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In Vitro Antibacterial activity of Ethanolic - aqua extract of *Tagetes minuta* leaves harvested from The University of Eastern Africa, Baraton, Nandi County, Kenya

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Abstract

Tagetes minuta is a plant that has been used in traditional medicine for the treatment of various illnesses. In this study, the ethanolic extract of *T. minuta* was tested for its antibacterial activity against selected microorganisms of clinical significance. In an antibiotic susceptibility assay using the plant extract and DMSO control, the results from the study showed that the extract was active against *Proteus vulgaris* with zone of inhibition of 17.30±0.333mm, *Escherichia coli* 9.30±0.333mm, *Salmonella typhi* 11.83±0.44mm, *Bacillus cereus* 8.00±0.000 and *Enterobacter aerogenes* 16.67±0.882mm. The penicillin positive control showed high zones of inhibition while the DMSO negative control showed no zones of inhibition. An analysis of variance test on the results showed that there were significant differences in the zones of inhibition of the extract and penicillin against all the organisms ($p<0.0001$). These results have shown that the growth of these organisms can be controlled with the extract, hence making the extract components potential agents for incorporation into drug production.

Key-Words: *Tagetes minuta*, Asteraceae, Antibacterial activity, Ethanol, Extract, Leaves

Introduction

Medicinal plants are the "backbone" of traditional medicine; about 3.3 billion people in the less developed countries utilize medicinal plants on a regular basis (Davidson, 2000). There are nearly 2000 ethnic groups in the world, and almost every group has its own traditional medical knowledge and experiences (Liu, 2009 and Kebriaee, 2003). Medicinal plants are valuable source of natural active constituents that are used to maintain human health and also used for the treatment of many human diseases (Stary, 1998). Microbial infections are an important health problem throughout the world and medicinal plants are possible sources of antimicrobial agents (Bunchoo, 1995).

According to WHO, about 80% of population in some Asian and African countries still depend upon tradition herbal medicine for the prevention of many diseases (Abu, 2010). The use of medicinal plants as fundamental component of the African traditional health care system is perhaps the oldest and most assorted of all the therapeutic systems.

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In some part of rural Africa, traditional healers prescribing medicinal plants are the most easily accessible and affordable health resources available to the local community and at times, the only therapy that subsists (Fawzi, 2013). Many medicinal plants have been known to produce biologically active substances, some of which are related to special flavour or taste and others are found to be useful as antioxidants and or antimicrobial agents (Fiorentiao, 2006).

An impressive number of present medicines which is used today have a record of being used from the olden times. Plant based antimicrobials represent a enormous unexploited source of medicines even after their enormous therapeutic potential and effectiveness in the healing of infectious disease hence, further exploration of plant antimicrobials need to occur (Parekh et al., 2007).

Medicinal plants are the basis of many of the modern pharmaceuticals used today for various ailments, as it has a good source of natural antioxidants for medicinal use and related to radical mechanism (Kumplainen, 1999). The emergence of multiple drug resistance bacteria (MDR) has been a major cause of failure of treatment of infectious diseases (Mathias, 2000).

Tagetes minuta L. belongs to Asteracea family it is an annual species native of South American, although it has become widespread throughout the world. This species is especially well adapted to disturbed habitats. *T. minuta* oil found a good market in perfumery and flavour industry (Moghaddam, 2004). It is also known as southern cone marigold, stinking roger or black mint, is a tall upright marigold plant from the genus *Tagetes*, with small flowers, native to the southern half of South America. Since Spanish colonization, it has been introduced around the world, and has become naturalized in Europe, Asia, Australasia, North America and Africa, (http://en.wikipedia.org/wiki/Tagetes_minuta). It is a highly aromatic annual 1-2 m tall, leaves 7-15 cm long, pinnatisect, leaflets 11-19, 4 cm long linear or lanceolate, flower heads pale yellow in corymbose clusters and black achenes (Joy et al., 2002).

T. minuta has many medicinal benefits such as remedy for respiratory inflammations, anti-spasmodic, colds, stomach problem, anti-parasitic, sedative, and anti-septic and insecticide. It is used for coughs, chest infections, dilating the bronchi, facilitating the flow of mucus and dislodging congestion and skin infection. And also it has a healing effect on wounds, cuts, calluses and bunions (Nikkon, 2011; Kamaraj, 2011; Govindarajan, 2011; Aristatile, 2013; Maity, 2011 and Parastoo, 2014)

Infusions of *Tagetes* sp. leaves have been used in folk medicine to treat intestinal and stomach diseases and some of them have been found to possess biological activity (Tereschuk, 1997 and Broussalis, 1999). *T. minuta* oils are used as flavour components in food products and as perfumes, and have a suppressive biological activity against some insects and pathogens (Vasudevan, 1997).

According to Sehrish (2013), flowers of *T. minuta* are used for epileptic fits and fevers (Qureshi et al., 2007). Its flowers are also used as mild laxative, insect repellent, for gastritis, indigestion (Neher, 1968). Leaves are used for kidney trouble, piles, muscular pain and their juice is used for ophthalmia, earache (Qureshi et al., 2007), hemorrhoids and as a snuff (Neher, 1968). Leaves are also used locally to repel safari ants and mosquitoes and to kill mosquitoes larvae. Oil obtained from leaves is more toxic to mosquitos' larvae than DDT (Macedo et al., 1997).

The present study was carried out to evaluate the antibacterial activity of ethanolic extract of *Tagetes minuta* leaves against selected pathogenic organisms.

Material and Methods

Sample collection and Extraction procedure

The leaves of the *Tagetes minuta* were collected around Baraton University campus. The samples were identified by a taxonomist in the University of Eastern Africa, Baraton. The fresh leaves of the *Tagetes minuta* leaves were air – dried for three weeks; the dried leaves were ground into powder. Forty grams (40 g) of the powdered leaves were mixed with 400 ml of ethanol – water (70:30). The mixture was kept for 24 hours on a shaker for effective extraction of the plant components. The extract was filtered and the solvent was evaporated to dryness at a temperature of 40^{o}C using rotary vacuum evaporator. The extract was brought to dryness using vacuum and pressure pump. The yield was kept at 4^{o}C prior to use.

Bioassay study

Preparation of the Bacterial Suspension:

The turbidity of each of the bacterial suspension was prepared to match to a 0.5 McFarland standard. The McFarland standard was prepared by dissolving 0.5 g of $BaCl_2$ in 50 ml of water to obtain a 1% solution of Barium chloride (w/v). Sulphuric acid (1%) was prepared in a 100-ml volumetric flask. To prepare the 0.5 McFarland Standard, 0.5 ml of the 1% $BaCl_2$ solution was mixed with 99.5 ml of H_2SO_4 solution. Measure the turbidity of the 0.5 McFarland Standards with the aid of a spectrophotometer at a wavelength of 625 nm to read an optical density of between 0.08 to 1.0. At this absorbance, the McFarland standard represents a bacterial cell density of approximately 1.5 x 10^8 CFU/ml (1.0 x 10^8 – 2.0 x 10^8 CFU/ml). It was then transferred to a screw-capped bottle and sealed with parafilm to prevent evaporation due to exposure to air. The bacterial suspensions were then tested against the McFarland standards until they reached the absorbance of the McFarland standard and then they were ready for use.

Preparation of the Extract Concentrations and Antibiotic

Stock solutions for the extract were prepared by dissolving 500 mg in 1 ml of dimethylsulfoxide (DMSO). An antibiotic control was made by dissolving 1µg of penicillin in 1 ml of sterile distilled water. DMSO served as a negative control.

Screening for the antibacterial potential of the plant extract

The agar well diffusion procedure used in the experiment was similar to that used by Taye *et al.*., (2011) and Jeyachandran and Mahesh (2007). The microorganisms used for this study were laboratory strains of *Proteus vulgaris, Escherichia coli, Salmonella typhi, Bacillus cereus* and *Enterobacter*

aerogenes. A single colony for each of the organisms was picked from agar plate and dissolved in 5 ml of Mueller Hinton broth. The broth was incubated overnight at 37^0C. Five millilitres (5 ml) of plain Mueller Hinton broth was incubated alongside the organisms to ensure that the medium was not contaminated. The spectrophotometer was set to 625 nm wavelength and each of the microbial cultures was pipetted into cuvettes to measure the absorbance. A cuvette of plain Mueller Hinton broth was used a blank at 0.000 absorbance. The absorbance of the microorganisms was measured. The bacterial organisms exceeding 0.1absorbance were adjusted by adding bacterial suspension until the absorbance fell between 0.08 to 0.10, matching the McFarland Standard. The organisms falling below 0.08 absorbance were also adjusted until the McFarland standard absorbance was achieved. All the organisms, therefore, reached a cell density of 1 x 10^8 cfu/ml (Ngeny *et al.*, 2013). One hundred (100) µl of each of the organisms were then inoculated onto agar plates for the bioassay (Agyare *et. al.*, 2013). Three 6 mm wells were made into each agar plate using a sterile metal cork borer. One hundred micro litres (100 µl) of the standard drug penicillin was placed in one well, the extract in another well and dimethylsulfoxide (DMSO) was placed in the third well on each plate. The experiment was run in triplicate for each extract and each organism tested. The plates were incubated for 24 to 48 hours and the zones of inhibition were measured in millimetres with the aid of a meter rule.

Statistical Analysis

A random sampling procedure was done for the entire test and the experiment was conducted in triplicate assays on Mueller Hinton agar plates (Jeyachandran and Mahesh, 2007). The mean values and standard error were calculated for the zones of inhibition. Analysis of variance was used to determine if there was significant difference among the average zones of inhibition of the bacterial organisms by the extract and controls. The Tukey's honestly significant difference test was used to determine pair-wise comparisons between average zones of inhibition among the bacterial organisms by SPSS version 21.0.

Results and Discussion

Analysis of variance showed significant difference in the zone of inhibition of the extract among the organisms ($p<0.0001$). The highest value for the zone of inhibition is that of *P. vulgaris,* followed by that of *E. aerogenes, S. typhi, E. coli* and *B. Cereus* (Table 1). Since the organisms all had zones greater than or equal to 8 mm, the extract was considered active against all the organisms. The penicillin control inhibited growth at higher zones of inhibition than all the microorganisms. The DMSO negative control showed no zone of inhibition.

Tukey's multiple comparison test showed that the zone of inhibition that did not differ significantly were those of *P. vulgaris* and *E. aerogenes*, *E. coli* and *S. typhi*, and *E. coli* and *B. cereus.* All other pair wise comparisons showed significant different differences including the zones of the extract in comparison to its control (Table 2). *P. vulgaris* zone was significantly bigger than those of *E. coli, S. typhi* and *B. cereus*. *E. coli* zone was similar to those of *S. typhi* and *B. cereus* but significantly smaller than those of *P. vulgaris* and *E. aerogenes*. *S. typhi* zone was significantly lower than *P. vulgaris* and *E. aerogenes* but bigger than *B. cereus* and *E. coli.*

There have been previous reports of the essential oils from aerial parts of *Tagetes minuta* exhibited antibacterial activity, especially against Gram-positive bacteria. The minimum inhibitory concentrations (MIC) for the oil from UK greenhouse-grown plants were 6.25– 25 µg/mL for Gram-positive bacteria and 25–50 µg/mL for Gram-negative bacteria, with the lowest MIC of 6.25 µg/mL against *Streptococcus faecalis.* Oil from plants from South Africa had MICs of 50–100 µg/mL against Gram-positive and Gram-negative bacteria (Senatore, 2004).

The secondary metabolite flavonoids from leaves of *T. minuta* showed antibacterial activity against Gram-positive and Gram-negative bacteria (Tereschuk, 1997) and *T. lucida* leaf extracts are reported to be active against Gram-positive bacteria (Caceres, 1991).

According to Eguaras (2005), *Tagetes minuta* oil demonstrated *in vitro* antibacterial, antifungal and miticide activity, although this oil shows a moderate inhibitor effect compared with other essential oils of native plants from Argentina. However, this oil presents a ratio selection that would allow it to be used in field conditions with a good safety margin. It is possible that this oil can be used in combination with others, in integrated pest management strategies in bee colonies. The essential oil of *Tagetes minuta* had an oxidant effect and a strong inhibitory action on root of *Zea mays* than *S. areira* oil (Scrivanti *et al.*, 2003).

The essential oil of *T. minuta* is stronger larvicide against *An. stephensi*, rather than its methanolic extract (Hadjiakhoondi, 2005). n- Hexane fraction of *T. minuta* showed significantly better antimalarial activity than the ether fraction (Shahzadi *et al*., 2010). *T. minuta* leaf powder was applied to rice field soil and significantly reduced emergence and growth of two paddy weeds *Echinochloa crusgalli* and *Cyperus rotundus* in pots under green house and in rice field (Batish et al., 2007).

T. minuta extracts showed inhibitory effect on germination and growth of *Lactuca sativa* (Kil et al., 2002). The essential oil of *Tagetes minuta* had an oxidant effect and a strong inhibitory action on root of *Zea mays* than *S. areira* oil (Scrivanti *et al.*, 2003).

T. minuta essential oil has radical scavenging and anti-inflammatory activities and could potentially be used as a safe effective source of natural anti-oxidants in therapy against oxidative damage and stress associated with some inflammatory conditions (Parastoo, 2014).

Plants play the significant role in all traditional system of food and medicine. Plants contain rich source of variety of natural products, secondary metabolites such as tannins, terpenoids, phenols alkaloids and flavonoids which found in to have antimicrobial properties (Cowan, 1999).

The plant sample has maximum antibacterial activity against gram positive bacterial microorganisms than that of gram negative bacterial microorganisms. Plant-based antibacterial have huge therapeutic potential as they can serve the reason with lesser side effects that are often associated with synthetic antibacterial (Lwu et al., 1999).

According to Patricia (2012), *Salmonellosis* causes more disease burden than any other foodborne pathogen. An estimated 93.8 million cases of gastroenteritis caused by Salmonella species occur globally each year and of these, nearly 80.3 million cases are food borne (Majowicz et al., 2010). In the United States, an estimated 1 million incident cases of human salmonellosis occur annually (Scallan et al., 2011); however, only a small portion of these cases are recognized clinically. In industrialized countries as few as 1% of clinical cases are actually reported (Heymann, 2008). Collectively, Salmonella infections in the United States account for roughly 19,336 hospitalizations, 17,000 quality adjusted life years lost (QALYs), and $3.3 billion in total medical expenditures and lost productivity each year (Batz *et al.*, 2011).

Enterohaemorrhagic E. coli causes bloody diarrhoea (haemorrhagic colitis), non-bloody diarrhoea and haemolytic uremic syndrome (James et al., 2004). *Enterotoxigenic E. coli* causes watery diarrhoea, which can range from mild, self-limiting disease to severe purging disease (Nataro, 1998). The evolution of pathogenic *E. coli* that has resulted in formation of distinct pathotypes capable of colonizing the gastrointestinal tract, urinary tract or meninges illustrates how key genetic elements can adapt a strain to distinct host environments. (James *et al.*, 2004).

Proteus vulgaris meningitis is relatively uncommon. The causative organism is a gram-negative, aerobic, non-sporulating, actively motile bacterium, usually occurring as a saprophytic non-pathogen in the upper respiratory, gastrointestinal, or genito-urinary tracts. Occasionally it produces severe infection and death (Leon, 1950).

Proteus species are the major cause of diseases acquired outside the hospital, where many of these diseases eventually require hospitalization (De Champs et al., 2000). *P. mirabilis* causes 90% of Proteus infections. *Proteus* species, particularly *P. Mirabilis*, is believed to be the most common cause of infection-related kidney stone, one of the most serious complications of unresolved or recurrent bacteruria (Coker et al., 2000). *P. mirabilis* has been implicated in meningitis, empyema, osteomyelitis and gastroenteritis. Also, it frequently causes nosocomial infections of the urinary tract (46%), surgical wounds (24%) and lower respiratory tract (30%). Less frequently, proteus species cause bacteraemia (17%), most often in elderly patients (Mansy, 2001).

B. cereus in association with food poisoning and eye infection, recognition and appreciation for the multitude of other serious infections such as fulminant sepsis and devastating central nervous system infections are lacking. Clinicians and clinical microbiologists must both give serious consideration to the significance of a *B. cereus* isolate from a clinical specimen, especially if the patient is immunosuppressed (Edward, 2010).

Enterobacter aerogenes is a Gram – negative, catalase positive, indole negative, rod shaped bacterium (Sanders, 1997). *E. aerogenes* is a nosocomial and pathogenic bacterium that causes opportunistic infections including most types of infections. The majority are sensitive to most antibiotics designed for this bacteria class, but this is complicated by their inducible resistance mechanisms, particularly lactamase which means that they quickly become resistant to standard antibiotics during treatment, requiring change in antibiotic to avoid worsening of the sepsis.

According to Gabriel, some of the infections caused by E. *aerogenes* result from specific antibiotic treatments, venous catheter insertions, and/or surgical procedures. *E. aerogenes* is generally found in the human gastrointestinal tract and does not generally cause disease in healthy individuals. It has been found to live in various wastes, hygienic chemicals, and soil.

Enterobacter aerogenes causes disease in humans through inadvertent bacteria transfer in hospital settings. A selection of enteric bacteria like *E. aerogenes* are opportunistic and only infect those who already have suppressed host immunity defences. Infants, the elderly, and those who are in the terminal stages of other disease or are immunosuppressed are prime candidates for such infections (Janda, 2006).

The genus *Enterobacter* is more specifically a nosocomial opportunistic pathogen and is sought out to be one of the many key causes for extraintestinal infections next to *E. coli*. Infections commonly attributed to *E. aerogenes* are respiratory, gastrointesntinal, and urinary tract infections, specifically cystits, in addition to wound, bloodstream, and central nervous system infections (Brooks, 2007; Lederberg, 2000 and Sankaran, 2000). Furthermore, *E. cloacea* and *E. aerogenes* are the species most commonly associated with aduilt cases of meningitis. Colonies of *Enterobacter* strains may be slightly mucoid.

Conclusion

Various reports describing the antimicrobial activity of *T. minuta* have proved that the results obtained in this study are in conformity with them. The plant therefore has potential for its incorporation into the health care system as alternative medicine where conventional medicines are both inaccessible and unavailable. For incorporation into conventional medicine in pharmaceutical industries, further analysis is required to describe the active ingredients of the extract and prove their separate activities against clinical isolates of microorganisms.

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Table 1: Zone of inhibition (mm ± S.E.) of Ethanolic extract of *Tagetes minuta* against selected bacterial organisms

Microorganism	Zone of Inhibition (mm ± S.E.)	Penicillin Control	DMSO control
Proteus vulgaris	17.30±0.333	30.33±0.333	0.00±0.000
Escherichia coli	9.30±0.333	42.33±1.202	0.00±0.000
Salmonella typhi	11.83±0.441	25.33±1.202	0.00±0.000
Bacillus cereus	8.00±0.000	40.33±0.333	0.00±0.000
Enterobacter aerogenes	16.67±0.882	39.00±0.577	0.00±0.000

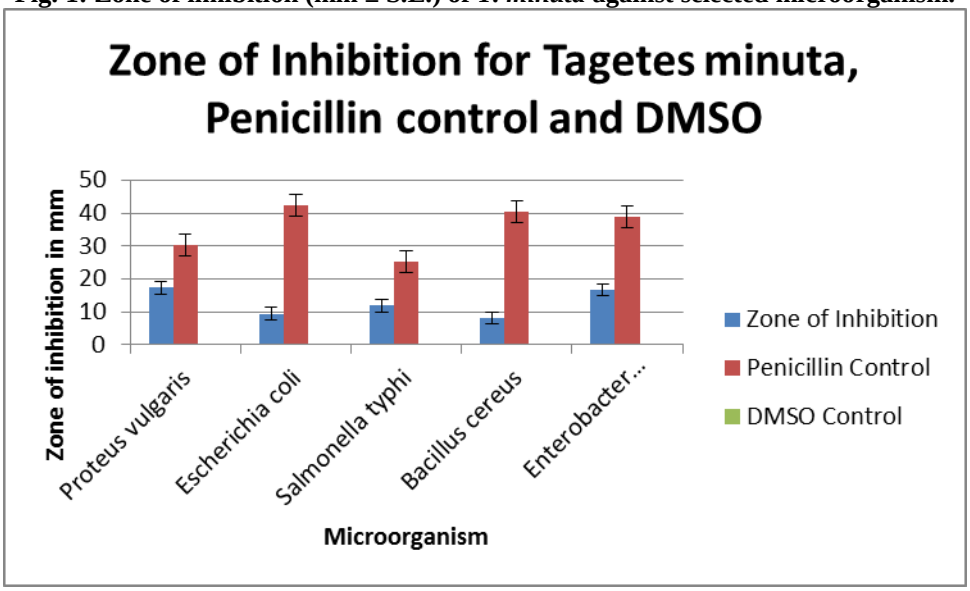
Key: S.E. = standard error; DMSO = dimethylsulfoxide

Table 2: Tukey's honestly significant difference test for the zone of inhibition of Ethanolic extract of *Tagetes minuta* against selected bacteria organisms.

COMPARISON	P-VALUE	SIGNIFICANCE
P. vulgaris vs E. coli	0.000	S
P. vulgaris vs S. typhi	0.000	S
P. vulgaris vs B. cereus	0.000	S
P. vulgaris vs E. aerogenes	0.999	NS
P. vulgaris vs P. vulgaris control	0.000	S
E. coli vs S. typhi	0.281	NS
E. coli vs B. cereus	0.918	NS
E. coli vs E. Aerogenes	0.000	S
E. coli vs E. coli control	0.000	S
S. typhi vs. B. cereus	0.020	S
S. typhi vs E. Aerogenes	0.002	S
S. typhi vs S. typhi control	0.000	S
B. cereus vs E. Aerogenes	0.000	S
B. cereus vs B. cereus control	0.000	S
E. aerogenes vs E. aerogenes control	0.000	S

S = significant; NS = not significant

Fig. 1: Zone of inhibition (mm ± S.E.) of *T. minuta* against selected microorganism.



***Tagetes minuta* plant**

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