, Zurich). They were then put in an electric blender (Ohms, Internationalfzc, China) and 125ml of organic solvent was added. The mixture was blended to make a paste. More garlic cloves and organic solvent were added in the same proportions of 100g cloves in 125ml solvent to the paste, to fill up to three-quarters' of a 1L flat bottomed flask. The procedure was done eighteen times to yield a total weight per volume of 1800 g of garlic in 2250 ml of ethyl acetate and kept in a dark cabinet for 24h. Shaking was done in the morning and in the evening to mix all the flask contents. The content was filtered using Whatman's paper No.1 to remove debris and the resulting filtrate was evaporated using rotary evaporator (Rotor Vapour Pump, Laboratoriums-Technic Ag, Buchi) at 50°C to remove the solvent. After evaporation of the resulting filtrate using rotary evaporator (Rotor Vapour Pump, Laboratoriums-Technic Ag, Buchi) at 50°C, the process yielded 710 g of the extract.

The prepared crude extract was put in a beaker for fractionation with solvents. It was dissolved in 4 litres of water and mixed thoroughly. A portion of 100 ml of the mixture was measured and mixed with 300 ml of ethyl acetate. Mixing was done in a 2 litres separation funnel by shaking to separate the two phases as ethyl acetate is immiscible with water. Compounds soluble in the upper ethyl acetate phase (ethyl acetate being lighter than water) were collected and the lower aqueous phase was extracted thrice with ethyl acetate. All fractions of ethyl acetate were combined and poured into round bottomed flask of rotary evaporator and ethyl acetate was isolated from the fraction leaving behind semisolid ethyl acetate fraction. The total concentrate was air dried for one week, stored in the glass vials, then freeze dried at -20°C to confirm total solvent removal (Padìas and Anne, 2011). The concentrate was weighed using a calibrated weighing balance and yielded 10.76 g of the ethyl acetate crude extract.

Identification of compounds in garlic extract

The compounds in the ethyl acetate extracts were identified using GC-MS analysis and their identity and concentrations are shown in Table 1 The GC-MS analysis was done using Agilent 6890 series GC system interfaced with Agilent 5973 mass selective detector. Chromatographic column used was Rex-5 column (30 m × 0.25 mm × 0.2 μm film thickness). Helium was used as carrier gas at a flow of 2.97 ml·min−1. For temperature programming, the column temperature was maintained at 40 ^{0}C for 2 min, programmed at 8 ^{0}C/min to 90 ^{0}C, then 4 ^{0}C /min to 120 ^{0}C and then at 10 ^{0} C/min to 200 ^{0}C, which was held for 5 min. The mass spectrometer was operated in electron impact (EI) ionization mode with electron energy of 70 eV and temperature 230 ^{0}C. The transfer line temperature was 200 ^{0}C. Scan range was 45 - 450 m/z.

Table1: Name, structure and percentage of compounds identified in ethyl acetate extract

S.NO	NAME	Structure	Percentage of Compounds in mixture w/w
1	Diallyldisulphide		2.89
2	6-(Methylthio) hexa-1,5-dien-3-ol		2.79
3	Trisulfide, di-2-propenyl		5.11
4	2-Vinyl-[4H]-1,3-dithiane		23.43
5	Tetrasulfide, di-2-propenyl		2.00
6	Hexadecanoic acid,2,3-dihydroxy propyl ester		2.58
7	Oleic acid		1.34
8	5-cyano-7-methyl-6(methylthio)benzo[c] carbazole		5.44
9	Tetrasulfide, di-2-propenyl		10.17
10	Abietic acid		5.75

RESULTS

Identified compounds in garlic extract

The names, structures and percentages of the active compounds identified in garlic ethyl acetate extract are given in Table 1 They were: Diallyl-disulphide (2.89%), 6-(Methylthio) hexa-1,5-dien-3-ol (2.79%), Trisulfide di-2-propenyl (5.11%), 2-Vinyl-[4H]-1,3-dithiane(23.43%), Tetrasulfide, Hexadecanoic acid,2,3-dihydroxy propyl ester (2.58%), Oleic acid (1.34%), 5-cyano-7-methyl-6(methylthio)benzo[c] carbazole (5.83%), 5-cyano-7-methyl-6(methylthio)benzo[c] carbazole (5.44%), Tetrasulfide, di-2-propenyl (10.17 %)

and Abietic acid (5.75%). Eight of the 10 compounds identified contain a sulfur group, while three (3) of them, that is; Hexadecanoic acid, Oleic acid and Abietic acid did not.

DISCUSSION

This study determined compounds in garlic ethyl acetate extract, which were responsible for its antimicrobial activity. Ethyl acetate extract was found to have the following compounds; Diallyl-disulphide (2.89%), 6-(Methylthio) hexa-1,5-dien-3-ol (2.79%), trisulfide di-2-propenyl (5.11%), 2-vinyl-[4H]-1,3-dithiane(23.43%), tetrasulfide, di-2-propenyl (2.00%), hexadecanoic acid, 2,3-dihydroxy propyl ester (2.58%), oleic acid (1.34%), 5-cyano-7-methyl-6(methylthio)benzo[c] carbazole (5.44%), tetrasulfide, di-2-propenyl (10.17 %) and abietic acid (5.75%) Table 1. Seven of the ten compounds identified had sulphur group and formed 90% of the total compounds. However, three of them, hexadecanoic acid, oleic acid and abietic acid did not have the sulfur group. This is in agreement with Sendl (1995), who concluded that organosulfur in *Allium species* constitute 75–90 %. The compounds in garlic ethyl acetate have well established biological activities and applications in medicine and elsewhere in technology.

All organosulfur compounds derived from garlic are isomers of allicin (Block, 2010). Allicin breaks down *in vitro* to form fat-soluble organosulfur compounds like diallyl trisulfides, diallyl sulfides and diallyl disulfides. It is very unstable even at room temperature. Studies by Bagiu *et al*., (2010) showed that after 20 h at 20 ºC it decomposed completely resulting in di-2-propenyl disulfide, di-2-propenyl trisulfide, di-2-propenyl sulfide, 6-(Methylthio) hexa-1,5-dien-3-ol, 5-cyano-7-methyl-6(methylthio)benzo[c] carbazole and sulfur dioxide and other volatile and non-volatile compounds. According to Itakura *et al*., (2001), GC-MS analysis of garlic produced the entire mentioned organosulfur compounds but not the acids, hexadecanoic, oleic and Abietic. Zhou, (2010) and Wang, *et al* (2012) reported that garlic produced higher amount of trisulfide (22.46 %) than tetrasulfide (1.79%) which contrasts with this study. According to Lim, (2014) the type of garlic compounds produced by GC-MS analysis depends on method of extraction, soils where grown and time of harvesting. In Kenya no research cited indicating analysis of garlic compounds from other parts of the country except determination of allicin.

The organosulfur and non organosulfur compounds are responsible for the antibacterial effects reported for garlic ethyl acetate extract in this study. This is in contrast to (Tsao *et al*., 2003), who suggested that it is only organosulfur compounds which make microorganisms not to be resistant. The compounds may have enhanced each other and as a result, microorganisms can never develop resistance because this would require modifying the very enzymes that make their activity possible (Tsao *et al*., 2003).

Along with diallyl trisulfide (allyl trisulfide; diallyl trisulfide; diallyl trisulphide) and diallyl tetrasulfide, it is one of the principal components of the distilled oil of garlic. When in human body, it decomposes into other compounds such as allyl methyl sulfide. All these compounds have sulfur group which is behind the antmicrobial activity of garlic (O'Gara *et al*., 2000).

Diallyl disulfide acts against the stomach ulcer germ *Helicobacter pylori*, however not as efficiently as allicin (Avato *et al*., 2000: O'Gara *et al*., 2000). Diallyl disulfides inhibit the growth of molds and bacteria in garlic oil. Because of its antimicrobial effects, diallyl disulfide is included in preparations which are used for selective decontamination of the organs (for example, gut) before surgical operations. Diallyl disulfide is an efficient agent for detoxication of the cells. It significantly increases the production of the enzyme glutathione S-transferase (GST), which binds electrophilic toxins in the cell.

Garlic organosulfur compounds control E*. coli*, *S. aureus*, *C. botulinum* S*. typhimurium* and molds and there is no need for refrigeration (Sloans, 2000). They are also ant carcinogen (FAO, 2002). They support the detoxification function of liver cells *in vitro* and protect nerve cells from oxidative stress, also *in vitro* (Fuko *et al*., 2004; Lemar *et al*., 2007). The detoxification effect may prevent symptoms of inflammation. This was confirmed by a study on rats where prolonged administration of diallyl disulfide protected poisoning of their intestinal cells (Fukao *et al*., 2004).

Oser, (1978) reported that 6-(Methylthio) hexa-1,5-dien-3-ol has an antimicrobial properties and can be used as an additive in meat while Campaigne *et al*., (1996) reported 5-cyano-7-methyl-6(methylthio) benzo[c] carbazole as colorless crystals with a melting point of 173-174ºC. They have antitumor properties

Vinyldithiins (2-Vinyl-(4H)-1,3- dithiin), 3-vinyl-4*H*-1,2-dithiin and 2-vinyl-4*H*-1,3-dithiin, are organosulfur phytochemicals products of degradation of allicin from crushed garlic using solvents less polar than 2-propanol (Sendl, 1995). In presence of oil or organic solvents Allicin breaks down to vinyldithiins (Block, 2010). They are only found in garlic oil that are made by incubation of crushed garlic in oil (Lawson, 1998). They have been reported to prevent cardiovascular disease and to have antioxidant characteristics (Keys, 1980).

Vinyldithiins in garlic have been shown to significantly lower the incidence of platelet aggregation (Rahman and Billington, 2000). Ingestion of foods containing vinyldithiins and other organosulfur compounds may decrease the risk of gastric and colon cancer (Fleischauer *et al*., 2000). They also have antibiotic properties against microorganisms (Hermes, 1990).

Abietic acid is a organic compound, yellow, crystalline, water –insoluble acid that occurs widely in trees like pine. It is the primary component of resin acid. It is the primary irritant in pine wood and resin. It is isolated from rosin and is the most abundant of several closely related organic acids that constitute most of rosin, the solid portion of the oleoresin of coniferous trees. The pure material is a colourless solid, but commercial samples are usually glassy or partly crystalline yellowish solid that melts at temperatures as low as 85 °C (185 °F) (Hoiberg *et al*., 2010). It belongs to the abietane diterpene group of organic compounds (compounds derived from four isoprene units).

Rosin has been used for centuries for caulking ships (Hoiberg *et al*., 2010). It is also rubbed on the bows of musical instruments to make them less slippery (Hoiberg *et al*., 2010). In modern times methods have been developed for improving the properties of the rosin acids, which are otherwise soft, tacky, and low-melting and subject to rapid deterioration by oxidation in air. Stability is greatly increased by heat treatment.

In vitro 50% ethanol extracts from *Resina pini* of Pinus sp. (Pinaceae) showed inhibitory activity against testosterone 5α-reductase prepared from rat prostate. The fraction responsible for this activity was purified, and the active constituent was isolated and identified as abietic acid which exhibited potent testosterone 5α-reductase inhibitory activity *in vitro* (Seong soo *et al*., 2010). Abietic acid is found in garlic yet no citation for its antimicrobial activity has been made.

Oleic acid is a fatty acid and a major source of n-6 polyunsaturated fatty acids (PUFAs) that come from vegetable oils (Chifu *et al*., 2011). The n-3 PUFAs have previously been identified to exhibit strong antibacterial activity against various oral bacteria (Kapoor and Huang, 2007). A range of n-6, n-7, and n-9 fatty acids of various alkyl carboxyl lengths were found to have antimicrobial activities against oral microorganisms. The data supported that some omega fatty acids and their ester derivatives effectively killed *S. mutans, A. actinomycetemcomitans, C. albicans, P. gingivalis*, *F. nucleatum*, and *S. gordonii*, although this effectiveness varied with the fatty acids. Some fatty acids have adjunctive antimicrobial agents and able to treat or prevent diseases caused by microorganisms. Oleic acid inhibits the bacterial enoyl-acyl carrier protein reductase (Fab1), an essential component of bacterial fatty acid synthesis which has served as a promising target for antimicrobial drugs (Chang *et al*., 2005). Oleic acid is an anti-inflammatory fatty acid playing a role in the activation of different pathways of immune competent cells (Carrillo *et al*., 2012). Oleic acid has not been reported to have antimicrobial activity in garlic before.

Hexadecanoic acid or Palmitic acid is the most common fatty acid (saturated) found in animals, plants and microorganisms (Gunstone *et al*., 2007). It is a major component of the oil from palm trees (palm oil), but can also be found in meats, cheeses, butter, and dairy products. Because it is inexpensive and adds texture to processed foods, hexadecanoic acid finds wide use including foodstuffs as an additive (US, 2015). Hexadecanoic acid, one of the carboxylic acids, has been reported to have antimicrobial activity against *Bacillus cereus*, *Staphylococcus aureus* and *Mucobacterium smegmatus (*Latifa *et al*., 2012; Arunkumar & Muthuselvam 2009; Yang *et al*., 2003). Extract of *Carduus pycnocephalus L.* growing in Saudi Arabia contains Hexadecanoic acid. However, its' antimicrobial activity in garlic is not known.

The antimicrobial compounds identified in garlic extract from Laikipia County were Diallyl-disulphide 6-(Methylthio) hexa-1, 5-dien-3-ol, Trisulfide di-2-propenyl. 2-Vinyl-[4H]-1, 3-dithiane, Tetrasulfide, di-2-propenyl, Hexadecanoic acid,2,3-dihydroxy propyl ester, Oleic acid, 5-cyano-7-methyl-6(methylthio)benzo[c] carbazole, 5-cyano-7-methyl-6(methylthio)benzo[c] carbazole, Tetrasulfide, di-2-propenyl and Abietic acid. Ninety percent (90 %) of compounds identified in garlic extract are sulfur containing compounds which have been associated with antimicrobial activity.

CONCLUSIONS

We therefore conclude that:

1. Garlic from Laikipia County has organosulfur and non organosulfur compounds known for their antimicrobial activity.
2. Concentration of vinyl-dithiane(23.43%) and tetrasulfide, (10.17 %) compounds from garlic extract were higher compared to others.

RECOMMENDATIONS

From the study, it is imperative to recommend the following”

1. Organosulfur and non organosulfur compounds can be effective in control of meat pathogens.
2. Further work is required to evaluate, isolate and purify pure compounds to test antimicrobial activity.

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