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Aims and scope
The Kenya Veterinarian is a peer-reviewed scientific journal published by the Kenya Veterinary Association on research and clinical practice of veterinary medicine. The main contents of the journal entails articles on research, case reports, retrospective studies, reviews or short communication. Authors may submit articles in the following main disciplines; Anatomy and Histology, Animal Physiology, Biochemistry and Molecular Biology, Animal Sciences, Pathology, Microbiology, Parasitology, Public Health, Pharmacology and Toxicology, Food Animal Practice, Companion Animal Practice and Wildlife Sciences.

Submission of Manuscripts
Manuscripts should be addressed to: The Editor, Kenya Veterinarian, Journal of the Kenya Veterinary Association, P O BOX 29089 00625 – Kangemi, Nairobi, Kenya.

Electronic copies should be submitted as word documents as email attachments addressed to the following email addresses: kvanational@yahoo.com and jdmande@uonbi.ac.ke.

Authors are advised to consult the instructions to authors before submitting their manuscripts. All manuscripts must be accompanied by a covering letter signed by the first author. The letter should state that the paper has not been published elsewhere and is not simultaneously being considered for publication by another journal.

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Three copies must be provided in English, double-spaced, Times New Roman throughout on one side A4 paper with a wide margin all round. The manuscript should follow the conventional format but must include:

Title
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and the address of the corresponding author should be provided. Only authors who have significantly been involved in the study should be included. The email addresses of all authors should be provided.

The article should be presented under the following headings; abstract, introduction, materials and methods, results, discussion, conclusions, and references. The Abstract should be 150-200 words and concise.

Introduction
The introduction should be brief but contain sufficient information reviewing pertinent information in the area of study. A statement of the objectives of the study should follow this.

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This section should contain sufficient detail to enable replication of the procedures. However, previously established procedures should only be referenced rather than described in the article. Where animal experimentation is involved, proof that the authors adhered to conventional protocols and guidelines on the welfare of the study animals should be provided.

Results should be appropriately detailed. Tables and figures should be used only when they add value to the narrative.

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Acknowledgement
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Submission
Authors should submit 3 hard copies of the manuscript and a diskette of the windows Microsoft Word formatted article after the paper has been corrected and accepted for publication.
Articles will be sent peer-reviewed by two experts in the subject area, as long as they conform to acceptable format. Articles will only be published based of a favorable report by both reviewers. The final decision on whether the article is published on rejected will be determined by the Editor In Chief. The editorial board provides advice on the journal policy and standards. The corresponding author will be notified whether their article have been accepted within 2 months. Original manuscripts of articles not accepted will be returned to the corresponding author.

Subscription
The Kenya Veterinarian is published two issues in a year. Subscription runs for a full calendar year. The prices are given per issue and include delivery

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Abstract
Animal diseases are a major constraint to livestock production, drought animal power and the acceptability of companion animals. These diseases also impact negatively to the food security in our country. The use of synthetic drugs for disease management is always a challenge because of the unavailability of these drugs especially in rural areas, shortage of foreign exchange to import them, lack of finance to purchase them, drug resistance, misuse due to paucity of knowledge and environmental pollution. Medicinal plants products are part of the natural products that have been in use in traditional medicine and also a source of novel drugs. Therefore, the use of medicinal plant products would be a rational alternative to the synthetic drugs. Ethnobotanical surveys carried out in many parts of Kenya have revealed a lot of plants being used in animal disease management. Specific plant extracts have been identified and screened by many researchers for their antimicrobial, anthelmintic, acaricidal, antiprotozoal activity and also their toxicity. There is therefore the need to look for ways on how these plants products will be available in the market and be integrated in the overall veterinary medicine practice in Kenya.

Introduction
The livestock sector in Kenya plays a key role in provision of food and income and it is the only economic activity for 25% of the country's population living in arid areas (Grandin et al., 1991). However, this sector is faced by a number of constraints that affect productivity which include among others; paucity of management skills, nutrition and diseases. Diseases of the livestock and companion animals continue to be a major cause of losses to owners. Disease management using the conventional medicine has achieved a lot in Kenya and other Africa countries. However, despite many years of intensive research and subsequent control measures some diseases continue to be a menace especially to livestock production and therefore a threat to food security.

The major constraints to the use of conventional methods include; shortage of capital to import drugs and purchase them locally, development of drug resistance (Gakuya et al., 2007; Kapila, 2005; Olliaro and Bloland, 2001; Runyoro et al., 2006), unavailability and inconsistent supply of drugs in rural areas, paucity of knowledge on drug use by livestock owners, environmental pollution, unscrupulous manufacture of drugs with little or no efficacy at all, shortage of field transport facilities and low staffing levels especially in ASALS where most of the livestock are kept. This then calls for a review of our animal health care provision and possible mitigations.

The use of ethnoveterinary medicine products especially medicinal plants has been in existence thousands of years before the advent of conventional medicine. Medicinal plant tissues are part of the natural product compounds that usually have a pharmacological or biological activity and therefore have an active principle. Some of these compounds have been used in traditional medicine in crude form while refined compounds have been in use in pharmaceutical drug industry for both human and veterinary medicine. They have also been used as lead compounds in the manufacture of modern drugs.

According to the World Health Organization, 80% of the world’s population depends on plants for the primary health care (Farnsworth et al., 1985). Although there is no similar data on the overall usage in veterinary practice, it would be expected to be higher. Most communities manage both human and animal diseases using herbal remedies. The increased interest in usage of medicinal plant products is because they are viewed as having less side effects, environmentally friendly, locally available, cheap and easily accessible to local communities who have used these plants since antiquity. This then offers a possible alternative to the usage of conventional drugs and a possibility for their integration in overall veterinary medicine practice in Kenya. However before they can be integrated there is need for their validation in terms of efficacy and safety, legislations to regulate their usage by practitioners, conservation and training.
Background on the use of medicinal plants
been used for both medicinal and ritual purposes (Bodeker, 1999). Medicinal plant usage has long been in (Samuelsson, 2004). There is archeological evidence on the use of medicinal plants in China dating back to 2,800 years (Jiang et al., 2007). Some of the plant material unearthed in Yanghai tombs in Torpan District in Xinjiang includes the seeds of Capparis spinosa L and shoots, leaves and fruits of Cannabis sativa L. Similarly, in the old and new testament of the Bible, five herbs were used as medicine namely; Aloë, Anise seeds, leaves and stems, oil from the bark, leaves and berries of balsam tree, a garden herb Rue and yellow dye of Saffron (Packer et al.,1980).

Plants have been in use since the beginning of life and are important for the survival of man and other animals. Plants are the biggest source of natural products used in folk veterinary and human medicine (Dery et al., 1999). Indeed the use of plant anthelmintics just faded recently in Britain. The British Veterinary codex (1953, 1965) lists the oil of Chenopodium for use against in horses and pigs and Strongylus in horses. Other plants listed include; the male fern Dryopteris filix-mas against Monezia, Ascaridia and other gastrointestinal nematodes, Artemisia cina and other Artemisia spp. used against Ascaris suum, Toxocara and tapeworm in poultry. In the British Pharmacopoeia of 1863 there are descriptions of 187 crude drugs which include Digitalis, Datura, Belladona and Hyoscyamus (Melero et al., 2000). Similarly, several authors have documented many medicinal plants and herbs used by different communities in Kenya (Bussman,2006; ITDG and IIRR,1996; Kaendi,1997; Kareru et al.,2007; Kokwaro,1993; Lindsay,1978; Miaron et al., 2004; Nguta any plants used as anthelmintics in various parts of the world including Kenya. Plants which have been scientifically evaluated or used as anthelmintics in many parts of the world have been reviewed (Gakuya et al., 2004; Githiori, 2004; Hussain, 2008; Iqbal and Jabbar, 2010). About 150 plants have been validated for their anthelmintic effects in animals using standard parasitological techniques (Iqbal and Jabbar, 2010). These plants have been tested both for their in vivo and in vitro anthelmintic activity against the various gastrointestinal helminths of cattle, sheep, goats, chicken, pigs, dogs and laboratory animals.

In Kenya, five plants namely; Dryopteris inaequalis, Albizia anthelmintica, Albizia gummi flora, Olea africana Mill and Myrsine africana have been listed as used as plants validated and their anthelmintic efficacy reported though with variation between authors include; Chrysanthemum cinerariaefolium (pyrethrum) (Mbaria et al.,1998), Albizia anthelmintica (Gakuya et al., 2000; Gathuma et al., 2004), Maerua edulis ( Gakuya et al., 2000), Maerua subcordata (Gakuya et al.,2000), Myrsine Africana ( Gathuma et al.,2004), Hildebrandtia sepalosa (existence and is widely documented in China, India and Egypt et al., 2010a,b). The plants with certain potent compounds which deter animals or insects from eating them have been naturally screened to survive and therefore would be a source of drugs. The trial and error method was the ancient way of discovering cures. Currently, quarter of all prescribed drugs in developed world use active ingredients derived from plants (Cox and Balick, 1994). Most of the lead compounds that have been sourced from plants include; morphine, cocaine, digitalis, quinine, nicotine and muscarine. Recently the anticancer agent paclitaxel from yew tree and antimalarial drug artemisinin from Artemisia annua have been isolated from plants. Drug discovery and their chemistry are therefore related to the plant kingdom and the process of drug derived from plants is not new (Parfitt, 1978).

The use of and search for drugs and dietary supplements have accelerated in recent years. Some of the herbal preparations include concoctions, decoctions, infusions, dried powders, ointments, tinctures and macerates. Many medicinal plants are currently used as unregulated crude preparations and their use by the public is increasing rapidly. As their usage is increasing, issues of efficacy and safety concerns are also coming into the picture. Plants have been screened for their biological activity, cytotoxicity, antimicrobial, anthelmintic, acaricidal, antiprotocoal and toxicity in the world and even here in Kenya.

Scientific evidence of the efficacy of some plants
Plants with anthelmintic activity
Several authors have reported anthelmintics among the Marakwets of the Kerio valley (Lindsay, 1978). ITDG and IIRR (1996) listed eighteen plants used as anthelmintic namely; Lantana trifolia, Albizia anthelmintica, Alibizia coriavera, Diospyros scabra, Trichilia emetica, Rhus vulgaris, Cassia spectabilis, Tamarindus indica, Raphanea melanophloes, Cariisa edulis, Cucurbita maxima, Launaea cornuta, Ricinus communis, Ocimum basilicum, Myrsine africana, Tedea nobilis, Allium sativum and Hagenia abyssinica. Kokwaro (1993) listed 79 plants used as general anthelmintic, 21 used for hookworm infestations, 6 for roundworms and 22 for tapeworms. There are a few plants in Kenya that has been scientifically evaluated locally for their anthelmintic activity against gastrointestinal helminths. Some of the Gathuma et al.,2004), Tephrosia vogelli and Vemonia amygdalina (Siamba et al.,2007), Jasminum abyssinicum (Komen et al.,2005) and Embelia schimpen Vatke (Bogh et al.,1996).

Some plant used locally in Kenya and have also been reported to have anthelmintic activity in other parts of the

**Plants with antimicrobial activity**

A number of plants has been reported to treat various ailment associated with disease causing microbes. Table 1 shows the plants tested against microbial culture using various in vitro methods.
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<th>Plant Name</th>
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<td>Acacia mellifera</td>
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<td>Ajuga remota</td>
<td><em>Micrococcus lutea</em>, <em>Bacillus cereus</em>, <em>E.coli</em></td>
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<td>Aloe secundiflora</td>
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<td>Amaranthus hybridus</td>
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<td>Aspilia mossambicensis</td>
<td><em>Streptococcus pyogenes</em>, <em>S. typhi</em>, <em>Aspergillus niger</em></td>
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<td>Carisa edulis</td>
<td><em>Herpes simplex virus type 1</em></td>
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<td>Tetracera boviniana</td>
<td><em>B. cereus</em>, <em>E. coli</em>, <em>P. aeruginosa</em>, <em>Salmonella typhimurium</em>, <em>P. vulgaris</em>, <em>Corynebacterium pyogenes</em></td>
<td>Mbaria et al., 2005</td>
</tr>
<tr>
<td>Tithonia diversifolia</td>
<td><em>E.coli</em>, <em>S. aureus</em></td>
<td>Karenu et al., 2008</td>
</tr>
<tr>
<td>Vernonia amygdalina</td>
<td><em>S. aureus</em></td>
<td>Cheruiyot et al., 2009</td>
</tr>
<tr>
<td>Vernonia lasiopus</td>
<td><em>E.coli</em>, <em>B. subtilis</em></td>
<td>Karenu et al., 2008</td>
</tr>
<tr>
<td>Vitex strickeri</td>
<td><em>E. coli</em>, <em>S. aureus</em>, <em>B. subtilis</em></td>
<td>Karenu et al., 2008</td>
</tr>
<tr>
<td>Ziziphus abyssinica</td>
<td><em>B. cereus</em></td>
<td>Wagate et al., 2009</td>
</tr>
</tbody>
</table>
Plants with antiprotozoal and anti-rickettsial activity

Hemoparasitic diseases mainly transmitted by ticks and tsetse flies are of economic importance due to the losses they cause in productivity of livestock. These diseases include; Babesiosis, Trypanosomosis, East Coast Fever, Anaplasmosis and Heart water. Various plants have been reported to have activity against these blood parasites though only a few has been validated. There are three plants that are available in Kenya and reported to be used for the treatment of Babesiosis in cattle namely; the stem bark of Acacia exocephalia, Mimosa ceaeae, roots of Erythrina abyssinica Lam. Fabaceae and the bark of Prunus africana (Hook.f.)Kalkman, Rosaceae (Githiori and Gathumbi, 2010).

Plants reported for the treatment of trypanosomosis in Kenya include; Acacia reficiens, Adenia volkensii, Fagara chalybea, Salvadora persica and Terminalia brownii (Githiori and Gathumbi, 2010). Other plants tested for their antitrypanosomal activities in Uganda are Albizia guamifera, Ehretia amoena, Entada abyssinica, Securinega virosa and Vernonia suligera (Freiburghaus et al., 1996). In Tanzania 10 plants, were reported to have antitrypanosomal activity namely; Asteranthe asterias, Commiphora emenii, Diospyros verrucosa, Enantia kummeriae, Hymenocardia ulmoides and Zanthoxylum chalybeum (Freiburghaus et al., 1997a, 1997b). The medicinal plants reported by many authors for the treatment of East coast fever in Kenya and the rest of East Africa are reviewed by Githiori and Gathumbi (2010) and include; Adansonia digitata (boabab tree), Aerva javanica, Croton megalocarpus, Euphorbia triaculeata, Synadenium compactum, Euphorbia candelabrum, Agave americana, Cissus quadrangularis, Clerodenu um myricoides, Gerrardanthus lobatus, Ibizo multiflora, Plecnthrus barbatus, Albitizia coriaria, A.zygia, Ananas comosus, Aristolochia elegans, Asparagus racemosus, Boerhavia diffusa, Maytenus senegalensis and Milicia excelsa. Anaplasmosis is treated using the roots, leaves and bark of Ficus sycomoros while heart water is treated with a combination of Abrus precatorius and Catha edulis (IIRR and ITDG, 1996).

Plants used as biopesticides

Several plants have been reported in Kenya and other parts of the world as being used as biopesticides. There are 25 plants documented to have activity against ectoparasites and the leaves are the most commonly used part (Githiori and Gathumbi, 2010). Some of these plants include; Tephrosia vogelii Hook F which is available in Kenya is cultivated in some areas. It is also used as an insecticide against lice, fleas and ticks and also as a molluscicide. It has rotenone which has acaricidal activity against ticks and mites (Dzenda et al., 2008). Other plants that have been reported as biopesticides include; Nicotiana spps.(Duke 1990), Nicandra physaloides (Crombie,1999), Rosemarinus officianalis, Chrysanthemum cinerariaefolium(Crombie,1999), Azidarachta indica (Boeke et al.,2004), Ricinus communis, Tagetes spps (Duke,1990), Acalypha fruticosa, Adenia veneta and Adenia multiflorum (Githiori and Gathumbi,2010).

The strengths of medicinal plants usage

Medicinal plants products are affordable especially for the rural people. Most of the plants are locally available and the herbalists are accessible and inexpensive. The other aspect is that of the plants being biodegradable and therefore causing minimum environmental pollution as opposed to the conventional drugs where drug spillage and their containers are part of the environmental pollution. The medicinal plants are also socially accepted by many communities especially in the rural areas as they have been in use for a long time. Furthermore, the use of medicinal plants is part of the livelihoods of majority of the people who are involved in conservation, harvesting, marketing and even the traditional practitioners. The other advantage on the usage of medicinal plants is that they have little side effects as compared to conventional drugs. There is also the existence of local expertise on the usage of these plants. Medicinal plants are also the raw materials for both traditional medicine and conventional medicine and 90% of all traditional medicines are estimated to be plant based (Murende, 2003).

Constraints to the usage of medicinal plants

Although there are a lot of medicinal plants and products being used in ethnoveterinary medicine and a few of which have been shown to be efficacious, there are very few plant products that are available in the conventional Veterinary practice in Kenya. This is due to a number of challenges facing the use of medicinal plants. The first challenge is the attitude or perception of the conventional veterinary practitioners to the traditional practitioners on the usage of medicinal plants and their products. There is a misconception that the use of medicinal plants is related to witchcraft which is opposed to the Christian beliefs leading to lack of interest, stigmatization and even opposition. There is also lack of guidelines, policies and regulatory framework on the medicinal plants usage and on how they can be integrated to the national animal health care. This is required to harmonize research, conservation, harvesting and marketing on medicinal plants. It will also identify genuine practitioners and control quacks who take advantage of desperate people and animal owners.
The other challenge is lack of validation, standardization and safety assessment of the medicinal plant products. For further development on the use of medicinal plants in ethnoveterinary medicine, there is need for information on the efficacy and safety of the products. Research has shown evidence that some medicinal plants used against veterinary diseases are efficacious, some up to 100% (Amin et al., 2009; Satrija et al., 1994). However other medicinal plants claimed to be efficacious have also been reported to have no efficacy at all (Ibrahim et al., 1984; Klicks, 1995). Other medicinal plants that have been in use have also been reported to be toxic (Mugera, 1970; Kellerman et al, 1988; Shone and Drummond, 1965). Validation will result to more acceptability of the use of medicinal plants in animal treatments and possibility of commercialization.

The availability and sustainability on the use of medicinal plants is a major concern. Some plants which are efficacious are becoming scarce. China and India harvest 80% and 90% of the medicinal plants from natural resources respectively (Lange, 1996). This is the same picture even in Kenya and unless emphasis on conservation and cultivation is undertaken many plants will become scarce. The overexploitation of economically valuable medicinal plants by herbalist and commercial traders due to increased demand by growing population, together with other deforestation activities leads to their scarcity. Some preferred species which carry the burden of treating by either being added to mixtures of others (being the key species) or added to enhance the potency of others are becoming threatened. Some of these plants include Trichilia emetica in Western Kenya and Rhamnus prinoides among the Maasai people (Maundu et al., 2004).

Plants previously found in the backyards are now scarce and can only be found in the wild and are difficult to locate as noted by Minja (1989). Population increase and need for more land for food growing has led to bush clearing for crop production and therefore affecting the availability of medicinal plants (Gakuya, 2001). Biodiversity of plants is affected by change of soil texture and preference of cash and food crops to indigenous plants and hence scarcity of medicinal plants. The methods for harvesting these plants parts especially the roots would also make the usage of some plants unsustainable.

**Future prospects of medicinal plants**

The first step is to ensure that we have an inventory on medicinal plants utilization as this knowledge is fast disappearing. Efforts are being made to carry out extensive ethnobotanical surveys and botanical identification of plants relevant to animal health though a sharing of benefits from medicinal plants.

The other aspect is increasing awareness through introduction of ethnoveterinary medicine in education curriculum. Ethnoveterinary information needs to be included in secondary schools, certificate and diploma in animal health training schools and the veterinary medicine training at the University. This will increase awareness and interest in medicinal plants usage and further research. There is a need for collaboration between researchers in various institutions and also other stakeholders like the department of culture, traditional medicine practitioners, processors, marketers, cultivators and communities owning the medicinal plants. This will enhance mutual trust in all these groups as well as commercialization and eq
Conclusion
Medicinal plant products have an important role to play in animal disease control as it is the only option in some rural areas of Kenya. There are plants which have already been tested and have appreciable activity and further product formulation would make them available in the market. These products would be integrated in the veterinary medicines to be used for disease control. Animal health service providers need to enhance their knowledge and skills on medicinal products utilization. The data on ethnoveterinary medicine practitioners and animals treatment need to be generated to assess the impact of ethnoveterinary medicine on animal health care.

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Research Article: Pharmacology and Toxicology

_in vitro_ and acute _in vivo_ toxicity of the aqueous and chloroformic extracts of _Rapanea melanophloeos_ (L) Mez

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Abstract

This study was conducted to generate a toxicological profile of _Rapanea melanophloeos_, a medicinal plant widely utilized in traditional medicine to treat helminthiasis, using brine shrimp (_Artemia salina_) and Sprague Dawley (SD) rats. The aqueous extract showed potent _in vitro_ toxicity to brine shrimp with a median lethal concentration (LC50) of 59.37 µg/ml, while the extract chloroformic extract did not exhibit potent _in vitro_ toxicity (LC50 of 1250 µg/ml), after exposure for 24 h. The acute toxicity study in rats indicated the no-observed-adverse-effect-level (NOAEL) and the lowest-observed-adverse-effect-level (LOAEL) of the aqueous extract to be 300mg/kg and 500mg/kg respectively, 24 h after single dosing. These values were lower than those of the chloroformic extract which had a NOAEL of 500mg/kg and a LOAEL of 1000mg/kg. The median lethal doses (LD50) of both extracts were above 7500mg/kg. The most overt signs of toxicity for both the aqueous and chloroformic extracts were depression, inactivity and somnolence, delayed reaction to stimuli, lethargy, piloerection and lackluster eyes. Recovery was complete in 24 h. Necropsy of the animals that died at the highest dose of both extracts revealed general congestion and enlargement of the liver, spleen and kidneys. There was mucoid content in the gastrointestinal tract of the animals dosed with the aqueous extract. At histopathology, the aqueous extract caused modest congestion of the kidney, liver and lungs, while the chloroformic extract did not change the histological appearance of any of the organs. The study shows that both extracts are harmless at the therapeutic doses used. This supports safe medicinal use of _Rapanea melanophloeos_ and there is need to study prolonged toxicity of the plant.

Key words: _In vitro_ toxicity, _in vivo_ toxicity, LOAEL, NOAEL.

Introduction

Herbal medicines have been used in traditional medicine since antiquity. Up to 80% of the population in the developing regions of the world relies solely on herbal products sold by traditional healers (Fouche et al., 2006). While the antimalarial activity of the leaf extracts was demonstrated by Clarkson et al. (2004), the antimalarial activity of the leaf extracts was demonstrated by Clarkson et al. (2004). The antimycobacterial activity has been analyzed by Lall and Meyer, 1999. Despite the wide utilization of _R. melanophloeos_ in traditional medicine, reports on its toxicity are scanty.

Materials and methods

Plant material

A specimen of _R. melanophloeos_ was collected from Narok, positively identified at the Department of Range Chloroform extraction was done in a Soxhlet, for 48 h. Chloroform was removed in a rotary evaporator at 50°C.

remedies for the provision of primary healthcare (WHO, 2008). In the recent past, there has been recrudescence in the utilization of medicinal plants in both conventional and folkloric medicine, coupled with an escalation of scientific probing of plants as potential sources of phytopharmaceuticals (WHO, 2005; CTA, 2007). _Rapanea melanophloeos_ (Myrsinaceae) is a tropical tree that is widely used in traditional medicine mainly to treat helminthiasis in man and animals (Mauandu and Tengnas, 2005; Nanyingi et al., 2008; Muthee et al., 2011). It is also used as an expectorant, emetic and astringent (Watt and Brandwijk, 1962), as an anti-inflammatory and analgesic (Chinemana et al., 1986), as a purgative (Lindsay and Hepper, 1978) and in the treatment of tuberculosis (McGaw et al., 2008). _R. melanophloeos_ is also used to cure heart-water in small ruminants (Dold and Cocks, 2002). In South Africa, the bark is the most utilized part of the plant in herbal medicine (Dold and Cocks, 2002). _R. melanophloeos_ is common in highland forests, especially in Mts. Kenya and Elgon, the Aberdare ranges, and most of tropical Africa to South Africa (Mauandu and Tengnas 2005).

_R. melanophloeos_ contains the benzoquinones embelin and rapanone (Midowo et al., 2002), and tannins (Watt and Brandwijk, 1962) as its major bioactive compounds. _R. melanophloeos_ is known to have anthelmintic activity (Watt and Brandwijk, 1962; Kokwaro, 1976; Mauandu and Tengnas, 2005), molluscicidal activity due to its epoxyoleanane saponins (Hostettmann et al., 2000) and fungicidal activity through the triterpene glycosides (Ohtani et al., 1993; Hostettmann and Marson, 1994; Hostettmann et al., 2000). Moderate anticancer activity has been described for the brine shrimp toxicity of the chloroformic and aqueous extracts to Sprague Dawley rats and brine shrimp (_Artemia salina_) in order to support its application in traditional medicine.

Management, University of Nairobi, and a voucher specimen was preserved in the department’s herbarium. The stem-bark was then air dried, pulverized using a mill, extracted separately using chloroform and distilled.

(Heidolph VV2000, Schwabach, Germany) and then oven-dried at 40°C for 24-72 h.
The aqueous extract was prepared by boiling 10g batches of the pulverized bark in 100 ml water for 5 min. The mixture was filtered through cotton gauze and then through double layer Whatmann’s (45µm) filter paper. The filtrate was centrifuged at 5000 rpm for 5 min and the supernatant freeze-dried. Both extracts were stored at -20°C until use.

The aqueous extract was dissolved in physiological buffered saline while the chloroformic extract was dissolved in olive oil before administration.

Experimental animals
Sprague Dawley (SD) rats aged 6-8 weeks were obtained from the Central Veterinary Research Laboratories (Kabete, Nairobi, Kenya). They were randomly allocated and housed in polycarbonate cages, each containing 5 rats; males and females separately with bedding changed twice a week to maintain hygiene. Commercial rat chow (Unga LTD, Nairobi, Kenya) and drinking water were provided ad libitum and animals were allowed 7 d to acclimatize before commencement of the experiments. This study was conducted in accordance with the internationally accepted principles for laboratory animal use and care as outlined in the European Community guidelines (EEC Directive of 1986; 86/609/EEC).

Brine shrimp lethality test
The eggs of Artemia salina (JBL, Germany) were obtained from an aqua pet shop (Aquacare, Nairobi, Kenya). They were randomly allocated to deriving and hatched within 48 h in a marine salt solution (33g/l) supplemented with yeast (6mg/l). Ten nauplii were transferred to 7 sets of 5 tubes each, containing varying concentrations of extracts and a control. The chloroformic extract was dissolved in dimethylsulfoxide (DMSO) to a stock concentration of 40mg/ml. Aliquots of the stock solution were dispensed in each set of the tubes containing 5ml of brine solution to final concentrations of 2000µg/ml, 1500µg/ml, 1000µg/ml 500µg/ml, 100µg/ml, 25µg/ml and 10µg/ml per tube and a control to which the highest dose of DMSO used was added. For the aqueous extract, a stock solution containing 10mg/ml of the extract was prepared in a marine salt solution. Aliquots of the stock solution were dispensed in each set of the tubes containing 5ml of brine solution to final concentrations similar to those of the chloroformic extract. The nauplii were observed at 24 h for mortality under a magnifying glass and survival data recorded (Kanwar, 2007). The LC_{50} was determined by the Finney computer program. LC_{50} values below 250µg/ml were considered as potently toxic (Pisutthanan et al., 2004; Maridass, 2008), while those above 1000µg/ml were considered not toxic.

Acute toxicity study
The dose range finding study was based on the Acute Toxic Class Method (2001) of Organization for Economic Cooperation and Development (OECD) and involved administration of single doses processing for histopathology (Culling, 1974). A complete histopathologic evaluation and photography of the tissues was done at x100, x400 and x1000 magnification under the microscope (Leica, Wetzlar, Germany).

Results
Extract characteristics and yields
After drying in vacuo, the chloroformic extract was a brown solid residue that was insoluble in water but dissolved in dimethylsulfoxide and corn oil after warming and agitation. The extraction yield was 2.5% (w/w). The freeze dried aqueous extract was a light brown, fluffy powder that was readily soluble in water, and its yield was 12.2% (w/w).

Brine shrimp lethality of the aqueous and chloroformic extracts of R. melanophloeos
The aqueous extract demonstrated a potent in vitro toxic activity to brine shrimp nauplii with a median lethal concentration (LC_{50}) of 59.37µg/ml while the chloroformic extract had no potent cytotoxic activity (LC_{50} of 1250 µg/ml) at 24 h post exposure (Table 1).
Table 1: Mortality of brine shrimp after exposure to the aqueous and chloroformic extracts of *Rapanea melanophloeos*

<table>
<thead>
<tr>
<th>Dose level (µg/ml)</th>
<th>Mortality for the aqueous extract</th>
<th>Mortality for the chloroformic extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number dead</td>
<td>%mortality</td>
</tr>
<tr>
<td>10</td>
<td>0.25</td>
<td>2.5</td>
</tr>
<tr>
<td>25</td>
<td>4</td>
<td>40</td>
</tr>
<tr>
<td><strong>LC₅₀</strong></td>
<td><strong>59.37 (43.56-65.67)</strong></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>7.25</td>
<td>72.5</td>
</tr>
<tr>
<td>500</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>1000</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td><strong>LC₉₀</strong></td>
<td><strong>1250 (1103.57-1542.53)</strong></td>
<td></td>
</tr>
<tr>
<td>1500</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>2000</td>
<td>10</td>
<td>100</td>
</tr>
</tbody>
</table>

Values are expressed as means ± standard deviation (n=4), % mortality = (no dead/10)*100

**Acute toxicity of the aqueous extract of *R. melanophloeos* in rats**

**Clinical signs**

In the dose range finding study, no adverse signs of toxicity were observed up to the 300 mg/kg dose (no-observed-adverse-effect-level, NOAEL) while the lowest observed adverse effect level (LOAEL) was considered to be 500mg/kg body weight (Table 2). A limit test was conducted at 7500 mg/kg, where only 3 out of 10 animals died (approximately the LD₅₀) (Table 2). Therefore, the LD₅₀ could not be determined but is possibly higher than 7500 mg/kg. The most prevalent signs at 7500mg/kg bodyweight were depression, inactivity, somnolence, delayed reaction to stimuli, piloerection and lackluster eyes (Figure 1). The clinical signs were evident from 30 min, they peaked at 3-4 h and were followed by partial recovery after 24 h and complete recovery in 48 hours.

Table 2: Symptoms following a single oral dose of *Rapanea melanophloeos* aqueous extract in rats

<table>
<thead>
<tr>
<th>Dose level (mg/kg)</th>
<th>Number of animals</th>
<th>Duration of signs (h)</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>3</td>
<td>-</td>
<td>None</td>
</tr>
<tr>
<td>50</td>
<td>3</td>
<td>-</td>
<td>None</td>
</tr>
<tr>
<td>300</td>
<td>3</td>
<td>-</td>
<td>None</td>
</tr>
<tr>
<td>500</td>
<td>3</td>
<td>2-4</td>
<td>Hypo-activity, dull eyes, slight piloerection.</td>
</tr>
<tr>
<td>1000</td>
<td>3</td>
<td>3-7</td>
<td>Inactivity, somnolence, dull eyes, piloerection.</td>
</tr>
<tr>
<td>2000</td>
<td>3</td>
<td>10-24</td>
<td>Inactivity, somnolence, dull eyes, piloerection, slow reaction to pain</td>
</tr>
</tbody>
</table>
Figure 1: Frequency of signs of toxicity exhibited by rats dosed with 7500 mg/kg aqueous *Rapanea melanophloeos* extract.

**Pathological changes**

Necropsy of the three animals that died at the highest dose revealed congestion of the kidneys, congestion and modest enlargement of the liver and spleen; and mucoid content in the lower gastrointestinal tract.

At histopathology, there was mild congestion of the kidney and the lungs. The liver was also congested, but maintained its histoarchitectural integrity (Plate 1). The other organs were unaltered in comparison with organs from the control animals.
Plate 1: Lesions in various organs of a rat dosed with 7500 mg/kg *Rapanea melanophloeos* aqueous extract (x400, H&E). (A) The kidney exhibits congestion, (B) The liver displays sinusoidal congestion, and (C) the lung exhibits mild congestion.

**Acute toxicity of the chloroformic extract of *R. melanophloeos* in SD rats**

**Clinical signs**

After administration of dosages up to 2000 mg/kg, there were no adverse clinical signs at 500 mg/kg (no-observed-adverse-effect-level) and the lowest-observed-adverse-effect-level (LOAEL) was at 1000 mg/kg body weight (Table 3).

A limit test was conducted at 7500 mg/kg, and no mortality was recorded, therefore implying that the oral LD50 of the chloroformic extract of *R. melanophloeos* in rats is higher than 7500 mg/kg. The most prevalent clinical signs at this dose level were depression, inactivity, somnolence, delayed reaction to stimuli, lethargy and piloerection. All the animals completely recovered in 24 h (Figure 2).

<table>
<thead>
<tr>
<th>Dose level (mg/kg)</th>
<th>Number of animals</th>
<th>Duration of signs (h)</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>3</td>
<td>-</td>
<td>None</td>
</tr>
<tr>
<td>50</td>
<td>3</td>
<td>-</td>
<td>None</td>
</tr>
<tr>
<td>300</td>
<td>3</td>
<td>-</td>
<td>None</td>
</tr>
<tr>
<td>500</td>
<td>3</td>
<td>-</td>
<td>None</td>
</tr>
<tr>
<td>1000</td>
<td>3</td>
<td>3-5</td>
<td>Hypo-activity, dull eyes, piloerection, slow reaction to pain</td>
</tr>
<tr>
<td>2000</td>
<td>3</td>
<td>4-24</td>
<td>Inactivity, somnolence, dull eyes, piloerection, slow reaction to pain</td>
</tr>
</tbody>
</table>
Figure 2: Frequency of signs of toxicity exhibited by rats dosed with the chloroformic *Rapanea melanophloeos* extract at 7500 mg/kg bodyweight

**Pathological changes**

On postmortem, the most striking pathological findings at dosage level 7500 mg/kg were enlargement and congestion of the liver. The spleen and kidneys were congested but there was no reaction in the other body systems. At histopathology, modest congestion of the kidneys, liver and spleen occurred at dosage level 7500 mg/kg bodyweight but the organ histoarchitecture and cellular detail were preserved (Figure 3, 5), when compared to the organs from control animals (Figure 4, 6).

**Figure 3:** The kidney of a rat dosed with 7500 mg/kg *Rapanea melanophloeos* chloroformic extract showing moderate congestion (x400, H&E)

**Figure 4:** The kidney of a control rat with normal histoarchitecture (x400, H&E)
Discussion
Toxicological studies are paramount in the development of botanical products to safe and efficacious drugs, particularly those herbs that are claimed or intended for cure, prevention or treatment of human disease (Wu et al., 2004). The brine shrimp lethality assay is a rapid, easy and effective bioassay technique for predicting the bioactivity, anticancer, cytotoxicity, toxicity, pesticidal activity, gastroprotective action among other pharmacological effects of plant extracts (Kanwar, 2007). It is also effective for predicting oral acute toxicity of plant extracts to mice (Lagarto et al., 2001). In addition, toxicity testing in rodents using standard toxicity testing protocols is a highly effective preliminary assay to approximate the likely adverse reactions to toxicants in man (Monosson, 2007), and it provides reliable toxicity profiling results.

This study tested the acute in vivo toxicity of the aqueous and chloroformic extracts of R. melanophloeos bark, to SD rats and in vitro toxicity to brine shrimp nauplii. In the brine shrimp lethality test, the aqueous extract demonstrated a potent in vitro toxic activity (LC50=59.37µg/ml) than the chloroformic extract (LC50 of 1250 µg/ml) probably because of the poor solubility of the chloroformic extract, or a difference in fractionation of the active phytoconstituents. The potent cytotoxic activity of the aqueous extract can be ascribed to the main benzoquinones, embelin and rapanone that have demonstrated powerful cytotoxic activity in cancer cell lines (Cordero et al., 2004; Podolak et al., 2005). It also reveals that Artemia salina may have a less elaborate system for biotransformation of the phytocomponents to non toxic moieties as compared to rats, since the aqueous extract did not cause severe toxicity in the rats.

In the acute oral toxicity study, aimed at comparing the toxicity of the polar and non polar fractions, both the chloroformic and aqueous extracts can be classified as harmless (OECD, 2001) or practically non toxic (Kennedy et al., 1986) because their LD50 are supposedly much higher than 5000mg/kg. However, the aqueous extract demonstrates a lower NOAEL and LOAEL than the chloroformic extract, probably because of the difference in fractionation of bioactive compounds; and its higher solubility when compared the chloroformic extract. Desta, 1995, obtained similar findings in an acute toxicity study of Embelia schimperi(Myrsinaceae), Myrsine africana(Myrsinaceae) and Maesa lanceolata (Myrsinaceae), where the aqueous extracts had lower lethal doses than the hydroalcoholic extracts. In addition, this finding is in line with the brine shrimp lethality test, where the aqueous extract showed a high in vitro toxic activity as compared to the chloroformic extract of R. melanophloeos.

The clinical manifestations of depression, limited locomotor activity, somnolence, delayed reaction to stimuli, lethargy, piloerection and lackluster eyes exhibited by the animals were less severe with the chloroformic extract. It can be argued that the extracts possess some depressive activity on the central nervous system (Atsamo et al., 2011). From the histopathological findings, it was evident that the aqueous extract at 7500
mg/kg caused mild renal, pulmonary and hepatic congestion but no change in cellular architecture. This contrasts a toxicity study of embelin where it caused severe hepatotoxicity after 6 weeks of administration (Prakash, 1994). This congestion of the liver, kidneys and the lungs that was evident in the acute toxicity studies might not have been due to the extracts but might have resulted from the diethyl ether euthanasia.

The general atoxic nature of *R.melanophloeos* extracts can be related to the very low toxicity of its main bioactive components, embelin and rapanone (Johri et al., 1990). This fact is further supported by acute toxicity studies by search for ethnomedical cure from this plant. Further testing is underway to fully support and promote the application of this plant in ethnomedicine, especially in the less economically endowed populations of sub-saharan Africa.

**Acknowledgement:**
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**Conflict of interest**
The authors declare that there are no conflicts of interest

**REFERENCES**


Desta, 1995 where members of Myrsinaceae had high LD50 values. Additionally, the results of this study confirm the fact that there are no reports in literature of any adverse effects due to administration of *R.melanophloeos* in traditional medicine.

The present study therefore shows that the plant is highly unlikely to cause adverse effects at the therapeutic doses used and can be utilized safely in traditional medicine as an anthelmintic, analgesic and against heart water in small ruminants, without posing a risk of acute toxicity to the patients. The results further supports any plausible


Lindsay RS and Hepper FN 1978: Medicinal plants of Marakwet, Kenya. Royal Botanic Gardens, United Kingdom, pg49.


Research Article: Pathology, Microbiology and Parasitology

Modulation of cytokine production profiles in splenic dendritic cells obtained from SAG1 transgenic mice infected with Toxoplasma gondii.

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Abstract

We examined the role of splenic dendritic cells in immune response to Toxoplasma gondii infection in SAG1 (P30+) transgenic mice by investigating the kinetics of intracellular cytokines expression of IL-4, IL-10, IL-12 and IFN-γ by intracellular cytokine staining (ICS) using flow cytometry, and compared the results to those of their P30- littermates. Th2 cytokines, IL-10 and IL-4 were expressed at higher levels compared to Th1 cytokines IL-12 and IFN-γ with IL-10 showing the highest expression in both groups of mice. Moreover, peak cytokine levels in P30+ mice were on day 5 post-infection and on day 7 post-infection in P30- mice. 30% of the P30+ mice succumbed to infection by day 7 post-infection whereas all the P30- littermates survived. The co-stimulatory molecule, CD80 and their MHC phycoprotein, were up-regulated both in P30+ mice and their P30- littermates following T. gondii infection. Our results suggest that the immune response to T. gondii infection demonstrates a concomitant Th1 and Th2 cytokine intracellular and mRNA expression with a crucial role for IL 10/IL 4 in the resolution of infection.

Key words: Dendritic cells, intracellular cytokines, Toxoplasma gondii, SAG1 transgenic mice.

Introduction

Cytokines are extracellular factors that affect cell proliferation and differentiation during a cell-mediated immune (CMI) response. They are produced by virtually all leukocytes that trigger off the initial steps of the CMI response, e.g. macrophages, neutrophils and dendritic cells (Gazzinelli et al., 1993; Reis e Sousa et al., 1997). Of these, the dendritic cells (DC) are the most potent (Knight et al., 1992; Makala et al., 2001a). The principal function of the DC appears to be the activation of T cells (Fearon and Locksley, 1996; Banchereau and Steinman, 1998; Makala et al., 2003a). After capturing antigen, dendritic cells migrate to the draining lymphoid organs and after a process of maturation, present the processed antigen to specific T cells, thus initiating clonal immunity (Palucka and Banchereau, 1999). Some of the cytokines produced by dendritic cells in detectable amounts include interleukin-16 (IL-16), IL-12, IL-15, IL-18, TNF and chemokines (Makala and Nagasawa, 2002). Of these, IL-12 appears to be the most important. Recent studies show that IL-12, a heterodimeric cytokine, is essential for stimulation of Natural Killer (NK) cells, differentiation of Th1 cells and induction of IFN-γ production, all of which enhance protection against Toxoplasma gondii infection (Koyama et al., 1999). Previously, it was believed that macrophages were responsible for producing IL-12 as the initial signal of the immune response to T. gondii infection but now it is known that it is actually the dendritic cells that are primary producers (Reis e Sousa et al., 1997; Makala et al., 2001b). In vitro studies have revealed that IL-4 acts as an enhancer of IL-12 production (Hochrein et al., 2000), whereas in vivo, a concomitant Th2 response, consisting of IL-10 and IL-4 secretion down-regulates the systemic type 1 cytokine production (Candolfi et al., 1995; Sher et al., 1998). Toxoplasma gondii induces a strong cell-mediated immunity which is characterized by a highly polarized Th1 cell response (Nagasawa et al., 1996; Ely et al., 1999). Humoral response has also been reported to be mounted during the active phase of infection (Nguyen et al., 1998), which is strong enough to protect against infection. Upon initial encounter with the immune system, T. gondii rapidly induces production of type 1 promoting pro-inflammatory cytokines by antigen presenting cells. Cytokines such as IL-6 (Lyons et al., 2001), IL-8 (Denney et al., 1999) and IL-18 (Guifang et al., 2000) have been shown to play a role in T. gondii infection. However, SAG1 transgenic mice have a higher susceptibility to T. gondii infection (Seng et al., 1999), which may be associated with a lower level of IFN-γ production.

In the present study, we investigate the cytokine production profile by splenic dendritic cells in SAG1 transgenic mice infected with a virulent strain of T. gondii. The objective of the study was to investigate the cytokine production profile by dendritic cells in SAG1 transgenic mice infected with a virulent strain of Toxoplasma gondii.

Materials and methods

Animals

P30+/B6C3F1 transgenic mice (Seng et al., 2000) and their P30+/B6C3F1 littermates were randomly picked from the stock maintained by the Immunology Laboratory at the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, Japan. All experimental mice 6-10 weeks of age and of both sexes were kept in cages (6-8 mice/cage) and fed on a standard commercial diet (CLEA, Japan, Inc). Sterilized tap water was provided ad libitum.

Sterilized tap water was provided ad libitum.
Parasite culture, purification and infection of mice

Toxoplasma gondii tachyzoites of the RH strain were maintained in Vero cells cultured in Minimum Essentia Medium, MEM-Eagle (Sigma, Poole, Dorset, UK), supplemented with 8% heat-inactivated fetal bovine serum (FBS) (BroWhittacker, MD, USA) and 100μg Kanamycin. The tachyzoites were freed from host cells and purified by shearing them through a 27-gauge needle and filtering the suspension through a 5.0μm-pore filter (MILLIPORE). Twenty four SAG-1 (P30) transgenic mice and 24 of their P30− littermates were divided into 3 groups of eight and infected intraperitoneally with 5 x 10^2 tachyzoites of the RH strain of T. gondii per animal as described by Seng (1999). The mice were sacrificed on days 3, 5 and 7. The day of infection was taken as Day 0. Spleens were collected and dendritic cells isolated. Non-infected mice were sacrificed and used as controls. All experiments using mice were carried out in accordance with rules and ordinances of the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine and are in line with acceptable international standards. (Moore and Mepham, 1995; Polites and Pinkert, 2002; Brown and Murray, 2006).

Monoclonal Antibodies

Anti-mouse monoclonal antibodies (mAb) used in this study are summarized in Table 1.

### Table 1. Anti-mouse monoclonal antibodies used in this study. All antibodies were purchased either from BD Pharmingen, USA or Serotec Ltd, England.

<table>
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<td>Kraal et al., 1986</td>
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<td>2.4G2</td>
<td>CD16/CD32</td>
<td>IgG2b</td>
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<tr>
<td>JES5-16E3</td>
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<td>IgG2b</td>
<td>Rat</td>
<td>Sander et al., 1993</td>
</tr>
<tr>
<td>XMG1.2</td>
<td>IFN-γ</td>
<td>IgG1</td>
<td>Rat</td>
<td>Prussin &amp; Metcaife, 1995</td>
</tr>
<tr>
<td>C15.6</td>
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<td>Prussin &amp; Metcaife, 1995</td>
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<tr>
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<td>IL-4</td>
<td>IgG1</td>
<td>Rat</td>
<td>Sander et al., 1993</td>
</tr>
</tbody>
</table>

Abbreviations: CD-Cluster of Differentiation; IL-Inter Leukin; IFN-γ-Interferon-gamma

Isolation of dendritic cells from the spleen

Spleen cells were isolated as described by Makala, 1996; Makala et al. (1998); Makala et al. (2000); Makala et al. (2003b) with some modifications. Briefly, mice were slaughtered and the spleens removed aseptically. Spleens were then cut up into tiny pieces and disrupted mechanically by passing through a nylon cell strainer (70μm-pore) (Becton Dickinson, USA). Resultant cells were suspended in tissue culture medium and layered onto 14.5% w/v analytical grade metrizamide (Nycomed AS, Oslo, Norway) in RPMI-1640 without bicarbonate and supplemented with 2% FBS, at 10ml of cells to 2ml of metrizamide, and centrifuged at 600g (with brakes off) for 10 minutes at room temperature. Low-density cells were removed from the interface, washed twice and re-suspended in tissue culture medium. 0.83% ammonium chloride was used to lyse contaminating red blood cells by incubating for 5 minutes at 37°C and finally washing in PBS containing 5% FBS (BroWhittacker, MD, USA). The cells were incubated for 1 hr on ice with an Fc blocking agent, CD16/32 antibody and FITC-labeled anti-CD205 (NLDC-145) as FITC control in PBS containing 5% FBS. PE-labeled anti CD80 (16-10A1) was used as the control for the PE staining. Cells were then washed twice in PBS with 5% FBS, and re-suspended 1ml of PBS.

Isolation of Unfractionated splenocytes

One or two spleens were isolated from the mice aseptically and then cut up into tiny pieces and disrupted mechanically by passing through a nylon cell strainer (70μm-pore) (Becton Dickinson, USA). 0.83% ammonium chloride was used to lyse contaminating red blood cells by incubating for 5 minutes at 37°C and finally washing in PBS containing 5% FBS (BroWhittacker, MD, USA). Splenic dendritic cells (DC), unfractionated splenocytes (UF) and leukocytes of the discrete Peyer's patches (DPP) were prepared for intracellular cytokine assay by incubation with Brefeldin A, followed by the
parafomaldehyde-saponin procedure previously described (Pala et al, 2000; Huang et al, 2001) with modifications. Briefly, 2μg/ml of Brefeldin A were added to 1ml cell suspension in PBS and incubated in a water 10 min, and washed twice in PBS/FBS/EDTA. For intracellular cytokine staining, cells were washed and stained in PBS/EDTA/1% FCS buffer containing 0.1% saponin. PE-conjugated Anti-IL-12, Anti-IL-10, Anti-IL-4 and Anti-IFN-γ antibodies (BD Pharmingen, USA) were used for staining. After incubation, cells were washed, analyzed and fluorescence quantified using a Coulter EPICS-XL flow cytometer at 488nm. Cells were electronically gated on forward angle light scatter to exclude debris and on 90º light scatter to exclude granulocytes.

RNA detection by Reverse transcriptase-Polymerase Chain Reaction
Unfractionated spleen cells and splenic dendritic cells from each of the mice groups were re-suspended in RNAzol and frozen at -70ºC. The cells were then homogenized and processed for mRNA isolation and amplification as described by Gazzinelli et al (1994). RNA detection for the cytokines IL-4, IL-10, IL-12 and IFN-γ, as well as the house-keeping gene β-actin were performed using a One-Step RNA PCR kit (Takara Biomedical, Japan) and sets of specific primers for each of cytokine. The primers used for amplification were as follows: IL-4 (sense): 5'- ACC CCC AGC TAG TTG TCA TC-3’;(antisense): 5’-ATG GGC TCC CTT CTC TG-3’;
IL-10 (sense): 5’- GGG GGA TTT AGA GAC TTG CT-3’;(antisense): 5’-CAC AGG GGA GAA ATC GAT GA-3’;
IL-12 (sense): 5’- TGG AAT GGC GTC TCT GTC TG-3’;(antisense): 5’-GGC GGG TCT GGT TTG ATG AT-3’;
IFN-γ (sense): 5’- AAC GCT ACA CAC TGC ATC T-3’;(antisense): 5’-ATG GCC TGA TTG TCT TTC A-3’, and
β-actin (sense): 5’- ATG GAT GAC GAC ATC GCT-3’;(antisense): 5’-ATG AGG TAG TCT GTC AGG T-3’. One μl of each test sample was added to 49μl of amplification mixture whose constitution as follows (according to manufacturer’s recommendations): 5μl of 10X One Step RNA PCR buffer, 10μl of 5mM MgCl2, 5μl of 1mM dNTP, 1μl each of RNase Inhibitor (0.8U/μl), AMV RTase XL (0.1U/μl), AMV-Optimized Taq (0.1U/μl) and 1μl Specific Primer-sense and -antisense (0.4μM). 24μl of RNase water was finally added to give a total mixture of 50μl. The RT-PCT was carried out using the following protocol on the automated thermocycler (GeneAmp PCR System, Perkin Elmer, USA); reverse transcription was carried out for 30 minutes at 50ºC before the amplification conditions were adjusted appropriately for each of the cytokines. In general, the conditions consisted of denaturing at 94ºC for 2 minutes, followed by 40-50 cycles of amplification at 94ºC for 30sec, 55-56ºC for 30sec and 72ºC for 1 minute. Finally, the extension period was carried out at 72ºC for 7-10 minutes before resting the reaction at 4ºC. The products were then visualised on an ethidium bromide-stained 1.5% agarose gel for 30 minutes and photographed under ultraviolet (UV) light.

Results
Yield, purity and viability of dendritic cells
The yield of the splenic cell population of 6-8 non-infected and Toxoplasma gondii-infected P30+ mice and their P30- littermates after mechanical disruption ranged from 0.7 x 10^8 – 1.1 x 10^8. The cell suspension of the low density population obtained after metrizamide centrifugation consisted of approximately 0.7 x 10^7 - 1 x 10^7 cells. The gradient cells consisted of a cell population characterized by expression of constitutively high levels of the DC marker, CD205 which was always greater than 85% in both non-infected and T. gondii-infected mice. Moreover, MHC Class II products expression showed high levels in both non-infected and T. gondii-infected mice. The yield of the splenic cell population of 6-8 non-infected and Toxoplasma gondii-infected P30+ mice and their P30- littermates after mechanical disruption ranged from 0.7 x 10^8 – 1.1 x 10^8. The cell suspension of the low density population obtained after metrizamide

Table 1. Expression of surface membrane markers CD80, CD205 and MHC Class II on splenic dendritic cells (DC) and unfractionated from non-infected (Day 0) and Toxoplasma gondii-infected P30+ve mice and their P30-ve littermates 3, 5 and 7 days post-infection. The DC were isolated from the unfractionated splenocytes by mechanical tissue disruption followed by metrizamide gradient centrifugation. Data are expressed as percentages from a log-scale.

<table>
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<tr>
<th>Specificity</th>
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<th>P30 Positive mice</th>
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<td><strong>MHC II</strong></td>
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</table>

Abbreviation: CD- Cluster of differentiation; DC-dendritic cells; MHC-Major histocompatibility complex.

**Morphology**

It is well established that expression of CD205 and MHC Class II alone are not enough criteria to identify DC subsets. We therefore analyzed additional criteria of morphology and surface antigens. Using the well established criteria of selective size, cytoplasmic:nucleus ratio, shape and position of nucleus and cytoplasmic processes of the low density cells, the morphological features of the resultant gradient cells were analyzed.

Mouse spleen macrophages were used as controls (data not shown). Subjectively, the putative DC were smaller than macrophages and cells showed evidence of cytoplasmic processes or dendrites (Figure 1). Under light microscopy, these cells were observed to continually extend, retract or re-orientate their cellular processes when observed over a long period of time (data not shown).

**Expression of surface antigens**

Several studies have demonstrated that MHC Class II products and co-stimulatory ligands in DC are essential for DC activation and consequently, antigen presentation (Makala, 1996; Makala et al., 1998). We were therefore prompted to analyze their expression in the isolated cells as additional DC identifying criteria by flow cytometric analysis. Flow cytometry of the low-density cells (LDC) from both P30+ transgenic mice and their P30- littermates showed (Table 1 and Figure 2) that over 85% expressed the pan-DC marker CD205 (Kraal et al., 1986). The co-stimulatory ligand CD80 was significantly up-regulated in both P30+ and P30- mice following infection with T. gondii (Table 1, Figure 3 and 4). Moreover, flow cytometric analysis showed that MHC Class II products on DC increased significantly following infection.
Figure 2. One parameter histograms showing expression of CD205 on splenic dendritic cells in mice infected with *T. gondii*. The expressions were carried out in spleen cells before (unfractionated splenocytes) and after metrizamide gradient centrifugation. Percentages of positive cells are given in each panel. The Y-axis represents frequencies showing numbers of positive cells and the X-axis is the log intensity of green (FITC) fluorescence. Data show the respective control staining (filled histograms) and specific monoclonal antibody binding (open histograms).

Figure 3. One parameter histograms showing expression of CD80 on splenic dendritic cells of non-infected and *Toxoplasma gondii*-infected P30+ transgenic mice and their P30- littermates. Percentages of positive cells are given in each panel. The Y-axis represents frequencies showing numbers of positive cells and the X-axis is the log intensity of red (PE) fluorescence. Data show the respective control staining (filled histograms) and specific monoclonal antibody binding (open histograms).
Infection status of the mice

Based on clinical and post mortem signs, the P30-infected mice appeared normal and unaffected by the parasites on day 3 post-infection whereas about 10% of the P30+ mice were dull and had starry coats. On dissection, the spleens in all the mice were enlarged and there was some ascites in the P30+ mice. The discrete and ascites, together with congestion on the intestinal serosa and enlargement of the discrete Peyer’s patches. On day 7 post-infection, 30% of the P30+ mice had succumbed to the disease while those that had survived had died. On dissection, there was splenomegaly, severe ascites, and generalized congestion of the abdominal organs. The intestinal lumina were devoid of faecal material and the abdominal fat was gelatinous. The discrete Peyer’s patches in the P30+ mice were not prominent. The infection status was ascertained by Peyer’s patches and mesenteric lymph nodes were more prominent in the P30+ than in the P30- mice. On day 5 post-infection, all the mice were dull and had not fed well nor drank much water, but the P30+ mice appeared more affected than their P30- littermates. Most had ruffled fur and they were cuddled up together. On dissection they all had splenomegaly were severely emaciated, dull and weak and had ruffled fur. The mortality was principally associated with pneumonia (Francis and Yves, 1991). Their P30-littermates were not as severely affected and none Indirect Fluorescent Antibody Test (IFAT) whose results are shown in Figure 5. The high virulent RH tachyzoites were detected in lungs on day 2-4 post infection, in brain and blood on day 4-6 and the parasitic loads remained consistently at a higher level in lung than brain until slaughter or death (data not shown).

Expression of intracellular cytokines in splenic dendritic cells of T. gondii-infected SAG1 transgenic mice

Flow cytometry of splenic dendritic cells dually stained with FITC-labeled CD205 and PE-conjugated IL-4, IL-10, IL-12 and IFN-γ antibodies showed that IL-10 was the most highly expressed cytokine during the course of toxoplasmosis in both P30+ and P30- transgenic mice (Table 2.). However, whereas in P30+ mice the
expression for IL-10 peaked at Day 5 post-infection, in P30- the levels remained low but rose sharply on Day 7 post-infection. A similar trend was evident for the other three cytokines, but at a lower level of expression (Table 2 and Figure 5.). In both P30+ and P30- mice, the Th2 cytokines IL-10 and IL-4 exhibited higher levels of expression during the course of infection, compared to the Th1 cytokines IL-12 and IFN-\( \gamma \). On the contrary, IFN-\( \gamma \) levels were higher in the non-infected animals compared to IL-4 levels. However, in P30+ mice, showed a slight increase in cytokine expression on Day 3 post-infection whereas no response was observed during the course of infection.

Table 2. Single and dual percentage expression of intracellular cytokines on splenic dendritic cells (DC) and unfractionated splenocytes from non-infected (Day 0) and Toxoplasma gondii-infected P30+ve mice (A) and their P30-ve littermates (B) 3, 5 and 7 days post-infection. Intracellular cytokine transport was blocked with Brefeldin A, after which cells were fixed in paraformaldehyde, permeabilized with saponin and stained first with FITC-labeled anti CD 205 antibody and followed by PE-labeled anti IL-4, anti IL-10, anti IL-12 and anti IFN-\( \gamma \) antibodies. Data are expressed as percentage on a log scale.

(A)

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Abbreviations: IL-interleukin, IFN-\( \gamma \)- Interferon gamma, DC-dendritic cells.
Figure 6. Kinetics of intracellular cytokines IL-4 ($\nu$), IL-10 ($\lambda$), IL-12 ($\sigma$) and IFN-$\gamma$ ($\nu$) in P30+ transgenic mice (A) and their P30- littermates (B) splenic dendritic cells obtained from non-infected (Day 0) and Toxoplasma gondii-infected mice 3, 5 and 7 days post infection (dpi). Surface marker staining with FITC-labeled anti-CD205 antibody was carried out before blocking intracellular cytokine transport with brefeldin A. Cells were thereafter fixed in paraformaldehyde, permeabilized with saponin and stained with PE-labeled anti of IL-4, IL-10, IL-12 and IFN-$\gamma$ antibodies. Data are expressed as percentages of a log scale.

A cytokine-to-cytokine comparison of expression levels between the P30+ and P30- mice shows that the levels of expression were generally higher in the P30+ mice, except on Day 7 post-infection when there was a drastic fall in expression to levels below those expressed in the P30- mice (Figure 7). The exception was however in IFN-$\gamma$ levels which, although were exhibited at peak levels on Day 5 post-infection in the P30+ mice, expression levels were slightly higher compared to their P30- littermates on Day 7. IFN-$\gamma$ levels in P30+ were generally higher when compared to those in the P30- mice.

Figure 7. A comparison of the kinetics of intracellular cytokines IFN-$\gamma$, IL-12, IL-4 and, IL-10 in P30+ transgenic mice ($\nu$) and their P30- littermates ($\lambda$) splenic dendritic cells obtained from non-infected (Day 0) and Toxoplasma gondii-infected mice 3, 5 and 7 days post infection (dpi). Surface marker staining with FITC-labeled anti-CD205 antibody was carried out before blocking intracellular cytokine transport with brefeldin A. Cells were thereafter fixed in paraformaldehyde, permeabilized with saponin and stained with PE-labeled anti of IL-4, IL-10, IL-12 and IFN-$\gamma$ antibodies. Data are expressed as percentages of a log scale.

Cytokine gene detection by RT-PCR

Retgressive studies were carried out to ascertain the presence of genes responsible for the production of the specific cytokines by isolating mRNA from splenic dendritic cells in each of the infected and non-infected P30+ mice groups. The house keeping gene $\beta$-actin was tested in the samples along with the cytokines of interest to prove that RNA had indeed been isolated. All the samples showed an intense band around the 500bp after amplification for $\beta$-actin (Figure 8). The splenic dendritic cells from non-infected P30+ mice showed a fairly strong band for IL-4 at about 650bp and another one at about
900bp. A weak band was visible at about 500bp. In the infected mice, however, there was no visible band. The unfractionated splenocytes did not show any bands in both the infected and non-infected groups. IL-10 showed clear bands at about 350bp in both unfractionated was even weaker in the infected groups. The unfractionated splenocytes apparently did not display this band. However, another band was present in both unfractionated splenocytes and dendritic cells at about 1000bp and this band was more prominent in the infected than in the non-infected mouse groups, especially so in the unfractionated splenocytes. There were clear bands for IFN-γ at 300bp in both unfractionated splenocytes and splenic dendritic cells from the infected and non-infected splenocytes and dendritic cells of the infected and non-infected P30+ mice. The bands were more prominent in the infected than the non-infected mice groups. IL-12 showed a weak band at about 450bp in the splenic dendritic cells from non-infected P30+ mice, but the band P30+ mice groups. This band was bigger in the infected than in the non-infected mice groups and the difference was more striking for the splenic dendritic cells Another prominent band was evident at 800bp in the dendritic cells from non-infected P30+ mice groups but absent in the unfractionated splenocytes. This same band was however, present in both unfractionated splenocytes and splenic dendritic cells in the infected mice groups.

Figure 8. Qualitative RT-PCR amplification of mRNA of inflammatory intracellular cytokine genes in unfractionated splenocytes and splenic DC obtained from non-infected (lanes 1 and 2) and Toxoplasma gondii-infected P30+ adult mice (lanes 3 and 4) respectively. mRNA was extracted from unfractionated splenocytes and DC as reported in materials and methods. Fifty amplification cycles were performed. The house keeping gene, β-actin was used as a control. Arrows point at the specific bands for the amplified β-actin or cytokine genes.

Abbreviations: M- 100 base pair ladder; IL-Interleukin; IFN-γ -Interferon gamma; DC- dendritic cells; mRNA-Messenger Ribo nucleic acid; RT-PCR-Reverse transcriptase polymerase chain reaction; β-actin- Beta actin

Discussion

Dendritic cells constitute only about 1% of all cells in the mammalian body, despite the fact that they are found in almost all tissues and organs (Makala and Nagasawa., 2002; Pillarisetty et al., 2003). Isolation and purification procedures must therefore aim at pooling the DC together while reducing the numbers of contaminating cells as much as possible. The cell population after mechanical disruption of the spleens from 6-8 mice ranged from 0.7 x 10^8 – 1.1 x 10^8. After metrizamide density centrifugation, the cell suspension consisted of approximately 0.7 x 10^7 - 1 x 10^7 cells. In a study carried complexity is enhanced by the fact that there is no single molecule known to be uniquely expressed by DC (Makala and Nagasawa., 2002). The combination of several markers defines a DC sub-population. For instance, in the spleen there are at least two sub-populations of DC, those that are of lymphoid origin and are CD8+, DEC-205^high, Mac-1^low, and those that are of myeloid origin and are CD8-, DEC-205^low Mac-1^high (Anjuere et al., 1999). The diverse anatomic distribution of members of the DC lineage indicates that they are a multi-potential end cells and express surface a difficult task. In this study, purification by metrizamide density centrifugation followed by DEC-205 (now
designated as CD205) and MHC Class II staining gave a purity of over 85% and 90% respectively (Figure 2 and Table 1). Taking expression of high levels of CD205 and MHC-Class II products as the main identifying criteria for DC, the majority of the isolated cells would be DC.

mice infected with Neospora caninum (a coccidian parasite that is morphologically similar to T. gondii) showed significant up-regulation of CD80 from day 3 post infection and which doubled up at day 7 post infection. The interaction of co-stimulatory molecules on T cells with B7 molecules on antigen presenting cells plays an important role in the activation of naive T cells. Full T-cell activation requires both an antigen-specific and a second co-stimulatory signal. Co-stimulation dictates the outcome for T-cells through the binding of B 7.1 (CD80) and B 7.2 (CD86) expressed on antigen-presenting cells to CD28 and CTLA4 on T-cells (Erbes et al., 2002). The unique capacity for DC to sensitize naive T-cells (Steinman, 1991) correlates with elevated expression of MHC antigens as well as co-stimulatory molecules. Primary humoral or cellular response is strictly B7 dependent and B 7.1 and B 7.2 mediate overlapping co-stimulatory functions, as either molecule alone is sufficient to initiate an immune reaction (Lespagnard et al., 1998).

Intracellular cytokine staining is a relatively new technique (Sander et al., 1991) which is yet to be perfected. However, it has an advantage over other tests such as ELISA in that with multi-colour staining, exclusive or mutual co-expression of different cytokines in individual cells can be obtained (Pala et al., 2000). In the present study, brefeldin A was found to be an efficient blocker of intracellular cytokine transport, resulting in expression of significantly higher levels of cytokines when compared to expression without brefeldin A action (data not shown). Indirect fluorescent antibody test (IFAT) results showed that the mice had antibodies against the T. gondii as early as day 3 post-infection (Figure 5) especially in P30+ mice. Moreover, Th2 cytokines IL-10 and IL-4, which are responsible for humoral response, were expressed at higher levels than Th1, pro-inflammatory cytokines IL-12 and IFN-γ, with IL-10 showing the highest expression in both groups of mice (Figure 6). Although it has been established that toxoplasmosis induces a primarily Th1 response by T-cells and macrophages, (Gazzinelli et al., 1994; Denkers et al., 1994; Nagasawa et al., 1996; Denkers and Gazzinelli, 1998; Ely et al., 1999), Gazzinelli and co-workers (1996) found that in the absence of endogenous IL-10, mice acutely infected with T. gondii readily succumbed. What they did not however ascertain was which cells are responsible for producing the IL-10 whose role appears not only to modulate both IL-12 and IFN-γ synthesis but also to stimulate humoral response. The results of the current study suggest that splenic dendritic cells may be important producers of IL-10. 30% of the T. gondii-infected P30+ mice succumbed whereas all their CD80 was up-regulated immediately after T. gondii infection of the P30+ mice, as compared to their P30-littermates in which the up-regulation was apparent on day 5 post infection (Figure 4). Similar work carried out by Veeraseatukul and Chutipongvivate (2005) in BALB/c similarly infected P30- littermates survived. Added to the fact that IL-10 and IL-4 peak expressions in P30+ mice were at day 5 post-infection, and at day 7 post-infection in the P30- group, it is probable that the premature production of these Th2 cytokines may have inhibited the pro-inflammatory response, resulting in higher mortality.

This in effect, implies that P30 transgenic mice are unresponsive to acute T. gondii infection (Seng et al., 1999; Seng et al., 2002) because their cell-mediated, pro-inflammatory immune response is inhibited by high level production of Th2 cytokines by splenic dendritic cells. This prevents the transgenic mice from producing a host protection against rapid tachyzoite growth, resulting in high mortality rates.

In conclusion, unresponsiveness to SAG1 of T. gondii by SAC1 transgenic mice may be caused by inhibition of Th1 cytokines production as a result of high level production of IL-10 by splenic dendritic cells. These results provide evidence that SAG1 is one of the important antigens involved in the induction of immune responses towards development of Th1-protective immunity during T. gondii infection. Transgenic mice may be a considerably valuable tool for dissecting the molecular mechanisms of the pathogenesis and host immune responses with regard to SAG1 function during T. gondii infection.

ACKNOWLEDGEMENTS

The authors acknowledge the Japanese Government, through the Japanese International Co-operation Agency (JICA) for funding and the Government of the Republic of Kenya, through the Director of Veterinary Services for facilitation of this study which was carried out at the National Research Center for Protozoan Disease, Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan.

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Inhibition of Listeria monocytogenes and Yersinia enterocolitica in minced beef by naturally occurring antimicrobial
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Department of Chemistry, Nutritional deficiency and Toxin Animal Health Research Institute, Egypt.

Abstract
The inhibitory effects of some plant extracts such as rosemary, thyme and garlic applied to minced beef were determined on L. monocytogenes and Y. enterocolitica at concentrations 10^5 cfu/g and 6×10^5 cfu/g, respectively and stored at 5°C for 16 days. The results showed that L. monocytogenes was more sensitive to rosemary and garlic extracts than Y. enterocolitica. The application of rosemary and garlic extracts to minced beef was found to significantly reduce (P< 0.05) the L. monocytogenes at 4th, 8th, 12th and 16th days, whereas the thyme reduced it only at 4th day as compared to pretreatment and control group. Rosemary and garlic extracts were effective at inhibiting growth of Y. enterocolitica at 4th and 8th days, while thyme can significantly reduced it at 12th and 16th days as compared to pretreatment and control groups.

Introduction
Microbial contaminations of food are often attributed to the microflora residing in ingredients that comprise the food, or originating from the food manufacturing practice during processing and storage. The proliferation of resident microorganisms not only causes food spoilage, but more seriously, results in food poisoning products may be necessary and useful to prolong their storage shelf life and to prevent meat-borne diseases (Fernandez-Lopez et al., 2005). Natural antimicrobials agent are defined as components that can exhibit antimicrobial activity in the foods in which they are commonly used as ingredients; they may also be used as additives in other foods (Pliego, 2007). Rosemary (Rosmarinus officinalis L.) originally grows in southern Europe. Its herb and oil are commonly used for spicing and flavoring food. It has high antioxidant activity and lately it has been used as antimicrobial agent. Rosemary plants are rich sources of phenolic compounds with high antimicrobial activity against both Gram-positive and Gram-negative bacteria. High percentage of the antimicrobial activity is attributed to carnosic acid and carnosol (Moreno et al., 2006). Thyme is an aromatic plant known by its an who named it allicin. These antimicrobial compounds are absent in intact garlic, but are generated from their common precursor, allicin, through enzymatic action when garlic is damaged. The antimicrobial activity of thiosulfimates-including allicin is due to a reaction between thiosulfinites and SH groups of essential cellular proteins (Shim and Kyung, 1999).

Materials and methods
Preparation of samples of meat samples
Whole sirloins were purchased aseptically in sterile polyethylene bags from a local retailer. Excess fat was trimmed with sanitized knife. Sirloin tips were placed on a rack, covered and roasted. Roasted sirloin tips were then cooled to 5°C in refrigerator. Roasted beef was sliced with sanitized knife, minced by a sterile glass
blender jar divided into 25 g and placed in sterile Petri dishes and immediately refrigerated (Hefnawy et al., 1993).

Bacterial strain
Listeria monocytogenes and Yersinia enterocolitica were obtained from department of food hygiene, Animal Health Research Institute (AHRI).

Preparation of bacteria
Bacterial cultures were maintained in culture agar at 5°C, then grown in tryptic phosphate broth at 30°C at least twice for 24 hour periods before being used in the experiment. Twenty-four hour culture of each bacterium was sedimented by centrifugation and plates were resuspended in 0.1 M potassium phosphate buffer (pH 7.0). The cell suspensions were serially diluted to an approximate final concentration of 10^6 CFU/g (Hao et al., 1998).

Antimicrobial plant extracts
The antimicrobial plant extracts used were rosemary, thyme and garlic. All extracts were acquired from commercial chemical and flavor vendors. All extracts were dissolved in ethyl alcohol (to make 20% solution), which allowed better distribution of extracts in the minced beef samples according to Hao et al., (1998).

Treatment:
One-tenth milliliter of extract was uniformly deposited on each 25g minced beef samples using a pipette and then spread over the surface with sterile bent glass rod. Control treatment consisted of minced beef samples to which only 0.1 ml ethyl alcohol was applied to that remained untreated. All samples were kept in a laminar flow biological safety cabinet for 15 minutes for alcohol to be evaporated before being inoculated with tested pathogens. The treated samples were then inoculated with 0.1 ml of 10^8 cfu/ml populations of Listeria monocytogenes and 0.6 ml of 10^6 cfu/ml population of Yersinia enterocolitica. Inoculated samples were held undisturbed for 30 minutes to allow residual moisture to be evaporated or absorbed. Samples were then placed into sterile polyethylene bags and kept at 50°C and the populations of Listeria monocytogenes and Yersinia enterocolitica after 0, 4, 8, 12 and 16 days were detected in duplicates (Hao et al., 1998).

Bacteriological examination:
Enumeration of Listeria monocytogenes was done according to McClaine and Lee (1988) and Curtis et al., 1989 and Yersinia enterocolitica according to Bodnaruk and Draughon (1998).

Statistical analysis:
Statistical analysis was done using SAS (Littell et al., 1991)

Results

Table 1. Listeria monocytogenes population (log_{10} cfu/g) in minced beef stored at 5°C (mean±SE).

<table>
<thead>
<tr>
<th>Day</th>
<th>Plant extracts</th>
<th>0</th>
<th>4th</th>
<th>8th</th>
<th>12th</th>
<th>16th</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5.00±0.01a</td>
<td>4.40±0.01Ab</td>
<td>3.54±0.02Ac</td>
<td>2.94±0.02Ad</td>
<td>2.00±0.01Be</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>5.00±0.01a</td>
<td>4.40±0.01Ab</td>
<td>3.54±0.02Ac</td>
<td>2.94±0.02Ad</td>
<td>2.00±0.01Be</td>
</tr>
<tr>
<td></td>
<td>Rosemary</td>
<td>5.00±0.01a</td>
<td>4.40±0.01Ab</td>
<td>3.54±0.02Ac</td>
<td>2.94±0.02Ad</td>
<td>2.00±0.01Be</td>
</tr>
<tr>
<td></td>
<td>Thyme</td>
<td>5.00±0.01a</td>
<td>4.40±0.01Ab</td>
<td>3.54±0.02Ac</td>
<td>2.94±0.02Ad</td>
<td>2.00±0.01Be</td>
</tr>
<tr>
<td></td>
<td>Garlic</td>
<td>5.00±0.01a</td>
<td>4.40±0.01Ab</td>
<td>3.54±0.02Ac</td>
<td>2.94±0.02Ad</td>
<td>2.00±0.01Be</td>
</tr>
</tbody>
</table>

Values with different superscripts in each row (a,b,c,d,e) and each column (A,B,C,D) differ significantly (P< 0.05).

Table 2. Yersinia enterocolitica population (log_{10} cfu/g) in minced beef stored at 5°C (mean±SE).

<table>
<thead>
<tr>
<th>Day</th>
<th>Plant extracts</th>
<th>0</th>
<th>4th</th>
<th>8th</th>
<th>12th</th>
<th>16th</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5.80±0.01b</td>
<td>5.74±0.02B,c</td>
<td>5.84±0.02A,b</td>
<td>5.94±0.02A,a</td>
<td>5.80±0.01A,b</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>5.80±0.01b</td>
<td>5.74±0.02B,c</td>
<td>5.84±0.02A,b</td>
<td>5.94±0.02A,a</td>
<td>5.80±0.01A,b</td>
</tr>
<tr>
<td></td>
<td>Rosemary</td>
<td>5.80±0.01b</td>
<td>5.74±0.02B,c</td>
<td>5.84±0.02A,b</td>
<td>5.94±0.02A,a</td>
<td>5.80±0.01A,b</td>
</tr>
<tr>
<td></td>
<td>Thyme</td>
<td>5.80±0.01b</td>
<td>5.74±0.02B,c</td>
<td>5.84±0.02A,b</td>
<td>5.94±0.02A,a</td>
<td>5.80±0.01A,b</td>
</tr>
<tr>
<td></td>
<td>Garlic</td>
<td>5.80±0.01b</td>
<td>5.74±0.02B,c</td>
<td>5.84±0.02A,b</td>
<td>5.94±0.02A,a</td>
<td>5.80±0.01A,b</td>
</tr>
</tbody>
</table>

Values with different superscripts in each row (a,b,c,d,e) and each column (A,B,C,D) differ significantly (P< 0.05).
DISCUSSION

Plant extracts differed significantly in their ability to inhibit growth of the two pathogens. In general *L. monocytogenes* organism were more sensitive to the plant extracts than *Y. enterocolitica*.

**Effect on *L. monocytogenes***. Samples treated with rosemary extract had bacterial populations that differed from control samples by nearly 1.36, 1.34, 1.00 and 0.62 log unit less *L. monocytogenes*, whereas samples treated with garlic extract differed by nearly 1.56, 1.30, 1.00 and 0.20 at day 4, 8, 12 and day 16 respectively. Moreover, thyme extract reduced the growth of *L. monocytogenes* by 2.28, 1.24, 0.54 and 0.59 log unit at the same days of preservation of samples (Table 1 and Fig.1).

The bacterial populations significantly reduced on days 4, 8, 12 and day 16 in treated samples with rosemary and garlic, but in samples treated with thyme significant reduction was observed on day 4 as compared to pretreatment values (P< 0.05) (Table 1 and Fig 1). Nearly, similar findings were reported by Burt (2004), Rožman and Jeršek (2009) and Pirbalouti et al. (2010).

**Effect on *Y. enterocolitica***. Plant extracts on minced beef were less effective against *Y. enterocolitica* than *L. monocytogenes*. *Y. enterocolitica* population on treated samples were < 1 log units lower than that on control samples, this difference in populations between treated and control samples persisted throughout the storage period (16 days) (Table 2 and Fig.2).

_Yersinia enterocolitica_ significantly reduced on day 4 and day 8 in treated samples with rosemary and garlic (P<0.05), while it deacreased significantly on day 12 and day 16 (P<0.05) in treated samples with thyme extract (Table 2 and Fig.2).

Nearly, similar findings were reported by Kumral and Sahin (2003), Fernández-López (2005) and Oral et al. (2007). From observations on the 2 bacteria, in this study, it may be extrapolated that the Gram positive bacteria were generally more sensitive to the plant extracts than Gram negative bacteria. However, Banerjee and Sarkar (2003) showed that garlic extract possesses a potent bacteriostatic principle against Gram-positive and Gram-negative food borne bacteria. It should be noted, also, that many factors in food could be responsible for the reduction of antimicrobial activity of plant extracts when applied on different types of food. A protective effect of high fat levels was observed when antimicrobial activity of spices and oils diminished in foods as a result of the solubilization of the antimicrobial agents into the food's lipid fraction (Hao et al., 1998). This observation agreed with our results.

Shan et al. (2007) showed that there is a highly positive relationship between antibacterial activity and phenolic content of the tested extracts against *L. monocytogenes*. According to a report of Rasooli et al. (2006), various concentrations of essential oils from *Thymus eriocalyx* and *Thymus x-porlock* tested on agar plates and in broth tubes showed very strong anti-listeria properties. Also, they reported that *Thymus x-porlock* oil was a stronger bactericidal agent than that of *Thymus eriocalyx* oil.

Rožman and Jeršek (2009) confirmed that antimicrobial activity of rosemary (*Rosmarinus officinalis* L.) extracts was depended on the following factors: selected rosemary extract, concentration of extracts, different species of *Listeria* and different strains of *Listeria monocytogenes*.

Sagdic (2003) indicated that two thyme (*Thymus vulgaris* L. and *Thymus serpyllum* L.) hydrosols at 50 and 75 ml/100 ml concentrations had a bactericidal effect against *Y. enterocolitica*. The essential oils of *Thymus daenensis* (Nickavar et al., 2005), *Thymbra spicata* (Hanci et al., 2003) and *Satureja bachtiarica* (Sefidkon et al., 2007) contained high levels of phenolics monoterpenes (thymol and carvacrol) and exhibited antibacterial activity. They
could be a potential source of inhibitory substances against some foodborne pathogens. Spices and herb extracts are used in the food industry, placed in the category of generally recognized as safe (GRAS). Hence both consumers and regulatory agencies are more likely to be comfortable with their use in food. This study has shown that rosemary, thyme and garlic extracts can suppress microbial growth in some situations. However, it must be kept in mind that application of these extracts on food might also adversely affect sensory quality; a possibility that must be investigated before an extract could be used.

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Abstract
Climate change is a phenomenon that is already happening and in some areas of Africa it is taking place at an alarming rate. Climate change is expected to cause an increase in weather-related disasters and extreme weather events, such as droughts, heat waves, floods, desertification, and vector-borne infestations. Long-term changes in climate exacerbate environmental degradation leading to loss of wildlife habitat in many vulnerable places. Furthermore, climate change will alter the location and nature of the geographical environment, and wildlife will be forced to migrate to new areas as a way of adapting or face extinction. As there are limited natural places left for wildlife to move to, this will likely bring wildlife into more densely populated human areas, and create situations of structure of terrestrial ecosystems thus making human wildlife conflicts inevitable in some regions with East African countries rising global average sea levels all point directly to a warmer planet (IPCC, 2007a & b). There is overwhelming evidence that humans are contributing to global warming. Most of the observed increase in temperatures since the mid-20th century is very likely due to the observed increase in anthropogenic greenhouse gas concentrations (IPCC, 2007a & b). Discernible human influences now extend to other aspects of climate, including ocean warming and acidification, change in continental average temperatures, temperature extremes and wind patterns. Anthropogenic greenhouse gas emissions are also one of the main contributing factors to sea-level rise (IPCC 2007). Climate change is increasingly recognized as among the greatest challenges human society will face over the coming century and is considered to be the greatest environmental challenge facing the world today (Sachs et al., 2004; DFID 2004). Climate change may be limited to a specific region, or may occur across the whole Earth. It is becoming evident that, climate change may affect everything from basic ecosystem processes to the spread of disease and change in biodiversity. Some of the greatest impacts are anticipated to occur due to increase in the frequency and intensity of extreme climate events that include storms, floods and droughts. Extreme weather events already account for over 70% of recorded disasters and are known to have a disproportionate impact on disadvantaged communities (Mwitubani & van Wyk, 2009). The recurrent losses due to such extreme events have, been identified as a major factor contributing to endemic poverty. Because of the lack of economic, development and institutional capacity, African countries are likely among the most vulnerable to the impacts of climate change (IPCC, 2007a & b; Brown et al., 2008). Climate change is likely to affect every part of the earth including all human and natural systems, but the degree to which such impact will produce damage will differ, depending on geographical circumstances, the capacity to withstand the impact and the nature of the economy. Though developing countries contribute the least in causing the problem, they are the most vulnerable to its impacts (Fredrick & Major, 1997; Thomas et al., 2008). Climatic change will have far-reaching, negative impacts on the availability of water resources, food and agricultural security, human health, tourism, coastal development and biodiversity (Fig 1).

Introduction
Climate change is real and happening now. The earth’s climate is projected to undergo significant changes during the twenty-first century. The Inter-governmental Panel on Climate Change (IPCC), the United Nation’s agency in charge of providing policymakers with an objective source of information about the phenomena, stated in its Fourth Assessment Report that ‘warming of the climate system is unequivocal’ (IPCC, 2007a & b). IPCC defines climate change as: “Any change in climate over time, whether due to natural variability or as a result of human activity”. It is now unequivocal that our Planet is warming,...
than 0.5°C per decade (high scenario) in the 21st century. The warmer temperatures in this region may lead to increase in rainfall for December to February (wet months) and decrease in rainfall from June to August (dry months). Rainfall changes and variations are not expected to be constant, but rather more sporadic and unpredictable, resulting in periods of prolonged droughts and periods of high rainfall leading to floods. Such extreme events like drought and floods associated to climate change have reduced the volume of river flow through evaporation and siltation (Figs 2 & 3). Furthermore, periods of both prolonged drought and of high rainfall changes and variations all of which led to famine in the region (Hulme et al., 2001; Nganga, 2006; Mwituru & van Wyk, 2009).

The recession and drastic decline of glacial melting on top of regional mountains (Kilimanjaro, Kenya and Ruwenzori) is another indicator of climate change in the region (Fig 4). It is speculated that complete disappearance of Kilimanjaro’s glaciers is expected by 2015 - 2020 (Thompson et al. 2002). The disappearance of the glaciers will affect agricultural activities, availability of water from both rural and urban population and wildlife/tourist activities.

In Eastern Africa, the conservation areas including the semi-arid areas and lakes are among the major victims of the deleterious effects of climate change. Shrinking and decline in lake and river levels are constant reminder of environmental changes due to climate change and other anthropogenic activities.

Other evidence of climate change include sea level rise along coastal areas of Kenya and Tanzania. This rise is likely to disrupt economic activities such as tourism, mining and fisheries. This rise and resulting coastal erosion due to coral reef loss is a major coastal management issue (Magadza, 2000). Sea-level rise will also threaten the availability of freshwater by causing salt water intrusion into coastal aquifers and deltas. Climate change is expected to significantly alter East African biodiversity as species struggle to adapt to changing conditions (Lovett et al., 2005). Biome sensitivity assessments in East Africa show deciduous and semi-deciduous closed-canopy forests may be very sensitive to small decreases in precipitation during the growing season. This illustrates that deciduous forests may be more sensitive to reduced precipitation than grasslands or savannahs (Erasmus et al., 2002). Invasive species and other species with high fertility and dispersal capabilities have been shown to be highly adaptive to variable climatic conditions. Climate change has the potential to alter migratory routes (and timings) of species that use both seasonal wetlands (e.g. migratory birds) and track seasonal changes in vegetation (Campbell et al., 2009). This may for instance increase conflicts between people and large mammals such as elephants, particularly in areas where rainfall is low (Thirgood et al., 2004).

A change in the intensity or duration of the rainy versus dry seasons could change relative breeding rates and, hence, genetic structures in these populations (Poole, 1989; Rubenstein, 1992; van Roomen & Wahl, 2008). Large changes in ecosystem composition and function because of regional climate change would have cascading effects on species diversity (Wrana et al 2006a & b; van der Wal et al., 2008). In sub-Saharan Africa, which includes parts of East Africa, several ecosystems, particularly grass and shrub savannas, are shown to be highly sensitive to short-term availability of water due to climate variability (Vanacker et al., 2005). Climate projections suggest that during already dry months, less precipitation will occur, reducing the resilience of plants (Vanacker et al., 2005). Species ranges will probably not shift in cohesive and intact units and are likely to become more fragmented as they shift in response to changing climate.

Indicators of East Africa’s vulnerability as outlined above show that many species of plants and animals are rapidly becoming extinct, tree density and floristic richness are decreasing, new species of plants and animals rarely showing up in the ecosystem, the disruption and reduction of the fruiting intensity of some trees, aberration in animal matings, changes in bird and animal migratory pattern (due to the need for new habitats or new food sources) and changes in fish spawning patterns, species of plants and animals. All these point to the fact that climate change will lead to failure of many systems including the environmental sector that includes wildlife and tourism which may jeopardize wildlife/tourism dependent- economies. Natural resource managers, therefore need to be well informed to enable them to either take precautionary measures against the destructive effects or combat the effects by devising adaptive measures.

**Causes of Human Wildlife Conflict (HWC)**

Human-wildlife conflict is considered to be any and all disagreements or contentions relating to destruction, loss of life or property, and interference with rights of individuals or groups that are attributable directly or indirectly to wild animals. Human-wildlife conflict in East Africa may be divided into two broad categories: 1) true problems between wildlife and people and 2) clashes of interest between people over wildlife, or interpersonal conflicts (centred on resources and the power to control wildlife benefits). HWC has escalated in recent years because of competition between growing human populations and wildlife for the same declining living spaces and resources. The transformation of forests,
savannah and other ecosystems into agrarian areas or urban agglomerates as a consequence of the increasing demand for land, food production, energy and raw materials, has led to a dramatic decrease in wildlife habitats. This is particularly true in Eastern Africa where the human population has almost tripled in the four decades from 1960 to 2010 and where as a consequence, settled agriculture has spread to more marginal rangelands leading to encroachment into wildlife habitats. Access to water is another essential human requirement that has compromised access to water for wildlife. Therefore as habitat gets fragmented, the length of ‘edge’ for the interface between humans and wildlife increases and the wildlife populations become compressed in insular refuges. Under these conditions, contact between human and wildlife is inevitable and conflict between wildlife and local communities inevitably increases as wild animals seek to fulfill their nutritional, ecological and behavioral needs (Muruthi, 2005). Climate change is expected to exacerbate the above situations.

Climate change impacts that create situations of HWC

Longer and more frequent dry periods (droughts) interspersed with intense but shorter & unpredictable periods of rainfall. These weather patterns are likely to deplete water and pasture resources, leading to natural resource scarcity, and increased competition for these resources. These factors spur the continuing migration of rural people and their livestock into areas occupied by wildlife, wildlife forced to migrate to new areas where water is accessible. In such situations, carnivores may kill domestic animals in villages near protected areas (Ogada et al., 2003; Patterson et al., 2004), wildlife destroy food crops as they wander away from the parks in search of food and water. An example is the case in Ladera in Northeastern Province in 2005 where warthogs attacked and killed goats and sheep to drink their intestinal fluids after the natural watering points dried up. In other instances, prolonged drought in certain areas can cause serious wild fires which reduce the quality of most wildlife habitats and force some animals to seek refuge in adjacent habitats, villages and farms.

Impact of erratic precipitations coupled with flash floods

These tend to erode and wash away grass, seeds and fertile soil leaving behind bear rocks. Consequently, the growth of vegetation and grass is inhibited even during good rainfall. Loss of wildlife habitats emerge as a secondary impact of these factors. Wildlife and livestock will be forced to move to areas where pasture can be found. Heavy rains can cause ungulates to disperse in an area and this leads predators to prey on easier targets like livestock found around communal land. For example a rise in lion attacks observed in the Tanzania in 1999 were attributed to the El Niño floods of 1997 and 1998. These heavy rains caused wildlife in many parts of the country to seek higher ground. As the floods receded in 1999, the wild ungulates returned to their normal ranges, leaving the lions with insufficient prey (Packer et al., 2005).

Extreme temperatures and heat waves

Melting of glaciers in key mountains in East Africa due to rise in temperature leads to water shortage and heavy evaporation. Water scarcity interferes with water availability leading to competition for water between livestock, human and wildlife. There are already serious conflicts over limited water resources in the semi arid areas of Kenya and Uganda. Water bodies (rivers and lakes) which have low levels are also areas of human-wildlife interactions. The fishermen who prefer to fish at periods of low water levels in some rivers and lakes because the catches are greater are more exposed to contact with crocodiles during the warm season and this is the period when more numerous crocodile attacks occur (Fergusson, 2002).

Disease incidence

Progressive drought interspersed with floods will increase disease incidence as the stagnant water and warm temperature will expand range and prevalence of vector-borne and water borne diseases. Warm temperature and stagnant surface water are essential for vector breeding (especially mosquitoes). Pastoralist’s encroachment into the protected areas during droughts exposes wildlife to livestock diseases and vice versa. In the droughts of 1993 and 1997, some pastoral communities moved their cattle to new areas., Since some cattle were infected with rinderpest, this escaped into the wildlife population and caused a devastating epidemic in buffalo and certain antelope. The resulting outcome was a conflict between humans and wildlife.

Consequences of Human wildlife conflict

The consequences of the human-wildlife conflict are more serious in the developing countries, especially East Africa where livestock holdings and agriculture are an important part of rural people’s livelihoods and incomes. In this region, local people with a low standard of living are particularly at risk, as are agropastoralists who depend exclusively on production and income from their land. Some of the consequences of HWC in East Africa are briefly described below.

Human injuries and deaths

Injuries to people mostly occur as a result of chance encounters with elephants, buffalo, hippopotamuses and lions, usually along paths between dwellings and a water source (Muruthi, 2005; Packer et al., 2005). Contact with crocodiles when bathing or collecting water more
have become true sylvatic maintenance hosts of a whole. For example, buffalos and certain antelopes the survival of wildlife populations or even the species as land, habitat fragmentation or pollution; all pose threats to domestic animals to wildlife, competition over grazing consequences. The transmission of diseases from Human-wildlife conflict also has several indirect Disease transmission

security.

dynamics or structure in some species (Bengis et al, prevalence rates is unknown, but preliminary evidence in various wildlife species. The long-term effects of this chronic progressive disease on wildlife host populations at sustained high prevalence rates is unknown, but preliminary evidence suggests that it may negatively affect population dynamics or structure in some species (Bengis et al, Wildlife authorities shoot between 50 to 120 problem elephants each year (WWF, 2007).

Food security and livelihoods
In most of rural Eastern Africa, food security is precarious, relying on a single cropping season or on the sale of livestock. Although on a national scale, the loss of two hectares of maize to elephants in a single night might not be noticed, it can mean the loss of their food supply for the year to the family concerned, and the difference between self sufficiency and destitution. This is particularly serious where governments do not have the capacity to pay compensation for losses. For example, owners of large farms situated on the edge of Kibale National Park in Uganda can employ guards or create a crop buffer zone to separate vulnerable yields from the forest edge, by cultivating less palatable plant species or using the land for pasture. These options are not available to subsistence farmers, who have less choice in their land use and cannot afford to pay for guards (Naughton-Treves, 1997). Elephant damage to food crops accounts for 75 to 90 percent of all damage caused by large mammals in the semi-arid areas of Zimbabwe and Kenya (Hoare and Mackie, 1993). Elephants can also damage food stores during the drier months following the main harvest. The loss of this stored food is considered far more disruptive to farmers than the raiding of crops while they are still growing in the fields, because so much damage can be done to a concentrated food source in a short space of time. Likewise, the loss of a family’s small herd of cattle to lions can effectively destroy that family’s wealth and way of life. For rural populations, domestic animals are not only their main resource through production of manure, milk, meat, and live sales, but are also their only source of wealth (means of saving, source of income, social role). Predators such as lions often kill numerous domestic animals such as cattle in one raid, and can devastate a household’s food supply. Canine distemper virus prevalent in domestic dogs has apparently crossed the species barrier in the Serengeti ecosystem, causing significant mortalities in lions. It is estimated that 30 percent of lions in the Serengeti died in this outbreak. The major population decline of the wild dog in this ecosystem may also be attributed in part to canine distemper (Bengis et al, 2002). The competition between growing human populations and declining wildlife populations for the same living space and resources has been demonstrated as being the underlying cause of the decline in the continent’s elephant populations (Parker and Graham, 1989).

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Health and employment
HWC can have repercussions on health and employment in those who encounter wildlife. Nuisance encounters

Killing of wildlife
The killing of wild animals in retaliation for incidents of human-wildlife conflict is a common reaction, even though the identification of the real culprit is seldom possible. This is particularly true for predators, but also for other species. Several species of large carnivores such as lions or hyenas have been eliminated from a large part of their former home ranges in response to human-wildlife conflict. Illegal killing of predators, including poisoning, shooting and trapping, is still one of the greatest threats to these species (Muruthi, 2005). In Northern Kenya, the number of predators killed by farmers has been reported to be equal to the number of livestock killed by lions, hyenas and leopards (Ogada et al., 2003). Kenyan pastoralists poisoned all the lions in Amboseli Reserve in 1990 and speared 27 out of 40 lions in Nairobi National Park in 2003. Pastoralists in several districts of Tanzania also poison lions (Packer et al., 2006; Shemwetta & Kideghesho, 2000; Kideghesho et al., 2005). When a crocodile kills or injures a human, the human response is to kill or remove not just the individual crocodile responsible, but the whole local population. In general more crocodiles are killed in retaliation than the number of people attacked (Wanjau, 2002). Elephants are often killed in retaliation for human deaths. Kenyan security.

Disease transmission
Human-wildlife conflict also has several indirect consequences. The transmission of diseases from domestic animals to wildlife, competition over grazing land, habitat fragmentation or pollution; all pose threats to the survival of wildlife populations or even the species as a whole. For example, buffalos and certain antelopes have become true sylvatic maintenance hosts of mycobacterial disease, and sporadic spill-over of the infection has been documented in various wildlife species. The long-term effects of this chronic progressive disease on wildlife host populations at sustained high prevalence rates is unknown, but preliminary evidence suggests that it may negatively affect population dynamics or structure in some species (Bengis et al, 1989).
with wildlife can expose one to zoonotic diseases, physical injury or even death. All these have high financial costs for individuals and society in the form of medical treatments.

Impact on tourism
HWC has a direct and indirect consequence on both local and international tourism. Increase in HWC will lead to loss of critical wildlife habitats and key species which serve as tourism attraction in East Africa. As a result of this loss there will be a decline in the rate of tourism visitation leading to loss of revenue. HWC puts the local community at loggerheads with conservation initiatives which might trigger hostility towards tourists. HWC might also affect community based tourism initiatives thus affecting the tourism and the income that could be ploughed back into the communities. For example, when a wild animal injures a human being, the humans respond by killing the animal or attacking the tourist who are visiting that particular area. Negative publicity by the media on the increased rate of HWC would scare tourists from visiting the affected areas.

Conclusion
Climate change acts in concert with other factors to affect environmental resources including wildlife and their habitat. Most of the hotspots for future HWC will be around the protected areas and conservation areas housing wildlife. It will create conflicts over the shared environmental resources that include water, grazing and land across the East African countries. In most areas severe drought interspersed with floods will be accompanied by increased violence and insecurity as people, animals including wildlife struggle to access scarce grazing resources.

Recommendations
Unless appropriate mitigation and adaptation strategies are employed, the result has and will continue being conflicts between humans themselves as well as conflicts between humans and wildlife.

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Fig 1: Diagram depicting effects of climate change with emphasis on global warming

Fig 2: Climate change effects on water bodies seen as decline in water levels and dry river
Fig 3: Climate change effects seen as rise in frequency and intensity of droughts and floods

Fig 4: Changes in the glacial cover for Mt Kilimanjaro between 1993 and 2000
Research Article: Wildlife Biology

Outdoor confinement does not compromise reproductive performance of the helmeted guinea fowl (Numidia meleagris)

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Abstract
Two types of housing management were employed in efforts to breed the semi domesticated helmeted guinea fowl: an open-air system (OAS) and a totally confined deep litter system (DLS). This was undertaken for a period of sixteen months spanning over four dry and three rainy seasons. Male courtship displays were evident especially during hot and dry months in both systems. Female displays and mating were observed in wet seasons but only in the OAS. There were three egg-laying phases in the OAS only each occurring during a wet season. Egg fertility ranged from 23 to 35% while hatch rate was between 70 and 89%. An analysis of weight of gonads and oviducts showed that these were significantly heavier (P<0.05) in wet months as compared with dry seasons in the OAS. There were no significant differences (P>0.05) in the weights in the DLS. Measurement of profiles of plasma estradiol, progesterone, testosterone and luteinizing hormone activity revealed adverse effects of the DLS on hormone levels as opposed to the OAS. Evidence is thus provided that outdoor confinement does not compromise reproductive performance of the helmeted guinea fowl. We therefore recommend this novel method as a substitute for rearing the helmeted guinea fowl in situations where the free-range system is not practicable.

Key words: Helmeted Guinea Fowl; Numida meleagris; Domestication; Rearing Methods; Egg Laying; Reproductive Hormones

Introduction
The helmeted guinea fowl (Numida meleagris) is a game bird that has been utilised as a source of protein in human food in Tropical Africa through trapping of adults and collection of eggs. Efforts to domesticate the bird have been successful in West Africa (Ayeni and Ayanda, 1982; Ayeni et al., 1983) where semi domesticated breeds have been developed. An egg fertility rate of about 53% and a hatch rate of up to 87% have been reported in some of the breeds (Ayorinde et al., 1989). However, the semi domesticated breeds still exhibit a seasonal breeding pattern typical of wild populations (Ayorinde and Ayeni, 1986). Reproductive performance of the home raised birds is also much lower than that of the wild ones. An egg fertility rate of 72.4% and a hatch rate of 97.2% have been found in a wild helmeted guinea fowl population in Kenya (Kimata, 2007). Guinea fowl have also been found to attain sexual maturity at about 28 weeks of age (Ogwuegbu et al., 1988) as compared with chicken that start laying much earlier. Despite these drawbacks, the helmeted guinea fowl possesses several advantages over conventional poultry. The bird is indigenous to Africa and is therefore likely to perform better in terms of resistance to drought and diseases. The cholesterol content of the helmeted guinea fowl egg is about 30% lower than that of the chicken egg (Oguntona and Hughes, 1988; Maurice et al., 1994). The guinea fowl egg is therefore much healthier for human consumption. There is also a need to diversify the sources of premium animal protein in human food by bringing in other species such as the East African helmeted guinea fowl. A major task in this effort is to devise practicable rearing methods for the helmeted guinea fowl. The free-range system is most popular in West Africa but is difficult to manage and control especially in densely populated areas. On the other hand, total confinement commonly used in conventional poultry farming has proven to be quite unsuccessful in rearing the helmeted guinea fowl (Ayorinde and Ayeni, 1987). The purpose of this work was to assess the suitability of rearing the East African helmeted guinea fowl in a semi confined outdoor system that is open to sunlight and rainfall. The rationale was to keep the birds in an environment half way between the free range and total confinement. The hypothesis was that semi confinement of the birds outdoors would not compromise reproductive performance. The study questions were; will a semi-confined open-air rearing system support the natural reproductive pattern of the helmeted guinea fowl just like the free-range system? Are there differences in reproductive hormone levels between semi-confined and totally confined helmeted guinea fowl?

Materials and methods
Sixteen (16) male and 24 female helmeted guinea fowl (Numida meleagris) were randomly selected from a 28-week-old semi domesticated stock in a suburban area of Nairobi City. Four males and six females were introduced into each of four wire mesh cages open to sunlight and rainfall. The sex ratio used was adapted from a one male to 1.6 females found in wild helmeted guinea fowl at a private ranch in Kenya (Kimata, 2007). The cage dimensions were 8 square meters on the ground and 4 meters high. This set up was designated open air system (OAS). A similar set up was established in four cages of the same dimensions in a roofed wooden house adjacent to the open cages. This was the deep litter system (DLS). The study site has a mean annual surface temperature of 18-20°C; mean annual rainfall of 1200-1600mm and 50-
of the number of eggs that were found to be fertile. Aradioimmunoassay system described for measurement of steroid hormone levels in human plasma (hRIA) (WHO, 1996) was validated for use with guinea fowl plasma and serum as follows. Samples of helmeted guinea fowl plasma and serum were treated with activated charcoal in order to strip them of steroid hormones. 300 and 1000 femtomoles of human estradiol, progesterone and testosterone were introduced into the charcoal treated samples. Hormone levels were measured by use of the hRIA system. Estradiol recovery in plasma was 88% at the 300 femtomoles dose and 68% at the 1000 dose. In serum, the recoveries were 40% and 60% respectively. Progesterone recovery was 70% and 80% respectively in plasma and 68% and 54% respectively in serum. Testosterone recovery was 73% and 77% in plasma and 68% and 72% in serum. The higher recoveries in plasma suggest that the hRIA system can be used reliably for the assay of estradiol, progesterone and testosterone in guinea fowl plasma. Serum samples are likely to yield inaccurate results.

Whole blood samples (2.5ml) were collected from the left brachial vein of every bird fortnightly for the entire 16 months. Plasma samples were prepared and preserved frozen at -20°C until when required for hormone measurement. Estradiol and progesterone levels were measured in the samples collected from the females by use of the hRIA system. Similarly, testosterone was measured in samples from the males and females. Data were calculated in picograms/ml (pg/ml). Luteinizing hormone (LH) activity was determined in samples from males and females by the indirect bioassay method described by Van Damme et al. (1974). Data were calculated as International Units per liter (IU/l). All data on hormone

2006). The birds were fed on a diet that Clutch size was calculated as total number of eggs per laying female. Number of laying females was obtained from an analysis of shape, size, colour and spotting of individual eggs as well as physical examination of the vent regions of the females. Laying rate was calculated as number of eggs per laying female per day. Random samples of eggs were taken from nests of actively mating helmeted guinea fowl and on the same day that they were laid. The eggs were incubated under chicken within seven days from the date of collection in order to minimize loss of viability that has been found to occur with storage (Brah and Sandhu, 1984; Ayorinde, 1987). Other batches of eggs were left in nests for incubation by the guinea hens. Eggs that failed to hatch after 28 days of incubation were broken and examined. Egg fertility was calculated as percentage of eggs with at least onset of embryogenesis out of all the eggs that were incubated. Hatch rate was the percentage of eggs that hatched out levels were computed as means ± s.e.m and were analysed by Student's t test and Analysis of Variance (ANOVA).

Six males and six females from the semi confined and the totally enclosed groups were decapitated and dissected during the dry spell that preceded the third rainy season. Testes, ovaries and oviducts were obtained and weighed in an analytical balance. The procedure was repeated with a similar number of birds during the third rainy season. Data on reproductive organ weight were subjected to Student's t test.

Results

General observations

Male courtship displays were conspicuous throughout the study period and especially during the dry spells in both the OAS and the DLS. Female displays were confined to the OAS but only during wet seasons. Three egg-laying phases were observed during the study period in the OAS but not in the DLS. Laying rate was 16, 44 and 56% in the three phases respectively. The first egg of the first phase was sighted 32 days after onset of the first rainy season. The birds were 35 weeks old at the time. Laying continued for 16 days. No mating was observed in the first phase. The second phase commenced with incidences of mating that were observed from 10 days after onset of the second rainy season. Close association and mating continued for 21 days. Egg laying commenced 16 days after onset of the rains and proceeded for 58 days. Towards the end of the rainy season, two hens became broody and sat on 32 eggs. The third laying phase started at 16 days after onset of the third rainy season and continued for 72 days. The first incidence of mating in this phase was observed 7 days after onset of the rains. Mating proceeded for about 28 days. Broodiness was evident in five females 40 days
after commencement of the third phase. Broodiness did not get broody continued to lay eggs under the broody ones. This resulted in an incubation of 18 eggs per hen. Clutch size was 3, 14 and 26 eggs in the three laying phases respectively. Egg fertility was 0.0, 24 and 35% in the three respective phases. Hatch rate of eggs was 89% under guinea hens and 70% under chicken. Hatching occurred after 28-30 days of incubation.

Weight of gonads and oviducts
The gonads and oviducts of the guinea fowl (Figure 1) in the OAS were significantly heavier (P<0.05) in the rainy season as compared to the weight in dry spell. There were no significant differences (P>0.05) in mean weight of the gonads and oviducts in the DLS during the dry and the rainy season. There were also no significant differences (P>0.05) in mean weight of the gonads and the oviducts between the OAS and DLS in the dry season. However, the gonads and the oviducts in the OAS were significantly heavier (P<0.05) than in the DLS in the wet season.

Hormone levels
Hormonal profiles obtained in the two systems are depicted in Fig. 2. During the rainy seasons mean plasma testosterone and LH activity in males in the OAS was significantly lower (P<0.05) than in the DLS. There were no significant differences (P>0.05) in mean plasma testosterone and LH in males between the two systems during dry spells. Mean plasma estradiol, progesterone and LH activity in females in the OAS was significantly higher (P<0.05) than in the DLS during the rainy seasons. There were no significant differences (P>0.05) in levels of the three hormones between the two systems during dry seasons. Mean plasma testosterone in females in the OAS was significantly lower (P<0.05) than in the DLS during rainy seasons. In the dry months mean plasma testosterone levels in the two groups of females were not significantly different (P>0.05).

Discussion
The results obtained strongly support the hypothesis that outdoor confinement does not compromise reproductive performance of the helmeted guinea fowl. Absence of egg laying in the DLS females underlines the importance of keeping helmeted guinea fowl in open air with exposure to sunlight and rainfall. Absence of displays in the semi confined females in dry weather and in the DLS strongly suggest that rainfall is a critical trigger of reproductive behaviour in the helmeted guinea fowl hen. Appearance of displays in the totally confined males and in OAS in dry weather is an indication that low humidity is responsible for induction of courtship displays in males. The male displays in dry weather/low humidity were closely associated with high plasma testosterone levels. This observation differs from an earlier report by Itoh et al. (1985) where plasma testosterone in the male guinea fowl was found to be high in wet breeding months and vice versa. However, similar findings have been reported in male pigeons (Haase et al., 1976), ringdoves (Feder et al., 1977) and ducks (Paulke and Haase, 1978). It is apparent from this study that high humidity is likely to be the cause of declines in plasma testosterone in males during the wet months paving way for mating. The erratic pattern of LH secretion in males in the DLS is a pointer to the adverse effects that persistently dry conditions can have on male guinea fowl reproductive physiology. The egg-laying pattern obtained in the OAS is similar to that described in the free ranging West African breeds (Ogwuegbu et al., 1988) and in the wild (Njiforti and Kortekaas, 1998). A key feature in this study is the relationship between length of rainy seasons and laying duration; the longer the rainy season, the longer the laying duration, the larger the clutch sizes and the higher the laying rate. This emphasizes the importance of rainfall in helmeted guinea fowl breeding. The absence of pairing and mating in the first phase probably implies that males were not synchronised with the females at this age or the males were not fully mature for breeding by then. This hypothesis needs to be explored further by introduction of older males to younger females. It is apparent that mating took place quite early in the second and third laying phases probably to ensure maximum egg fertility. Plasma estradiol, progesterone and LH activity were conspicuously high in the females in the OAS during the laying phases. This was more so for progesterone. This is attributable to the role of the hormone (and LH) in ovulation as has been observed in the domestic ostrich (Degen et al., 1994) and the turkey (Yang et al., 1997). Dry weather and total confinement in low humidity had the opposite effect. Testosterone profile in females was similar to that of the males in both systems: high in dry conditions and low in high humidity. This was contrary to a report made earlier by Ogawa et al. (1990). High plasma testosterone in dry weather prior to reproduction is likely due to the anabolic role of the hormone in readiness for breeding. Testosterone is also probably an inhibitor of reproductive behavior in guinea hens exposed to conditions of low humidity. The observed broody period of 28 days is similar to that reported in free-ranging and wild helmeted guinea fowl (Elbin et al., 1986; Crowe, 1978). There was a decline in LH activity during broodiness in the helmeted guinea fowl that occurred concomitantly with reduction in plasma estradiol, progesterone and testosterone. A similar reduction in plasma LH and sex steroids has been reported in the broody chicken (Sharp et al., 1979) and turkey (Harvey et al., 1981). Open-air confinement therefore did not adversely affect broodiness in the guinea hen. Results on weight of gonads and oviducts indicate that the reproductive organs of the semi confined helmeted
guinea fowl exhibit a growth phase in wet seasons and a regression phase in dry months. This is in agreement with earlier studies (Itoh et al., 1985; Ogawa et al., 1990). Fertility rate of eggs obtained is comparable to that reported in the free ranging West African helmeted that allow the helmeted guinea fowl hen to freely lay and incubate eggs with minimal human interference is therefore likely to boost hatch rates. Nevertheless, the chicken qualifies as a suitable alternative to the guinea hen since the former becomes broody more often. Guinea fowl breeders should therefore integrate guinea fowl domestication with chicken production for better productivity. Hatch rate of fertile eggs in this study was comparable to that obtained with the free ranging West African breeds (Nwagu et al., 1997).

It is apparent from the foregoing that reproductive behaviour and egg laying pattern obtained in outdoor confinement is similar to that reported in wild and free ranging helmeted guinea fowl. Egg fertility and hatch rate as well as seasonal growth and regression of reproductive organs are also not compromised by keeping the birds outdoors in systems that expose them to sunlight and rainfall. Seasonal patterns of reproductive hormone profiles are not affected by semi-confinement. Evidence is thus provided that outdoor confinement is as good as the free range in breeding the helmeted guinea fowl. The outdoor rearing system is therefore recommended for adoption in domestic helmeted guinea fowl production in situations where the free-range system is not practicable.

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guinea fowl breeds (Nwagu and Alawa, 1995; Nwagu, 1997). Results on hatchery characteristics show that the helmeted guinea fowl hen performs better in incubation of eggs as compared with chicken. Provision of suitable nesting facilities


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