In vitro Antibacterial Activity of Essential Oil from the Fruits of Toddalia asiatica (L) Lam. (Rutaceae)

Article · March 2017

DOI: 10.1080/22311866.2017.1299590

CITATIONS
0

READS
288

3 authors, including:

Martin Muthee Gakuubi
Mwenge Catholic University
12 PUBLICATIONS **17** CITATIONS
SEE PROFILE

Wycliffe Wanzala
Maasai Mara University
61 PUBLICATIONS **204** CITATIONS
SEE PROFILE

Journal of Biologically Active Products from Nature

ISSN: 2231-1866 (Print) 2231-1874 (Online) Journal homepage: http://www.tandfonline.com/loi/tbap20

In vitro Antibacterial Activity of Essential Oil from the Fruits of Toddalia asiatica (L) Lam. (Rutaceae)

Martin M. Gakuubi, Kiraithe N. Micheni & Wycliffe Wanzala

To cite this article: Martin M. Gakuubi, Kiraithe N. Micheni & Wycliffe Wanzala (2017) In vitro Antibacterial Activity of Essential Oil from the Fruits of Toddalia asiatica (L) Lam. (Rutaceae), Journal of Biologically Active Products from Nature, 7:1, 52-61

To link to this article: http://dx.doi.org/10.1080/22311866.2017.1299590

Published online: 03 Mar 2017.

Taylor & Francis
Taylor & Francis Group

ISSN Print: 2231-1866
ISSN Online: 2231-1874

In vitro Antibacterial Activity of Essential Oil from the Fruits of *Toddalia asiatica* (L) Lam. (Rutaceae)

Martin M. Gakuubi [1,3]*, Kiraithe N. Micheni [1], Wycliffe Wanzala [2]

[1] University of Nairobi, Biology, P.O. Box 30197-00100, Nairobi, Kenya
[2] Maasai Mara University, Department of Biological/Biomedical Sciences, P.O. Box 861-20500, Narok, Kenya
[3] Department of Biology, Faculty of Science, Mwenge Catholic University, P.O. Box 1226, Moshi, Tanzania

Received 20 November 2016; accepted in revised form 28 January 2017

Abstract: This study was aimed at evaluating the antibacterial prospective of essential oil (EO) extracted from *Toddalia asiatica* L. against four pathogenic bacteria. The essential oil was steam-distilled using a modified Clevenger-type apparatus and its chemical composition established by gas chromatography - mass spectrometry. Disc diffusion method was used to assess the antimicrobial activity of the essential oil against the test pathogens while tube dilution method was used for the determination of minimum inhibitory concentration and minimum bactericidal concentration of the EO on the test bacteria. The highest activity of the EO was observed against *E. coli* where a mean inhibition zone of 34.00 mm was recorded at a concentration of 100 % followed by *B. cereus* where a mean inhibition zone of 32.33 mm was recorded at an oil concentration of 50 %. The minimum inhibitory concentrations and minimum bactericidal concentrations of the EO on the test pathogens were in the range of 3.13 - 12.25 % and 12.25 - >50 %, respectively. When compared to tetracycline which was used as the standard, crude essential oil of *T. asiatica* had significantly higher activity in three out of four studied bacterial strains. These findings justify the continued use of *T. asiatica* extracts in traditional herbal medicine for the treatment of bacterial-based infections.

Key words: Antimicrobial agent, Disc diffusion, Essential oil, *Toddalia asiatica.*

Introduction

Infectious diseases caused by microorganisms such as bacteria and fungi represent a serious health problem world over. It is estimated that infectious diseases account for more than one-third of all deaths in the world today; with the World Health Organization (WHO) estimating that nearly fifty thousand people die each day throughout the world from infectious diseases [15]. The discovery of antibiotics in the early 20th century was one of the most significant medical achievements of modern times. Since their discovery, antibiotics have helped to save countless lives and initially provided some hope for a future free from infectious diseases [21]. However, many of the currently available antibiotics have been rendered inefficacious as a result of excessive and inappropriate uses and subsequent development of resistance [9,40]. Today, cases of multi-drug resistant microbial strains have increasingly reduced the effectiveness of available drugs, causing a global public health concern [44]. Examples include methicillin-resistant staphylococci, vancomycin-resistant enterococci and *Mycobacterium tuberculosis*

*Corresponding author (Martin M. Gakuubi)
E-mail: < bromarto@yahoo.co.uk >

and *Neisseria gonorrhoeae*, both of which are resistant to common antibiotics [2].

Plants and plant products have for many years served as the starting point for the discovery and development of new antimicrobial agents. Fossil records have revealed that human beings living in Mesopotamia more than 60 000 years ago were using medicinal plants to treat various infections [17]. This is an indication that faced with the challenge of microbial infections, plants and plant products were the first curative and preventive defense weapons that mankind turned to [1]. The World Health Organizations estimates that more than 80 % of the population of developing countries relies on traditional plant-based medicine for their primary healthcare needs [39]. Furthermore, at least 25 % of drugs in modern pharmacopoeia are derived from plants with many synthetic drugs also having been developed based on template compounds isolated from plants [11]. In recent years, much focus has been directed at plants as both sources of novel and more effective antimicrobial agents and as template for synthesis of new drugs [3]. The search for new botanical-based antimicrobials has been greatly enhanced by exploitation of the richness of ethnobotanical and ethnopharmacological survey data from various local communities around the world [4]. For example, ethnopharmacological research was the basis upon which the antiviral activity of prostratin, a candidate drug for the treatment of HIV was discovered [16].

Toddalia asiatica L. (Rutaceae), which is known by the English common name, orange climber, is a well known plant species in the evolutionary history of humanity for its high medicinal value, particularly among the Africans and Asians [41]. For example in East Africa, the plant has been prepared in various forms and used to relief stomach problems, malaria, cough, chest pain, food poisoning and sore throat [13,36]. The Maasai people use the fruits to treat cough problems, roots to treat indigestion and influenza while the leaves have been used for the treatment of lung diseases and rheumatism [13]. In addition, people in Madagascar use the plant's roots and barks to treat fever, malaria, cholera, diarrhea and rheumatism [43]. Scientific studies have revealed that the roots of the plant have coumarins (5,7-dimethoxy-8-(3'-hydroxy-3'methyl-1'-butene)-coumarin (1)), compounds with antiplasmodial activity [35], while Lu and co-workers [27] have shown that the plant's extracts have antiviral activity against H1N1 influenza.

Essential oil, which are products of plants' secondary metabolism have been recognized as some of the most promising candidates for development of novel, safe and eco-friendly pharmaceuticals. Essential oil isolated from different aromatic plants and their constituents have been shown to be active against a wide variety of microorganisms especially bacteria and fungi [25,30]. As a way of supporting the traditional use of *T. asiatica* for the treatment of bacterial-based infections, the current study was aimed at evaluating the antibacterial activity of steam-distilled essential oil from the fruits of *T. asiatica* against four clinically important bacteria strains, namely: methicillin-resistant *Staphylococcus aureus*, *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus*.

Materials and methods

Description of Toddalia asiatica L. (Rutaceae)

Toddalia asiatica (L) Lam. (Rutaceae) (Syn: *Paullinia asiatica* L., *Scopolia aculeata* Sm., *Toddalia aculeata* Pers.) is native to many countries in Africa and Asia, where it is found growing along forest margins, riverine habitats or as secondary regrowth in grassland thickets. It is a woody liana belonging to a monotipic genus, *Toddalia* [(a genus with only one species) [36]]. It grows as a twig to a height of 2-15 m with hooked prickles of about 5 mm (Figure 1A). However, the plant may grow into a large shrub when grown in full sun but generally grows well in areas with relatively high annual rainfall **(**Figure 1C). The corky stems are covered with knobby thorns and become yellow when cut. The plant has shiny green citrus-scented leaves which are alternate with leaflets appearing elliptic or slightly obovate with the base cuneate, apex obtusely acuminate, 3-8 by 1-3 cm, glabrous and margins sometimes crenulate. The small, greenish-yellow unisexual flowers (5-merous, stamens 5) give rise to berry-like drupe fruits measuring about 5-7 mm **(**Figure 1B).

Figure 1. A branch with hooked prickles of the plant specimen of *Toddalia asiatica* (A), fruits from which the essential oil was extracted (B) and the distribution of the plant in Kenya represented by black dots (C)

Collection of plant material

The fruits of *T. asiatica* were collected from Maseno area (0°0'10.39"S, 34°36'71"E; 1524 MASL) in Kisumu County, Kenya with the assistance of a Plant Taxonomist. In addition, other plant parts useful in preparation of voucher specimen were collected. A sub-sample of the collected plant materials was prepared, packaged and stored according to the herbarium rules and regulations for further identification, authentication and taxonomic studies. Authentication of the collected plant samples was performed by a Plant Taxonomist at the School of Biological Sciences, University of Nairobi and voucher Specimen (MMG2015) deposited at the University herbarium.

***Toddalia asiatica* essential oil**

The essential oil used in the current study was originally steam-distilled from fruits of *T. asiatica* as previously described [22]. The essential oil whose chemical composition was established using gas chromatography coupled to mass spectrometry (GC-MS) contained majorly a mixture of monoterpenes and sesquiterpenes and their analogues namely; oxygenated monoterpenes and sesquiterpenes. The most abundant constituents in the EO were sabinene (15.8 %), β-pinene (9.5 %) and Linalool (8.3 %) [22]. After extraction, the essential oil of *T. asiatica* was stored at -20°C in a refrigerator in a laboratory at the School of Biological Sciences, University of Nairobi, Kenya until when they were required for the antimicrobial bioassays.

Source and maintenance of test microorganisms

The test microorganisms used were: methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 1385, obtained from the Centre for Microbiology Research, Kenya Medical Research Institute (KEMRI), *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Bacillus cereus* ATCC 11778, which were obtained from the Department of Public Health, Pharmacology and Toxicology, University of Nairobi. The test microorganisms were maintained at -20°C on nutrient agar (NA) and their identity confirmed based on cultural, morphological and biochemical characteristics.

Retrieval of test pathogens and preparation of inocula

Stock culture of each of the test bacterial strain was retrieved by sub-culturing on Mueller Hinton agar (MHA). For each microorganism, three pure colonies of the same morphological type were selected from agar plates and aseptically transferred into test tubes containing 10 mL of Mueller

Hinton broth (MHB) using a sterile loop. The culture tubes were then incubated at 37°C for 24 hours to obtain fresh cultures. Bacterial suspensions were adjusted to match a turbidity of 1.5 × 10^8 colony forming units (CFU) mL^{-1} equivalent to 0.5 McFarland standard (prepared by mixing 0.05 ml of 1% barium chloride dehydrate with 9.9 5 mL of 1% sulfuric acid).

Antimicrobial assay-*Disc diffusion method*

Disc diffusion method as described by Balouiri *et al*.,[8] was used to evaluate antibacterial activity of two concentrations i.e. 100 % (crude EO) and 50 % (diluted with DMSO) of the essential oil *T. asiatica* against the test pathogens. Twenty milliliters of molten MHA was poured into sterile Petri plates (9 cm in diameter) and allowed to set. Lawn culture of the test microorganisms (20 µl) were made on the Muller Hinton agar plates using sterile cotton swab and the plates were dried for 15 minutes. Sterile Whatman filter paper discs (No. 1, 6 mm in diameter) were each impregnated with 10 µL of *Toddalia asiatica* essential oil and aseptically placed at the center of the inoculated plates using a sterile pair of forceps.

Commercial tetracycline susceptibility discs (30 µg/disc) were used as positive control. Tetracyclines are a broad-spectrum antibiotics exhibiting activity against a wide range of Gram-positive and Gram-negative bacteria. Dimethyl sulphoxide (DMSO) was used as a negative control. The labelled plates were refrigerated at 4°C for 2 hours to allow the essen-tial oil to diffuse into the agar medium and finally incubated upside down at 37°C for 24 hours.

Antimicrobial activity was detected by measuring zones of inhibition to the nearest millimeter at the end of the incubation period. The sensitivity of individual bacterial strain to the essential oil was classified based on the mean inhibition zones expressed as millimeters (mm) as follows: not sensitive (-) for total zone diameters ≤ 8 mm; sensitive (+) for diameters between 8 and 14 mm; very sensitive (++) for zone diameters between 15 and 19 mm and extremely sensitive (+++) for zone diameters ≥ 20 mm [7].

All the tests were run in triplicates in a biological safety cabinet and in accordance with the protocols of Clinical and Laboratory Standards Institute (CLSI) formerly National Committee for Clinical Laboratory Standards (NCCLS).

Determination of minimum inhibitory and minimum bactericidal concentration

Turbidimetric or tube dilution method as described by Dhiman *et al.,*[18] with some modification was used for the determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the EO on the test bacteria. Serial two-fold dilutions of the essential oil were made in Mueller-Hinton broth as follows: One milliliter of sterilized MHB was poured into a sterile screw-capped test tube. One milliliter of crude essential oil containing 0.5 % Tween 80 was added to the test tube and vortexed to homogenize the mixture. This was then serially diluted to give concentrations of 50, 25, 12.5, 6.25, 3.13, 1.56 and 0.78 % essential oil in test tubes containing 1 mL of sterile MHB. The tubes were then inoculated with 15 µL of standardized inoculum and incubated aerobically at 37°C for 24 hours. Tubes containing MHB but no essential oil were seeded with the test microorganisms to serve as controls. After incubation, the tubes were examined for growth by observing for any turbidity. The lowest concentration (highest dilution) of the essential oil that produced no visible growth (turbidity) when compared with the control tubes was regarded as the MIC of the oil on the test pathogen [6]. To determine the MBC, the tubes which had showed no visible growth were shaken to homogenize the content and 0.01 mL of the content of each tube subcultured on freshly prepared MHA plates. The plates were incubated at 37°C for 24 hours. Minimum bactericidal concentration was determined as the highest dilution (lowest concentration) at which no growth occurred following the subculturing onto MHA plates [28].

Data analysis

Data on inhibition of test pathogens by the two essential oil concentrations and the standard were analyzed using the PROC ANOVA procedure of GENSTAT version 15 and significant differences amongst means compared using Fisher's Protec-

ted LSD at 5 % probability level of significance. The inhibitory effects of the essential oil against the test pathogens were expressed as mean ± standard error of the mean inhibition zones diameter (mm).

Results and discussion

Antibacterial activity of Toddalia asiatica essential oil

The *in vitro* studies demonstrated strong antibacterial activity of the essential oil of *Toddalia asiatica* against the four test bacteria strains namely, *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli* and methicillin-resistant *Staphylococcus aureus* (Figure 2). There were variations in the antibacterial activity of the two studied concentrations of essential oil among the test bacterial strains (Table 1). The highest activity of the essential oil was observed against *E. coli* where a mean inhibition zone of 34.00 mm was recorded at a concentration of 100 %, followed by *B. cereus* where a mean inhibition zone of 32.33 mm was recorded at an oil concentration of 50 %. The lowest activity of the essential oil was observed against *S. aureus* where a mean inhibition zone of 19.00 mm was recorded at an oil concentration of 50 %. The undiluted crude essential oil had a significantly higher activity ($p \leq 0.05$) in *S. aureus*, and *E. coli* in comparison to the activity recorded at an oil concentration of 50

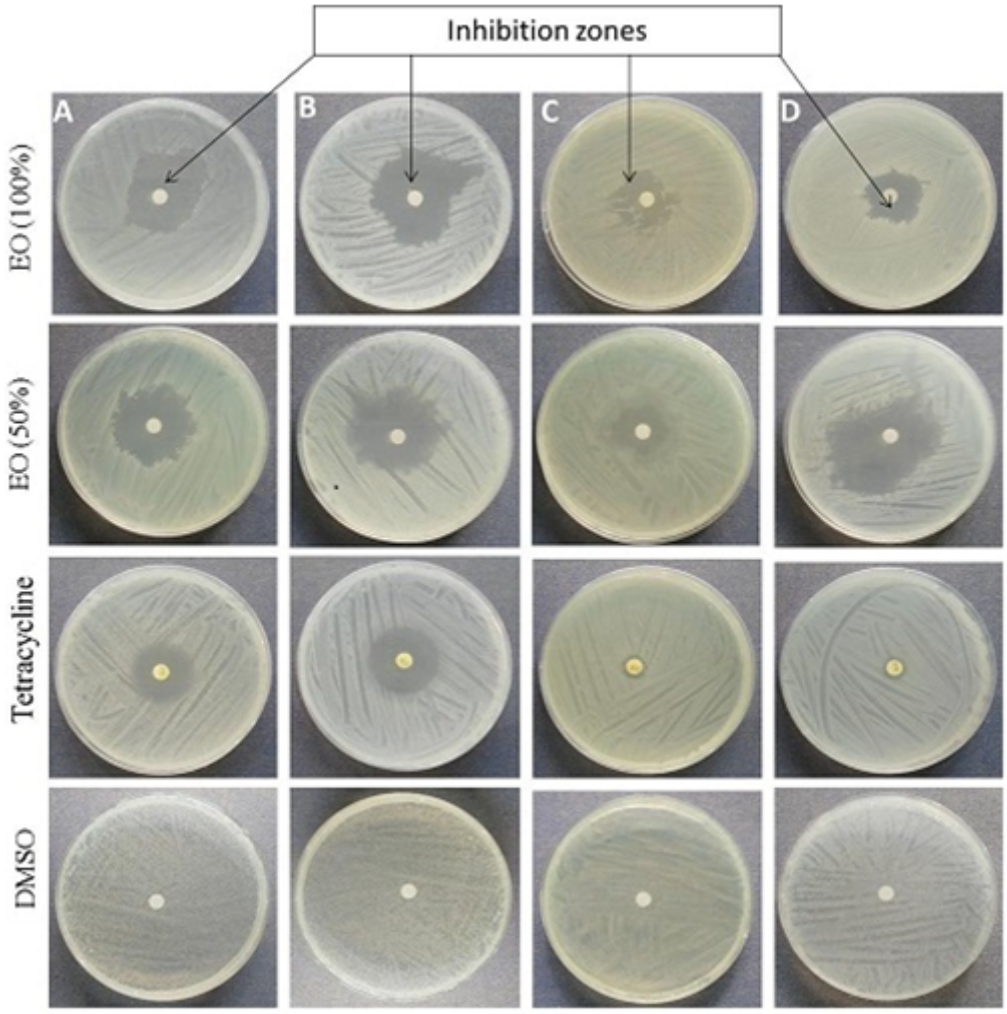

Figure 2. Inhibition zones of essential oil of *T. asiatica* at concentrations of 50 and 100 %, tetracycline (positive control) and dimethyl sulphoxide (negative control) after 24 hours incubation for. *E. coli* (A), *B. cereus* (B), *S. aureus* (C) and methicillin-resistant *S. aureus* (D)

Table 1. Mean inhibition zones (mm) of *Toddalia asiatica* essential oil, tetracycline (positive control) and dimethyl sulphoxide (negative control) on four pathogenic bacteria after 24 hours

Bacteria species	Essential oil (% concentration)		Tetracycline	DMSO
	50 %	100 %	(positive control)	(negative control)
Bacillus cereus	32.33±1.45^{a}	25.33±0.88^{bc}	31.67±1.20^{a}	0.00
Staphylococcus aureus	19.00±1.16^{d}	21.67±1.45^{cd}	11.67±0.88^{e}	0.00
Escherichia coli	21.00±2.08^{d}	34.00±2.65^{a}	26.00±0.58^{b}	0.00
MRSA	22.33±1.67^{bcd}	20.00±1.15^{d}	9.00±0.58^{e}	0.00

Values are mean ± standard error of the mean for bioassay conducted in triplicates.
Means followed by the same superscript alphabetical letter(s) are not significantly different (Multivariate analysis, Fisher's protected LSD at $p \leq 0.05$).
MRSA: Methicillin-resistant *Staphylococcus aureus*
DMSO: Dimethyl sulphoxide

%. The activity of the essential oil against *B. cereus* and methicillin-resistant *S. aureus* at a concentration of 50 % was significantly greater ($p \leq 0.05$) than that recorded in the undiluted essential oil. In the case of *B. cereus* and methicillin-resistant *S. aureus* therefore, higher inhibitory activity was observed in the diluted essential oil in comparison to the activity observed in the undiluted essential oil. Instances where a more concentrated essential oil produce smaller inhibition zones in comparison to less concentrated essential oil have been reported in literature [23]. Such observations are attributed to the fact that dilute essential oil diffuse more easily in the agar medium (i.e. aqueous environment) than the undiluted essential oil [23,32].

Based on the criteria that was adopted for categorization of sensitivity of individual bacterial strain to the essential oil [7], three (*B. cereus*, methicillin-resistant *S. aureus* and *E. coli*) out of four test bacteria fell within the category of extremely sensitive (mean inhibition zone ≥ 20 mm) at an essential oil concentration of 50 %. One test pathogen (*S. aureus*) was within the category of very sensitive (mean inhibition zone of 15-19 mm) at the same essential oil concentration. In the case of the crude essential oil (100 %), all the four bacteria strains were within the category of extremely sensitive. Generally, the activities of the two concentrations of the essential oil against the four bacterial strains were significantly different ($p \leq 0.05$). The activity of the essential oil at concentrations of 50 and 100 % was significantly ($p \leq 0.05$) higher (with the exception of *E. coli* at 50 % and *B. cereus* at 100 %) in comparison to the activity of tetracycline, which was used as a positive control. The highest activity of the standard antibiotic was observed against *B. cereus* and *E. coli* where mean inhibition zones of 31.67 and 26.00 mm, respectively were recorded. The lowest activity of the standard antibiotic on the other hand was observed in methicillin-resistant *S. aureus* and *S. aureus* in which the mean inhibition zones of 9.00 and 11.67 mm respectively, were recorded. These low values of mean inhibition zones are however, signals of confirmation of *S. aureus* being notorious for its ability to resist a wide range of antibiotics and antimicrobial agents. This limits the treatment options in healthcare systems for *Staphylococcus aureus*-based infections as previously reported [9,14,32,42]. These findings are in agreement with a number of previous studies that have showed promising antimicrobial activity of the essential oil, aqueous and organic extracts from *T. asiatica* against a wide range of pathogenic fungi and bacteria [31,34]. For instance, high to moderate activity of the essential oil of *T. asiatica* has been reported against clinically important bacterial and fungal pathogens such as *S. aureus*, *Candida albicans, Candida krusei*, *Cryptococcus neoformans*, *Trichophyton mentagrophyte* and *Microsporum gypseum* [5].

The current study is an important addition to previous diversity of studies on antiplasmodial, antiviral, analgesic, anti-inflammatory, antifungal, antidiabetic, tumor selective cytotoxicity, larvicidal, antioxidant, radical scavenging and antinociceptive activities of *T. asiatica* [12,26,27]. The study thus confirmed the widely claimed myriad folkloric ethnomedicinal and nutritional values of *T. asiatica* in Africa and Asia, where the plant species is prevalent and well acquainted with the local and native communities.

Minimum inhibitory and minimum bactericidal concentrations

The results of the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of the essential oil of *T. asiatica* on the test bacteria are shown in Table 2. The MICs of the essential oil of *T. asiatica* on the studied bacteria strains ranged from 3.13 to 25.00 % with *B. cereus* and *S. aureus* having the lowest and highest MIC values, respectively. The MBCs were in the range of 12.25 to >50 % of the essential oil concentration. *Escherichia coli* MBC was greater than 50 % of the essential oil. *Toddalia asiatica* crude essential oil produced the largest mean inhibition zone (34.00 mm) against *E. coli* in the disc diffusion bioassays. However, the tube dilution bioassay results revealed *E. coli* as the least susceptible bacteria species to the inhibitory action of the essential oil. This was shown by the fact that the essential oil had the highest MBC against *E. coli* (> 50.00). The findings that *E. coli* was least susceptible to the essential oil of *T. asiatica* in the current study are in accordance with previous studies [19,38]. It has been reported in literature that generally, Gram positive bacteria are more susceptible to essential oil than Gram negative bacteria [20,24,33]. The low susceptibility of Gram negative bacteria in comparison to Gram positive bacteria to essential oil is ascribed to the presence of an outer lipopolysaccharide (LPS) layer in Gram-negative bacteria. This hydrophilic LPS layer acts as a barrier to hydrophobic essential oil hence offering greater protection to Gram-negative bacteria against many essential oil and indeed other antimicrobial agents [24,37]. However an in-depth scientific study is needed to understand the underlying science and henceforth promote the use of essential oil in the management of bacterial-based infections.

Conclusions

The growth inhibitory effects of plant extracts and secondary metabolites such as essential oil against microorganisms of clinical importance remain a focal priority area for future research. The current study showed promising antibacterial activity of essential oil of *T. asiatica* against the four studied bacterial strains. The activity of the essential oil of *T. asiatica* was comparable to the commercial antibiotic, tetracycline. In fact on average the essential oil of *T. asiatica* had a greater inhibitory effect than the standard antibiotic. These findings therefore confirms the antibacterial properties of the essential oil of *T. asiatica* and thus justifies the continued use of extracts

Table 2. Minimum inhibitory and minimum bactericidal concentration of *Toddalia asiatica* essential oil on the test bacterial strains

Bacteria species	*Toddalia asiatica* essential oil concentration (%)	
	MIC	MBC
Bacillus cereus	3.13	12.25
Staphylococcus aureus	25.00	50.00
Escherichia coli	12.25	>50.00
MRSA	6.25	25.00

MIC: Minimum inhibitory concentration
MBC: Minimum bactericidal concentration
MRSA: Methicillin-resistant *Staphylococcus aureus*

from this plant in folkloric medicinal practices of local and native communities in the management of bacterial infections. However, further in-depth scientific studies on the toxicity and *in vivo* activity of the essential oil are required in order to understand the underlying science and position them as future sources of cheap, safe and effective antibacterial agents for possible deployment in the control and management of a wide range of microbial-based infections.

Acknowledgements

The first author wishes to acknowledge the support received from the World Federation of Scientists (WFS) for a one year research internship at the International Centre of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya.

Competing interests

The authors declare that there are no competing interests regarding the publication of this paper.

References

1. **Abdallah, E.M. (2011).** Plants: An alternative source for antimicrobials. Journal of Applied Pharmaceutical Science. 1(6): 16-20.
2. **Abreu, A.C., Borges, A., Malheiro, J. and Simões, M. (2013).** Resurgence of the interest in plants as sources of medicines and resistance-modifying agents. In: Méndez-Vilas, A. ed. Microbial Pathogens and Strategies for Combating Them: Science, Technology and Education. Badajoz, Formatex research center. pp. 1287-1297.
3. **Akinsulire, O. R., Aibin, I.E., Adenipekun, T., Adelowotan, T. and Odugbemi, T. (2007).** *In vitro* antimicrobial activity of crude extracts from plants *Bryophyllum pinnatum* and *Kalanchoe crenata*. Afr. J. Tradit. Complement. Altern. Med. 4(3): 338-344.
4. **Albuquerque, U.P., de Medeiros, P.M., Ramos, M.A., Júnior, W.S.F., Nascimento, A.L.B., Avilez, W.M.T. and de Melo, J.G. (2014).** Are ethnopharmacological surveys useful for the discovery and development of drugs from medicinal plants? Rev. Bras. Farmacogn. 24(2): 110-115.
5. **Amuka, O., Machocho, A.K., Okemo, P.O. and Mbugua, P.K. (2014)**. Antibacterial and antifungal activities of essential oils from *Satureja biflora* D. Don, Benth, Speng (Chepsagitiet). *Lippia javanica* Burm. F. (Labotuet) and *Toddalia asiatica* (L) Lam. Rutaceae (Chepindoruet). Global J. Sci. Front. Res. 14(4): 44-51.
6. **Andrews, J.M. (2001).** Determination of minimum inhibitory concentrations. J. Antimicrob. Chemother. 48(suppl 1): 5-16.
7. **Babu, A.J., Sundari, A.R., Indumathi, J., Srujan, R.V. and Sravanthi, M. (2011).** Study on the antimicrobial activity and minimum inhibitory concentration of essential oils of spices. Veterinary World. 4(7): 311-316.
8. **Balouiri, M., Sadiki, M. and Ibnsouda, S.K. (2016).** Methods for *in vitro* evaluating antimicrobial activity: A review. Journal of Pharmaceutical Analysis. 6(2): 71-79.
9. **Bandow, J.E., Brötz, H., Leichert, L.I.O., Labischinski, H. and Hecker, M. (2003).** Proteomic approach to understanding antibiotic action. Antimicrobial Agents and Chemotherapy. 47(3): 948-955.
10. **Bennett, P. M. (2009).** Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. British Journal of Pharmacology. 153(S1): S347-S357.
11. **Bodeker, G., Bhat, K.K.S., Burley, J. and Vantomme, P. (Eds.) (1997).** Medicinal plants for forest conservation and health care, Global Initiative for Traditional Systems (GIFTS) of Health (Vol. 92). Food and Agriculture Organization. 8 p.
12. **Borah, R., Kalita, M.C., Kar, A. and Talukdar, A.K. (2010)**. Larvicidal efficacy of *Toddalia asiatica* (Linn.) Lam against two mosquito vectors *Aedes aegypti* and *Culex quinquefasciatus*. African Journal of Biotechnology. 9(17): 2527-2530.
13. **Bussmann, R.W., Gilbreath, G.G., Solio, J., Lutura, M., Lutuluo, R., Kunguru, K., Wood,**

N. and Mathenge, S.G. (2006). Plant use of the Maasai of Sekenani valley, Maasai Mara, Kenya. J. Ethnobiol. Ethnomed. 2(1): 1-7.

14. **Chambers, H.F. and Frank, R.D. (2009).** Waves of Resistance: Staphylococcus aureus in the Antibiotic Era. Nat. Rev. Microbiol. 7(9): 629-641.
15. **Chanda, S. and Rakholiya, K. (2011)**. Combination therapy: Synergism between natural plant extracts and antibiotics against infectious diseases. In: Méndez-Vilas, A. ed. Science against microbial pathogens: communicating current research and technological advances. Badajoz Formatex Research Center. pp. 520-529.
16. **Clapp, R.A. and Crook, C. (2002).** Drowning in the magic well: Shaman Pharmaceuticals and the elusive value of traditional knowledge. The Journal of Environment & Development. 11(1): 79-102.
17. **Cowan, M.M. (1999).** Plant Products as Antimicrobial Agents. Clin. Microbiol. Rev. 12(4): 564-582.
18. **Dhiman, A., Nanda, A., Ahmad, S. and Narasimhan, B. (2012)**. *In vitro* antimicrobial status of methanolic extract of *Citrus sinensis* Linn. fruit peel. Chronicles of Young Scientists. 3(3): 204-208.
19. **Duraipandiyan, V., Ayyanar, M. and Ignacimuthu, S. (2006)**. Antimicrobial activity of some ethnomedicinal plants used by Paliyar tribe from Tamil Nadu, India. BMC Complementary and Alternative Medicine. 6(1): 1-7.
20. **Erkmen, O. and Özcan, M. (2004)**. Antimicrobial effects of essential oils on growth of bacteria, yeasts and molds. J. Essent. Oil Bearing Plants. 7(3): 279-287.
21. **Fernández, L., Breidenstein, E.B. and Hancock, R.E. (2011).** Creeping baselines and adaptive resistance to antibiotics. Drug Resist Update. 14(1): 1-21.
22. **Gakuubi, M.M. (2016).** Steam distillation extraction and chemical composition of essential oils of *Toddalia asiatica* L. and *Eucalyptus camaldulensis* Dehnh. Journal of Pharmacognosy and Phytochemistry. 5(2): 99-104.
23. **Hood, J.R., Wilkinson, J.M. and Cavanagh, H.M. (2003).** Evaluation of common antibacterial screening methods utilized in essential oil research. J. Essent. Oil Res. 15(6): 428-433.
24. **Inouye, S., Takizawa, T. and Yamaguchi, H. (2001).** Antibacterial activity of essential oils and their major constituents against respiratory tract pathogens by gaseous contact. J. Antimicrob. Chemother. 47 (5): 565-573.
25. **Joseph, B. and Priya, R.M. (2010).** *In vitro* antimicrobial activity of *Psidium guajava* L. leaf essential oil and extracts using agar well diffusion method. Int. J. Curr. Pharm. Res. 2(3): 28-32.
26. **Kariuki, H.N., Kanui, T.I., Yenesew, A., Patel, N. and Mbugua, P.M. (2013)**. Antinocieptive and anti-inflammatory effects of (*Toddalia asiatica* L) Lam. (Rutaceae) root extract in Swiss albino mice. The Pan African Medical Journal. 14: 1-5.
27. **Lu, S,Y., Qiao, Y.J., Xiao, P.G. and Tan, X.H. (2005).** Identification of antiviral activity of *Toddalia asiatica* against influenza type A virus. China Journal of Chinese Materia Medica. 30(13): 998-1001.
28. **Manisha, V., Neha, S. and Satish, S. (2009).** Antimicrobial activity of stem bark extracts of *Nyctanthes arbortristis* Linn. (Oleaceae). International Journal of Pharmacognosy and Phytochemical Research. 1(1): 12-14.
29. **McCarthy, H., Rudkin, J.K., Black, N.S., Gallagher, L., O'Neill, E. and O'Gara, J.P. (2015).** Methicillin resistance and the biofilm phenotype in *Staphylococcus aureus*. Front Cell Infect. Microbiol. 5 (1): 89-98.
30. **Miller, A.B., Cates, R.G., Lawrence, M., Soria, J.A.F., Espinoza, L.V., Martinez, J.V. and Arbizú, D.A. (2015).** The antibacterial and antifungal activity of essential oils extracted from Guatemalan medicinal plants. Pharm. Biol. 53 (4): 548-554.

31. **Munyendo, W.L., Orwa, J.A., Rukunga, G.M. and Bii, C.C. (2011).** Bacteriostatic and bactericidal activities of *Aspilia mossambicensis*, *Ocimum gratissimum* and *Toddalia asiatica* extracts on selected pathogenic bacteria. Res. J. Med. Plant. 5 (6): 717-727.
32. **Muyima, N.Y., Nziweni, S and Mabinya, L.V. (2004).** Antimicrobial and antioxidative activities of *Tagetes minuta, Lippia javanica* and *Foeniculum vulgare* essential oils from the Eastern Cape Province of South Africa. J. Essent. Oil Bearing Plants. 7(1): 68-78.
33. **Nazzaro, F., Fratianni, F., De Martino, L., Coppola, R. and de Feo, V. (2013).** Effect of essential oils on pathogenic bacteria. Pharmaceuticals. 6(12): 1451-1474.
34. **Nyahanga, T., Jondika, J.I., Manguro, L.O.A. and Orwa, J.A. (2010).** Chemical composition, antiplasmodial, larvicidal and antimicrobial activities of essential oils of *Toddalia asiatica* leaves and fruits. Int. J. Essen. Oil Ther. 4: 54-58.
35. **Oketch-Rabah, H.A., Mwangi, J.W., Lisgarten, J. and Mberu, E.K. (2000).** A new antiplasmodial coumarin from *Toddalia asiatica* roots. Fitoterapia. 71(6): 636-640.
36. **Orwa, J.A., Jondiko, I.J.O., Minja, R.J.A. and Bekunda, M. (2008).** The use of *Toddalia asiatica* (L) Lam. (Rutaceae) in traditional medicine practice in East Africa. J. Ethnopharmacol. 115 (2): 257-262.
37. **Parekh, J. and Chanda, S. (2007).** *In vitro* antibacterial activity of the crude methanol extract of *Woodfordia fruticosa* Kurz. flower (Lythraceae). Braz. J. Microbiol. 38(2): 204-207.
38. **Saxena, V.K. and Sharma, R.N. (1999).** Antimicrobial activity of the essential oil of *Toddalia asiatica*. Fitoterapia. 70(1): 64-66.
39. **Shanley, P. and Luz, L. (2003).** The impacts of forest degradation on medicinal plant use and implications for health care in eastern Amazonia. BioScience. 53(6): 573-584.
40. **Usha, P.A., Jose, S. and Nisha, A.R. (2010).** Antimicrobial Drug Resistance - A global concern. Veterinary World. 3(3): 138-139
41. **Usher, G. (1974).** A dictionary of plants used by man. London, Constable and Company Ltd., London. 117 p.
42. **Wang, L., Qiao, X., Ai, L., Zhai, J. and Wang, X. (2016)**. Isolation of antimicrobial resistant bacteria in upper respiratory tract infections of patients. Biotech. 6(166): 1-7.
43. **Watt, J.M. and Breyer, M.G. (1962).** The medicinal and poisonous plants of Southern and Eastern Africa, 2nd ed. London, Livingstone, Ltd. 817 p.
44. **Worthington, R.J. and Melander, C. (2013).** Combination approaches to combat multidrug-resistant bacteria. Trends Biotechnol. 31(3): 177-184.