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ANTIMICROBIAL ACTIVITES OF EXTRACTS FROM *GLEDITSIA TRIACANTHOS* L. AND *SCHINUS MOLLE* L.

Kemelo Sanett Matela[1], Sissay Bekele Mekbib[2], Manoharan Karuppiah Pillai[1]*

[1]Department of Chemistry and Chemical Technology, Faculty of Science and Technology, National University of Lesotho, Roma Campus, P. O. Roma 180, Kingdom of Lesotho, Southern Africa.
[2]Department of Biology, Faculty of Science and Technology, National University of Lesotho, Roma Campus, P. O. Roma 180, Kingdom of Lesotho, Southern Africa.

* kmharan@rediffmail.com

Abstract

The methanolic extracts from leaves and stem-bark of *Gleditsia triacanthos* L. and *Schinus molle* L., methanolic extract from thorns of *G. triacanthos* and chloroform extracts from seeds of *G. triacanthos* and *S. molle* were evaluated for their antibacterial activity against four bacterial isolates *viz*. *Proteus spp*., *Streptococcus spp*., *Escherichia coli* and *Entereobacter spp*. and one fungal isolate, *Candida albicans* by agar hole-plate diffusion method. All extracts showed a moderate to strong activity against all four bacteria ranging from 10.0 – 33.5 mm inhibition zones. The MIC values of these extracts were also determined against four bacteria and were found to be moderately to significantly active and in few cases, they were weakly active. Against the fungal isolate, *C. albicans*, these extracts exhibited no visible inhibition zone.

Keywords: *Gleditsia triacanthos L., Schinus molle L., antibacterial activity, methanolic extracts, chloroform extracts.*

Introduction

Called by other names such as honey locust and thorny locust, *Gleditsia triacanthos* L. is a deciduous tree belonging to Fabaceae family and is native to North America and Asia [1-4]. The species *Gleditsia* can tolerate a wide range of climate and soil conditions [5]. *G. triacanthos* can grow up to 15-30 m height [6, 7], its flowers appear as yellow with strong pleasant smell. *G. triacanthos* has single or branched thorns [6] which can grow up to 3-10 cm or even more. The fruits are legumes (pods) which are 15-40 cm long and 2.5-3.5 cm wide [6, 8, 9] and they are edible [10]. The pulp of legumes is rich in tannin and has strong sweet taste and hence the name honey locust is given to it. It has been reported that GreenTech S.A uses seed extracts of *G. triacanthos* in the cosmetic industry and sold in the name of *Gleditschia.* [11]. It has also been reported that *Gleditsia* species have been used in hair protection, used as local medicine for treatment of ache, whooping, measles, smallpox, skin diseases, asthma and difficult labour in the Native American etc. [12-14]. Miguel et al. reported that the seeds extract of *G. triacanthos* possesses antioxidant activity ranging between 18.77 and 71.59 % in DPPH radical scavenging assay. Mohammed et al. reported that ethanolic extract obtained from leaves of *G. triacanthos* possesses potent cytotoxic activity against liver, breast, cervix, larynx and colon cancer cell lines and also has potent antioxidant activity [1]. Mohamed et al. evaluated 70% ethanolic extract obtained from leaves of *G. triacanthos* and fractions obtained from this 70% ethanolic extract for their analgesic, anti-inflammatory, hepatoprotective and antimicrobial activities [15].

Known by other names such as Pepper tree, American pepper, Aguaribay, Peppercorn tree and Pervian pepper, *Schinus molle* L. is belonging to Anarcardiaceae family [16-19]. Native to South and Central America, *S. molle* is a small tree, grows up to 7-10 m height [18] and has been cultivated in Southern African countries [18]. The red or reddish pink fruits are edible which is about 5 mm diameter [18] and has a taste which is remarkably similar to pepper and hence the name pepper tree is given to *S. molle*. However, *S. molle* is unrelated to the true pepper plant, *Piper* nigrum [16] in the sense, for example, the former is a tree and the latter is a climber plant. It has been reported that *S. molle* has many biological and pharmacological activities such as cytotoxic, analgesic, antibacterial, antifungal, anti-inflammatory, antiseptic, antiinsecticidal activities etc. [21-26].

The Kingdom of Lesotho is one of the Southern African countries and is blessed with many natural resources including plant biodiversity. However, the biological and pharmacological activities of plants from Kingdom of Lesotho are not explored well. The aim of the present study is to evaluate *in vitro* antimicrobial studies of methanolic and chloroform extracts obtained from *G. triacanthos* and *S. molle* against four bacterial strains *viz*. *Proteus spp*., *Streptococcus spp*., *Escherichia coli* and *Enterobacter spp*. and one fungal isolate, *Candida albicans*. Although, several reports were available on the antimicrobial studies of extracts and essential oils from these plants, the methanolic and chloroform extracts were not explored and to the best of our knowledge this is the first report of this kind from these plants from the Kingdom of Lesotho.

Methods

Plant materials

The plant materials, *G. triacanthos and S. molle* were collected from trees growing inside the National University of Lesotho, Roma Campus and/or Botanical Garden, Department of Biology, National University of Lesotho, Roma Campus, Kingdom of Lesotho, Southern Africa. Both plant materials were collected in August 2017 and they were identified by Mr. Moretloa Polaki, Lecturer, Department of Biology, Faculty of Science and Technology, National University of Lesotho, Roma Campus, Roma P.O. 180, Lesotho, Southern Africa. From *G. triacanthos*, the following parts were used for this studies: leaves (337.000g), stem-bark (247.271g), thorns (165.169g) and seeds (217.705g). Similarly, the following parts of plant materials were used from *S. molle*: leaves (596.744g), stem-bark (241.246g) and seeds (115.126g). A voucher specimen for each part of plants (labelled as KMGTLS for leaves, KMGTSB for stem-bark, KMGTTS for thorns and KMGTSD for seeds of *G. triacanthos* and KMSMLS for leaves, KMSMSM for stem-bark and KMSMSD for seeds of *S. molle*) were kept at Organic Chemistry Laboratory, Department of Chemistry and Chemical Technology, Faculty of Science and Technology,

National University of Lesotho, Roma Campus, Roma P.O. 180, Lesotho, Southern Africa.

Processing of materials

All parts of the plant materials were allowed to air dry separately at room temperature for two weeks. The air-dried leaves, seeds and thorns of *G. triacanthos* were ground into powder using a commercial blender (Waring Blender, Blender 80119, Model HGB2WT93, 240V AC, 50-80 Hz, 3.6 AMPs, Laboratory and Analytical Supplies). The air dried stem-bark of *G. triacanthos* was chopped into small pieces and then crushed using a Woodworking Table Saw 250 mm machine (Serial Number: JFD1412109-13, Model Number: SAWLD001, Motor, 1500W, 220 V, 50 Hz, Blade Rising Range: 0-80 mm, Motor Speed: 4500 rpm, Max. Depth of Cutting (90° and 45°): 80 mm and 5 mm. The crushed materials were further ground into powder using the above mentioned Waring Blender. The same procedures mentioned above were repeated to get powder from the air-dried leaves, seeds and stem-bark of *S. molle*.

Preparation of plant extracts

The powdered leaves of *G. triacanthos* was extracted first with methanol with occasional shaking at room temperature for two days. Filtered the solution using Whatman No.1 filter paper and the solvent methanol was removed using water bath and/or rota-vapour. Repeated the same procedure once again. Finally, the materials were extracted with methanol by reflux condition for 5 hours and all three filtrates were combined; 47.51 g of extract was obtained. The same procedure was followed to for the powdered stem-bark and thorns of *G. triacanthos* and 8.44 and 7.51 g of extracts, respectively, were obtained. The powdered seeds of *G. triacanthos* was extracted first with chloroform with occasional shaking at room temperature followed by reflux condition for 5 hours. The extracts were combined and 8.94 g of resinous extract was obtained after removal of solvent methanol using water bath and/or rota-vapour. The same extraction procedures were repeated for *S. molle* and respectively, 63.00 and 7.19 g of methanolic extracts were obtained from leaves and stem-bark; 12.91 g of chloroform extract (oily/semi-solid) was obtained from seeds.

Evaluation of antimicrobial activity

The antimicrobial activities of various extracts were screened by *in vitro* by "hole-plate diffusion method" as described in literature [27, 28]. Solutions of various extracts were prepared separately at a concentration of 100 mg of extract in 1 mL of DMSO. The solutions were then filtered separately using a 0.20 µm filter and used for both antibacterial and antifungal activities. For antibacterial assay, each one of the test bacterium was maintained on nutrient agar slant separately and was recovered for testing by growth in nutrient broth for 14 hours at 37°C. The cultures were adjusted to a suspension of 1×10^6 to 2×10^6 colony-forming units (CFU)/mL. Instead of sterile paper discs, cylindrical cavities of size 4.00 mm height and 8.00 mm diameter were punched into the agar medium using a sterile cork-borer. 30 µL aliquots of extract were placed into the cylindrical cavities. The plates were then incubated at 37°C for 24 hrs. Ciprofloxacin at a concentration of 30 µg in 1 mL of DMSO served as positive control while DMSO served as negative control. For antifungal assay, the Petri dishes containing 25 mL of Sabouraud Dextrose Agar (SDA) medium were evenly seeded with 0.5 mL of adjusted fungal inoculum size of 2×10^5 CFU/mL. Holes of size 4.00 mm height and 8.00 mm diameter were punched into the agar medium using a sterile cork-borer and filled with 30 µL aliquots of the extract. The plates were incubated at 28 °C for 96 hrs. Miconazole nitrate at a concentration of 30 µg in 1 mL of DMSO served as positive control while DMSO served as negative control. The sensitivity of microorganism species to the various extracts of *G. triacanthos and S. molle* was determined by measuring the diameter of inhibition zones on the agar surface around the holes. All of the experiments were performed in duplicate and the results are reported as the average of two experiments. A clear zone > 10 mm are considered positive results [29].

Determination of minimum inhibitory concentrations (MICs)

The lowest concentration of the sample which inhibits the visible growth of a microbe after 24 hrs. is defined as MIC values [28, 30] and the MIC values < 100 µg/mL, 100 to ≤ 625 µg/mL and > 625 µg/mL were considered as significantly active, moderately active and weakly active, respectively [31, 32]. The MICs were determined by procedure as described in

literature [30, 33] with slight modifications. Briefly, a stock solutions at a concentration of 1000 µg/mL of various extracts of *G. triacanthos* and *S. molle* were prepared separately and two-fold serial dilutions such as 1000, 500, 250, 125, 62.5 and 31. 25 µg/mL were made from these slock solutions. The cultures were adjusted to a suspension of 1 x 10^6 to 2 x 10^6 colony-forming units (CFU)/mL and the cylindrical cavities of the size 4.00 mm height and 8.00 mm diameter were filled with 30 µL solution of various extracts. The plates were then incubated at 37°C for 24 hours; reference positive and negative controls were also assayed.

Microorganisms

The test microorganisms used in this study were four bacteria *viz*. *Proteus spp*., *Streptococcus spp*., *E. coli* and *Enterobacter spp*. and one yeast species *viz*. *C. albicans*. All these microorganisms were available in the Department of Biology, Faculty of Science and Technology, National University of Lesotho, Roma Campus, Kingdom of Lesotho, Southern Africa.

Statistical analysis

Data analysis was performed using the SPSS 17.0 statistics program by means of two-way analysis of variance. When $p \leq 0.05$, the differences were considered statistically significant.

Results

Table **1** summarises the antibacterial and antifungal activities of various extracts obtained from *G. triacanthos* and *S. molle*. GTMELS, GTMESB, GTMETS and GTCHSD are respectively, *G. triacanthos* methanolic leaves extract, *G. triacanthos* methanolic stem-bark extract, *G. triacanthos* methanolic thorns extract and *G. triacanthos* chloroform seeds extract. Similarly, SMMELS, SMMESB and SMCHSD are respectively, *S. molle* methanolic leaves extract, *S. molle* methanolic stem-bark extract and *S. molle* chloroform seeds extract.

Against *Proteus spp*., GTMELS, GTMESB, GTMETS and GTCHSD showed inhibition zones of 17.5±2.1, 11.5±3.5, 10.0±1.4 and 13.5±0.7 mm, respectively; SMMELS, SMMESB and SMCHSD showed inhibition zones of 15.5±0.7, 13.0±1.4 and 19.5±6.3 mm, respectively. The positive control ciprofloxacin showed an inhibition zone of 29.5±1.4 mm. This result showed that GTMESB and GTMETS, with inhibition zones of 11.5±3.5 and 10.0±1.4 mm, were weakly active and all other extracts showed inhibition zones between 12 to ≤ 20 and therefore moderately active.

Against *Streptococcus spp*., GTMELS, GTMESB, GTMETS and GTCHSD showed inhibition zones of 17.5±3.5, 12.5±3.5, 08.5±0.7 and 13.0±0.0 mm, respectively. SMMELS, SMMESB and SMCHSD showed inhibition zones of 16.0±2.8, 14.0±1.4 and 17.0±5.6 mm, respectively. The positive control ciprofloxacin showed an inhibition zone of 31.5±3.5 mm. This observation showed that GTMETS with inhibition zone of 08.5±0.707 mm is weakly active and the rest of the extracts are moderately active with inhibition zones greater than 12.0 mm but less than 20.0 mm.

Against *E. coli*, GTMELS, GTMESB, GTMETS and GTCHSD showed inhibition zones of 17.0±1.4, 15.5±0.7, 12.0±0.0 and 13.5±0.7 mm, respectively, whereas SMMELS, SMMESB and SMCHSD showed inhibition zones of 16.5±0.707, 14.5±0.707 and 20.5± 1.4 mm, respectively, against the same bacteria. The positive control ciprofloxacin showed an inhibition zone of 25.5±0.7 mm. These data showed that SMCHSD has strong activity with an inhibition zone of 20.5±1.4 mm. All other extracts exhibited moderate activity with inhibition zones greater than 12.0 mm.

Against *Enterobacter spp*., GTMELS, GTMESB, GTMETS and GTCHSD showed inhibition zones of 17.5±3.5, 19.5±6.3, 10.5±0.7 and 13.5±0.7 mm, respectively; SMMELS, SMMESB and SMCHSD showed inhibition zones 21.0±4.2, 13.5±2.8 and 33.5±0.7 mm, respectively. The positive control ciprofloxacin showed an inhibition zone of 22.0±0.7. GTMESB exhibited weak activity with an inhibition zone of 10.5±0.7 mm. On the other hand, SMCHSD exhibited remarkably very strong activity with an inhibition zone of 33.5±0.7 mm. All other extracts showed moderate activity with inhibition zones greater than 12.0 mm. In general, all extracts exhibited inhibition activity against all four bacteria but their relative inhibition activity varied from one extract to another as shown in Table **1**. In the case of SMCHSD, the activity is remarkably very strong with an inhibition zone of 33.5±0.7 mm. against *Enterobacter spp*. In other words, SMCHSD might have potent active ingredients against *Enterobacter spp*. All these extracts were evaluated for their antifungal activity against *C. albicans* and did not exhibit any visible inhibition zone while the positive

control miconazole nitrate showed an inhibition zone of 27.0±2.8 mm.

Table **2** summarises the minimum inhibitory concentrations (MICs) of various extracts of *G. triacanthos* and *S. molle* on four bacterial isolates tested. From Table **2**, it is observed that all extracts, except GTMETS, were significantly active against *Proteus spp.* and each one with with MIC value of 62.5 µg/mL. For GTMETS, the MIC value was found to be >1000 µg/mL meaning that it has very weak activity. Against *Streptococcus spp.* these extracts showed varied activity; GTMELS, SMMESB and SMCHSD were significantly active and each one with MIC value of 62.5 µg/mL; GTCHSD and SMMELS were moderately active and their MIC values were 125 and 250 µg/mL, respectively; GTMESB and GTMETS were weakly active and each one with MIC value >1000 µg/mL. Against *E. coli* also these extracts showed varied activity; GTCHSD and SMCHSD were significantly active and each one with MIC value of 62.5 µg/mL; GTMELS, GTMESB, SMMELS and SMMESB were moderately active and their MIC values were 250, 500, 250 and 125 µg/mL, respectively; GTMETS was weakly active with MIC value >1000 µg/mL. Against *Enterobacter spp.*, GTMETS was found to be weakly active with MIC value >1000 µg/mL and GTMESB and SMMELS were found to be moderately active with MIC values 500 and 250 µg/mL, respectively; all other extracts were found to be significantly active and each one with MIC value of 62.5 µg/mL. Ciprofloxacin served as positive control for which the MIC values were found to be 62.5 µg/mL against all four bacteria. GTMETS is the only extract which showed very weak activity against all four bacteria tested and it is noted that GTMETS also showed relatively weak activity against all four bacteria in the antibacterial inhibition assay (Table **1**). The MIC assay against the fungus, *C. albicans*, was omitted since all these extracts showed no visible inhibition zone in the antifungal inhibition assay (Table **1**).

Discussion

Previously, it was reported that 70% ethanolic extract from leaves of *G. triacanthos* and three fractions obtained from this 70% ethanolic extract were evaluated for antibacterial activity against *Staphylococci, Staphylococcus aureus, Escherichia coli, Proteus mirabilis, Klebsiella pneumonia* and *Pseudomonas auruginosa* [15]. However, the 70% ethanolic extract and its three fractions did not exhibit any activity against *E. coli* [15]. *E. coli* is the only microorganism common to our study also. However, the methonloic extract from our study showed strong inhibitory activity against *E. coli* with an inhibition zone of 17.0 mm. The reasons for this discrepancy may be due to improved extracting power of methanol relative to 70 % ethanol. Additionally, geographic location and/or region from where the plants are grown, season during which the plant materials are collected etc. will also play a significant role in the chemical constituents and subsequently to this biological activity.

The essential oil obtained by hydrodistillation of dry leaves of *S. molle* was evaluated for its antibacterial activity against *Proteus mirabilis, E. coli, S. aureus, P. aeruginosa* and *K. pneumonia* and against one fungal isolate, *C. albicans* [16]. This hydrodistilled essential oil showed week to strong antibacterial and antifungal activities [16]. It was reported that at 75% dilution, this hydrodistilled oil extract showed an inhibition zone of 13.00 against *E. coli* and and 18.33 mm against *C. albicans* [16]. These microorganisms, *E. coli* and *C. albicans* were common to our study also. However, the methanolic extract from leaves of *S. molle* in our study showed higher inhibition zone of 16.5 mm against *E. coli* and showed no activity against *C. albicans*. Again, these discrepancies are due to different extracting power of active ingredients by the solvents, methods of extraction used etc.

Belhamel et al. collected leaves of *S. molle* from Gouraya Park Station of Ecology, Bejaia, Algeria and obtained essential oil by hydrodistillation. This hydrodistilled essential oil was evaluated for antibacterial activity against four bacterial strains *viz. S. aureus*, *S. pyogenes*, *E.coli* and *Vibrio vulnificus*. This hydrodistilled oil extract showed an inhibition zone of 17.1 mm against *E. coli* without dilution and the diameter of inhibition zones decreased with increasing dilutions [34]. The only microorganism common to our study is *E. coli* and our methanolic extract from leaves showed almost same activity.

Pedro MMR et al. collected leaves and fruits of *S. molle* from Angel Gallardo, Argentina, crushed them together and obtained essential oil by steam distillation. This steam distilled essential oil was evaluated for their antibacterial activity against

three bacterial strains namely, *P. aeruginosa*, *S. aureus and E. coli* and exhibited inhibition zones of 16.0, 38.2 and 22.0 mm, respectively and the diameter of inhibition zones decreased with increasing dilutions [19]. Here again, the only microorganism common to our study is *E. coli* and our methanolic extract from leaves alone showed an inhibition of 16.5 mm against *E. coli* and the chloroform seeds extract alone showed an inhibition zone of 20.5 mm against the same microorganism.

We have evaluated antibacterial and antimicrobial activities of methanolic extracts from leaves, stem-bark and thorns of *G. triacanthos*, chloroform extract from seeds of *G. triacanthos*, methanolic extracts from leaves and stem-bark of *S. molle* and chloroform extracts from seeds of *S. molle*. Four bacterial isolates *viz. Proteus spp.*, *Streptococcus spp.*, *E. coli* and *Enterobacter spp.* were used to evaluate the antibacterial activities. In general, all extracts showed a moderate to strong activity against all four bacterial isolates ranging from 10.0 – 33.5 mm inhibition zones. All the above extracts were also evaluated for their MIC values against four bacteria and were found to have moderate to significant activities and in some cases, they have weak activity. All the above extracts were also evaluated for their antifungal activity against one fungal isolate, *C. albicans* and exhibited no visible inhibition zone.

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Table 1. : Inhibitory effect of methanolic and chloroform extracts of *G. triacanthos* and *S. molle* on selected bacterial and fungal isolates.

Extracts	Microorganisms/ Zone of inhibition (mm) (diameter)				
	Proteus spp.	*Streptococcus spp.*	*E. coli*	*Enterobacter spp.*	*C. albicans*
GTMELS	17.5±2.1	17.5±3.5	17.0±1.4	17.5±3.5	-
GTMESB	11.5±3.5	12.5±3.5	15.5±0.7	19.5±6.3	-
GTMETS	10.0±1.4	08.5±0.7	12.0±0.0	10.5±0.7	-
GTCHSD	13.5±0.7	13.0±0.0	13.5±0.7	13.5±0.7	-
SMMELS	15.5±0.7	16.0±2.8	16.5±0.7	21.0±4.2	-
SMMESB	13.0±1.4	14.0±1.4	14.5±0.7	13.5±2.8	-
SMCHSD	19.5±6.3	17.0±5.6	20.5±1.4	33.5±0.7	-
Positive Controls	29.5±1.4	31.5±3.5	25.5±0.7	22.0±0.7	27.0±2.8

GTMELS = *G. triacanthos* methanolic leaves extract; GTMESB = *G. triacanthos* methanolic stem-bark extract; GTMETS = *G. triacanthos* methanolic thorns extract; GTCHSD = *G. triacanthos* chloroform seeds extract; SMMELS = *S. molle* methanolic leaves extract; SMMESB = *S. molle* methanolic stem-bark extract; SMCHSD = *S. molle* chloroform seeds extract; *Proteus spp.* = *Proteus species; Streptococcus spp.* = *Streptococcus species*; *E. coli* = *Escherichia coli*; *Enterobacter spp.* = *Enterobacter species*; *C. albicans* = *Candida albicans*; (-) = inactive; Positive controls: Ciprofloxacin and miconazole nitrate for bacteria and fungus respectively; inhibition zones of < 12 mm, 12 to ≤ 20 and ≥ 20 are, respectively, week, moderate and strong inhibitory effects [19]. Data are expressed as the mean ± standard deviation (SD) of duplicate.

Table 2: The minimum inhibitory concentrations (MICs) of methanolic and chloroform extracts of *G. triacanthos* and *S. molle* on selected bacterial isolates.

Extracts	Minimum inhibition concentrations (MICs) (µg/mL)			
	Proteus spp.	*Streptococcus spp.*	*E. coli*	*Enterobacter spp.*
GTMELS	62.5	62.5	250	62.5
GTMESB	62.5	>1000	500	500
GTMETS	>1000	>1000	>1000	>1000
GTCHSD	62.5	125	62.5	62.5
SMMELS	62.5	250	250	250
SMMESB	62.5	62.5	125	62.5
SMCHSD	62.5	62.5	62.5	62.5
Positive Control	<31.25	<31.25	<31.25	<31.25

GTMELS = *G. triacanthos* methanolic leaves extract; GTMESB = *G. triacanthos* methanolic stem-bark extract; GTMETS = *G. triacanthos* methanolic thorns extract; GTCHSD = *G. triacanthos* chloroform seeds extract; SMMELS = *S. molle* methanolic leaves extract; SMMESB = *S. molle* methanolic stem-bark extract; SMCHSD = *S. molle* chloroform seeds extract; *Proteus spp.* = *Proteus species; Streptococcus spp.* = *Streptococcus species*; *E. coli* = *Escherichia coli*; *Enterobacter spp.* = *Enterobacter species*; Positive control: Ciprofloxacin; the MIC values < 100 µg/mL, 100 to ≤ 625 µg/mL and > 625 µg/mL were considered significantly active, moderately active and weakly active, respectively [31, 32].