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Synergistic Antibacterial Interaction between *Trachyspermum ammi*, *Senna alexandrina* Mill and *Vachellia nilotica* spp. Nilotica Extract and Antibiotics

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ABSTRACT

To evaluate the interaction between plants extract and antibacterial drugs then to confirm the rationale of the ethno-medicinal use of candidate plants. Methanolic extract of *Trachyspermum ammi* (Fruit), *Senna alexandrina* mill (Leaves) and *Vachellia nilotica* spp. nilotica (Fruit) individually and in combination with commonly used antibiotics (ampicillin, gentamycin and tetracycline) were tested by the Cup Plate method in crude for their antibacterial activity against four standard bacterial strains (*Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*). The highest antibacterial activity was recorded in *V. nilotica* extracts against *S. aureus* (IZ = 39 mm) and the lowest antibacterial activity was recorded in *T. ammi* extract against *P. aeruginosa* (IZ = 0 mm). When the standard antibiotics were tested, the IZ ranged from 0-31 mm. The lowest (MIC) of the three plants extracts against the standard bacteria were determined, the most potent antimicrobial plant was *V. nilotica* (MIC < 1.156-12.5 mg mL⁻¹). In synergistic results the three extracts showed synergistic interaction in combination with the tested antibiotics which differed according to the species of bacteria. The Inhibition Zones (IZ) ranged from 0-40 mm. The highest largest IZ of 40 mm was observed against *B. subtilis* where a combination of *V. nilotica* and Tetracycline were used. The least susceptible bacteria to the plant extract and combination was *S. aureus* organisms and the most susceptible bacteria was *B. subtilis*. The synergy of *T. ammi*, *S. alexandrina* and *V. nilotica* showed an overall increase in the activity of standard antibiotics against standard bacteria, thus, there is a scope to develop effective combinations of such antibiotics and purified forms of these medicinal plants.

Key words: Synergistic, antibacterial, *Trachyspermum ammi*, *Senna alexandrina*, *Vachellia nilotica* spp., antibiotics

INTRODUCTION

Plant species have been used for medicinal purposes throughout history. Their health properties are linked to a number of chemical constituents. In recent years, phytochemicals in plants have received a great deal of attention mainly for their role in preventing diseases that result from microbial infections. A large number of medicinal plants

used in Sudanese traditional medicine are attributed with antibacterial action (El-Kamali and El-Amir, 2010; El-Kamali *et al.*, 2005).

Bacterial resistance is beyond doubt the consequence of years of widespread indiscriminate use, incessant misuse and abuse of antibiotics (Peterson and Dahoff, 2004).

Because of the limited life span of antibiotics, it is of utmost importance to find appropriate solutions to impede, or

perhaps even reduce, the development of drug resistance associated with many microbial species and agents (Martini and Eloff, 1998).

Recent research on medicinal plants and their antimicrobial activity aiming to analyze the past, present and future of medicinal plants suggests fundamental findings on plant extracts' mechanism of action, interaction with antibiotics and with other medicinal plants (synergism) (Rios and Recio, 2005). Research on synergism is very limited and few studies have reported the existence of combined action. In Sudan, this is the first study reporting on synergism between commercial antibiotics and plant extracts.

Recently many researchers' studies have attempted combining plants and antibacterial drugs to avoid antibacterial resistance and allow for the discovery of novel drugs (Betoni *et al.*, 2006). Betoni *et al.* (2006) tested the synergy between 13 antimicrobial drugs and 8 plant extracts against *Staphylococcus aureus* strains. *In vitro* anti-*Staphylococcus aureus* activities of the extracts were confirmed and synergism was verified for all the extracts; clove, guava and lemon grass presented the highest synergist activity with antimicrobial drugs, while ginger and garlic showed limited synergistic capacities.

In vitro, the antibacterial activity of ethanolic leaf extract of *Vangueria spinosa* Roxb. (Rubiaceae) alone and in combination with antibiotics (doxycycline and ofloxacin) were tested against one gram-positive bacterium: *Staphylococcus aureus* and three gram-negative bacteria: *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. The synergistic actions were observed in all the cases especially against *P. aeruginosa* where an additive effect in combination with floxacilin was demonstrated when it was in combination with the medicinal plant extract (Chatterjee *et al.*, 2009).

In another study Ahmed *et al.* (2010) investigated the inhibitory effect of two antibiotics: Penicillin and tetracycline against *Staphylococcus aureus* separately and when they were combined it was found that the zone of inhibition was 23 mm for tetracycline and 18 mm for penicillin when they were tested individually. However, when used in combination they displayed a synergy and were much more effective and causing a zone of inhibition measuring 27 mm in diameter. These antibiotics were also added as a combination to various plant extracts including (ethanolic extracts) of *Salvadora persica*. The highest inhibition was noted to be (a zone of 31.5 mm) when *S. aureus* was exposed to tetracycline in combination with the *Salvadora* stem extract. This result was followed by tetracycline plus leaf extract of *Salvadora persica* which displayed a zone of inhibition measuring 30.0 mm. The combination of stem and leaf extracts with penicillin could not produce the same inhibitory effect as that of tetracycline and *Salvadora* stem and leaf extracts. However, synergy was still noted for the penicillin and stem extract of *Salvadora* combination which resulted in a zone of inhibition (IZ) of 21.0 mm thus surpassing the, 18 mm IZ recorded for both penicillin and stem extract of *Salvadora persica* when tested individually.

The possible *in vitro* interaction between ethanolic extracts of *Rhus coriaria* and Oxytetracycline HCl against *P. aeruginosa* was evaluated by using the microdilution method, The results of this study found strong bactericidal activity against *P. aeruginosa*. These results indicate that combinations between *R. coriaria* extract and oxytetracycline could be useful in fighting the emerging drug resistant *P. aeruginosa* (Adwan *et al.*, 2010).

In another study, *in vitro* antibacterial activity of *Tectona grandis* leaves with tetracycline was tested using the Kibry-Bauer method, maximum synergy was recorded against *Salmonella typhimurium*, *Klebsiella pneumoniae* and the lowest synergy was against *Pichia pastoris* and *Escherichia coli*. No synergistic activity was observed against *Citrobacter freundii* (Purushotham *et al.*, 2010).

Rakholiya and Chanda (2012) evaluated in *in vitro* interaction between methanolic extracts of *Terminalia catappa* and *Carica papaya* leaves and certain known antimicrobial agents such as penicillin G (P), ampicillin (AMP), amoxyclav (AMC), cephalothin (CEP), polymyxin B (PB), rifampicin (RIF), amikacin (AK), nilidixic acid (NA), gentamicin (GEN), chloramphenicol (C) and ofloxacin (OF). The combination were evaluated against five gram-positive and five gram-negative bacteria. The evaluation of synergy between plant extracts and antimicrobial agents was carried out using the disc diffusion method. They found that there is increased activity in case of combination with methanolic plant extracts and tested antimicrobial agents. The most combination potent was between the methanolic extract of *C. papaya* and the antibiotics against the tested bacterial strains. Interestingly, methanolic extract of *C. papaya* alone did not show any antibacterial activity (Purushotham *et al.*, 2010).

Synergistic activity of *Salvia officinalis* and *Cichorium intybus* extracts and commonly used antibiotics, amoxicillin and chloramphenicol were evaluated against *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 as well as against clinical isolates of *Staphylococcus aureus*, *Bacillus subtilis*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Escherichia coli* and *Proteus mirabilis*. Extract of *Salvia officinalis* showed increased synergistic capacity compare to *Cichorium intybus* (Sefanovic *et al.*, 2011).

The use of medicinal plants against certain types of illnesses is rooted in Sudan's traditional medicine. This study was undertaken to determine the possible inhibitory effects of some plants that are used against common infectious diseases in Sudan.

In a 2012 study conducted by Javed *et al.* (2012) the essential oil of *T. ammi* was subject to *in vitro* antifungal and antibacterial assays. Micro well dilution assays were adopted against three human pathogenic fungal strains that included *Aspergillus flavus*, *Aspergillus niger* and *Candida albicans* and four bacterial isolates: *Escherichia coli*, *Staphylococcus sp.*, *Pseudomonas syringae* and *Bacillus subtilis*. All aqueous; organic extracts particularly ethanol, n-hexane and essential oil displayed marked antimicrobial potential against all pathogens (Javed *et al.*, 2012).

In 2004, Shahidi reported that the methanol extract of *T. ammi*, when tested against some bacterial species, showed significant antibacterial activity (Bonjar, 2004).

Many medical research have been published, especially on the use of *S. alexandrina* as a laxative, in case of intestinal obstruction and acute intestinal inflammation (Bosch, 2007). Also Boch showed the ethanolic extracts of *S. alexandrina* leaves had inhibitory activity against *Bacillus cereus*, *Staphylococcus aureus* and *Staphylococcus epidermidis* but not against gram-negative bacteria.

In another study, the powder of *V. nilotica* spp. nilotica fruit was used externally as an antiseptic or mixed in with yogurt to treat dysentery (El-Kamali and Khalid, 1996). The powder of the fruit was also used externally as vulnerary remedy for skin pustules. An infusion is used to treat cough which is applied by fumigation. Fruits are also used to treat catarrh, fever and measles (El-Kamali and Khalid, 1998). The powder of the leaves and bark are used externally to treat eye diseases. The powder of the gum is taken to treat diarrhea. A decoction prepared from the bark is used as stimulant as well as to treat fever and indigestion. It is also used to treat sore throats, colds, bronchitis, pneumonia, ophthalmia, diarrhea, dysentery, leprosy, venereal diseases and hemorrhages (Abd El Nabi *et al.*, 1992).

The methanol and acetone extracts of *V. nilotica* showed significant inhibition against gram-positive and gram-negative bacteria (Kambizi and Afolayan, 2001). The ethanol extract of *V. nilotica* also showed activity against gram-positive and gram-negative bacteria (Khafagi, 1999). Aqueous extracts of *V. nilotica* fruits exhibited activity against *Candida albicans* and some gram-positive and gram-negative bacteria (Abd El Nabi *et al.*, 1992).

In 2005, Daowd, investigated methanol, ethanol, ethyl acetate and aqueous extracts of *Vachellia nilotica*. The most potent antibacterial activity was found in ethanol and methanol extracts (a large zone of inhibition was recorded against *S. aureus* amongst the gram-positive bacteria while maximum activity was observed against *E. coli* and *K. pneumoniae* amongst the gram-negative bacteria) (Daowd, 2005).

This study was conducted to compare the activity of the antimicrobial plants extracts with the activity of the commercial antibiotics, to determine the Minimum Inhibitory Concentrations (MICs) of the plants under investigation and to evaluate the synergistic activity between plants extract and antibacterial drugs.

MATERIALS AND METHODS

Collection and identification of the plants: The plants used in this study were collected from different parts of Sudan by herbalists. *T. ammi* was collected from Western Sudan, *S. alexandrina* was collected from North of Sudan and *V. nilota* was collected from an area near the Nile river in Khartoum state. They were authenticated by the researcher Dr. Haider Abdelgadir at the Medicinal and Aromatic Plant Research Institute (MAPRI). These candidate plants were selected based on literature review and their use in traditional Sudanese medicine.

Chemical and reagent: Mueller and Hinton Agar and Nutrient Agar, were obtained from Hi-Media, Mumbai, India and the methanol and DMSO from S.D fine-chemical company. All other chemicals and reagents used are of analytical grade.

Preparation of extracts: Two hundred fifty grams of each plant sample were extracted by soaking the powdered plant material in 500 mL of 80% methanol for 72 h with daily filtration and evaporation (Harborne, 1984). Solvent was evaporated under reduced pressure using a rotary evaporator. The yield percentages were calculated.

Preparation of bacterial suspension: Microorganisms were obtained from the department of Microbiology at the Medicinal and Aromatic Plant Research Institute (MAPRI) in Sudan. Two strains of gram positive bacteria *Bacillus subtilis* (NCTC 8236), *Staphylococcus aureus* (ATCC 29213) and two gram negative bacteria *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) were obtained and used in this study. The bacterial cultures were maintained in their appropriate agar slants at 4°C throughout the study and used as stock cultures. One milliliter aliquots of 24 h broth culture of the test organisms was aseptically distributed on to nutrient agar slopes and incubated at 37°C for 24 h. The bacterial broth was harvested and washed off with 100 mL sterile normal saline to opacity of matched barium chloride turbidity standard. The suspension was stored in the refrigerator at 4°C until it was used.

Testing of extracts for antibacterial activity: Screening of strains for antibacterial activity was done by Minimum Inhibitory Concentration (MIC) and it has been determined by the cup method assay (Kavanagh, 1972). Semi confluent growths of bacteria were obtained by matching the turbidity of the test and control inoculate with the turbidity standard of 0.5 Macfarland units, this turbidity was equivalent to approximately $1-2 \times 10^8$ CFU mL⁻¹ (Eman *et al.*, 2010). One milliliter of the standardized bacterial stock suspension of 10^8 CFU mL⁻¹ were thoroughly mixed with 100 mL of Muller Hinton agar which was maintained at 45°C. Twenty milliliters of aliquots of the inoculated Muller Hinton agar were distributed into sterile Petri dishes. The agar was left to set and in each of these plates, 4 cups (10 mm in diameter) were cut under aseptic conditions using a sterile cork borer and agar discs were removed.

Alternating cups were filled with 0.1 mL samples of each of the extracts using automatic microtitre pipettes and allowed to diffuse at room temperature for 2 h. The plates were then incubated in the upright position at 37°C for 18 h. After incubation the diameters of the resultant growth inhibition zones were measured.

Minimum Inhibitory Concentration (MIC) assay: The principle of the agar plate dilution is the inhibition of growth on the surface of the agar by the plant extracts incorporated in to the medium. Plates were prepared on the series of increasing concentrations of the plant extract and media. The

tested organisms were grown in broth overnight contain 10^8 CFU mL^{-1} . Loop full of diluted cultures was spotted with the standard loop that delivers 0.01 mL on the surface of segmented disposable Petri dish. The end point (MIC) is the last concentration of antimicrobial agent that completely inhibits the growth. Results were reported as the MIC in mg mL^{-1} (Blair *et al.*, 1970).

Determination of synergistic activity: The synergistic activity was calculated by combining each of the extracts (*Trachyspermum ammi*, *S. alexandrina* and *Vachellia nilotica*) with the standard antibiotics (ampicillin, gentamicin and tetracycline), by means of the Cup-Plate method (Kavanagh, 1972). The cups were filled with 0.05 mL of equal volumes of antibiotic and extract using the automatic microtitre pipette and allowed to diffuse at room temperature for 2 h. The plates were incubated in the upright position at 37°C for 18 h. After incubation the diameters of the resultant growth inhibition zones were measured (Adwan and Mhanna, 2008).

RESULTS

Trachyspermum ammi extract showed high antibacterial activity against *B. subtilis* and *E. coli*. Moderate activity was observed against *S. aureus* and no activity against *P. aeruginosa*. *Senna alexandrina* extract was found to have high activity against *B. subtilis* and *E. coli*, moderate activity against *S. aureus* and *P. aeruginosa*. The most active extract was *V. nilotica*, showed pronounced antibacterial activity against all tested organisms. The results of the Minimum Inhibitory Concentration (MIC) of evaluated plant extracts are presented in Table 1.

According to Van Vuuren and Viljoen (2008), natural products with MIC values below 1.0 mg mL^{-1} are considered noteworthy. In this study plant crude extract showing antibacterial activity with MIC is considered to have good activity.

The MICs of *Trachyspermum ammi* extract were low against *E. coli* (6.25 mg mL^{-1}), moderate MIC (12.5 mg mL^{-1})

against (*B. subtilis* and *S. aureus*) and very high ($>200 \text{ mg mL}^{-1}$) against *P. aeruginosa* i.e., the plant extract showed moderate activity against gram positive bacteria and high activity against *Escherichia coli*, whereas the MIC of the extract against *P. aeruginosa* was inactive.

The lowest Minimum Inhibitory Concentration (MIC) values as obtained by the micro-dilution assays were $<1.156 \text{ mg mL}^{-1}$ for the crude extract of *Vachellia nilotica* against *Staphylococcus aureus* and 6.25 mg mL^{-1} for the crude extract of *Senna alexandrina* and *Vachellia nilotica* against *Bacillus subtilis* and also for the crude extracts of *Senna alexandrina*, *Vachellia nilotica* and *Trachyspermum ammi* against *Escherichia coli*.

Synergism is observed when the effect of the combined substance is greater than the sum of the individual effects.

Synergistic activity *T. ammi* extract with different standard antibiotics against bacteria is shown in Table 2. The synergistic effect was found only against *B. subtilis* when the extract combined with tetracycline.

The combination of *S. alexandrina* with ampicillin was found to have synergistic activity against *S. aureus* and *B. subtilis*, whereas it was not found to have any synergistic activity against *P. aeruginosa* and *E. coli*. Gentamicin and tetracycline are presented good synergistic activity with *S. alexandrina* against *B. subtilis* only (Table 3).

Synergistic activity of *Vachellia nilotica* with all standard antibiotics were found to have high synergistic activity against all standard bacteria except *S. aureus*, *B. subtilis*. When gentamicin was combined with *V. nilotica* its activity against *B. subtilis*, *E. coli* and *P. aeruginosa* increased, proving, the presence of synergy (Table 4).

Table 1: Determination of minimum inhibitory concentration of crude extract against standard organisms

Plants	B.s	S.a	E.c	P.a
<i>T. ammi</i>	12.50	12.5	6.25	>200.0
<i>S. alexandrina</i>	6.25	25.0	6.25	50.0
<i>V. nilotica</i>	6.25	1.156	6.25	12.5

B.s: *Bacillus subtilis*, S.a: *Staphylococcus aureus*, E.c: *Escherichia coli*, P.a: *Pseudomonas aeruginosa*, *T. ammi*: *Trachyspermum ammi*, *S. alexandrina*: *Senna alexandrina*, *V. nilotica*: *Vachellia nilotica*

Table 2: Synergism between *Trachyspermum ammi* extract and antibiotics recorded by zone of inhibition

Bacteria antibiotics	Amp (mm)	Gen (mm)	Tet (mm)	<i>T. ammi</i> (mm)	<i>T. ammi</i> +Amp	<i>T. ammi</i> +Gen	<i>T. ammi</i> +Tet (mm)
B.s	16	25	20	20	-	-	30
S.a	22	31	29	18	-	-	-
E.c	-	30	-	30	-	-	-
P.a	-	22	14	-	-	-	-

Amp: Ampicillin, Gen: Gentamycin, Tet: Tetracycline, *T. ammi*: *Trachyspermum ammi*, B.s: *Bacillus subtilis*, S.a: *Staphylococcus aureus*, E.c: *Escherichia coli*, P.a: *Pseudomonas aeruginosa*, -: Synergistic activity

Table 3: Synergism between *Senna alexandrina* extract and antibiotics recorded by zone of inhibition

Bacteria antibiotic	Amp (mm)	Gen (mm)	Tet (mm)	<i>S. alexandrina</i> (mm)	<i>S. alexandrina</i> +Amp (mm)	<i>S. alexandrina</i> +Gen (mm)	<i>S. alexandrina</i> +Tet (mm)
B.s	16	25	20	24	25	30	25
S.a	22	31	29	16	23	-	-
E.c	-	30	-	25	-	-	-
P.a	-	22	14	14	-	-	-

Amp: Ampicillin, Gen: Gentamycin, Tet: Tetracycline, *S. alexandrina*: *Senna alexandrina*, B.s: *Bacillus subtilis*, S.a: *Staphylococcus aureus*, E.c: *Escherichia coli*, P.a: *Pseudomonas aeruginosa*, -: Synergistic activity

Table 4: Synergism between *Vachellia nilotica* extract and antibiotics recorded by zone of inhibition

Bacteria antibiotic	Amp (mm)	Gen (mm)	Tet (mm)	<i>V. nilotica</i> (mm)	<i>V. nilotica</i> +Amp (mm)	<i>V. nilotica</i> +Gen (mm)	<i>V. nilotica</i> +Tet (mm)
B. s	16	25	20	36	37	37	40
S. a	22	31	29	39	-	-	-
E. c	-	30	-	35	-	36	36
P. a	-	22	14	35	36	36	37

Amp: Ampicillin, Gen: Gentamycin, Tet: Tetracycline, *V. nilotica*: *Vachellia nilotica*, B.s: *Bacillus subtilis*, S.a: *Staphylococcus aureus*, E.c: *Escherichia coli*, P.a: *Pseudomonas aeruginosa*, -: Synergistic activity

DISCUSSION

The extract of *T. ammi* individually, showed lowest antibacterial activity when we compared with the other two plants, while all tested bacteria except *P. aeruginosa* showed sensitivity to the extract. These results were also noted in the work of Chauhan *et al.* (2012) and that was suggested to the use of same solvent. Whereas Tariq *et al.* (2014) disagree with our result when they used *T. ammi* seeds against *P. aeruginosa* were found good inhibition zone, this might be due to they used different part of plant from our part.

The medium antibacterial activity of the three plants extracts under study is *S. alexandrina*, showed good antibacterial activity against all tested bacteria. This finding coincided with finding obtained by Doughari *et al.* (2008).

Methanolic extract of *V. nilotica* showed pronounced antibacterial activity against all tested organisms. El-Kamali and El-Karim (2009) and Khan *et al.* (2009) were conformity with our results when they used pods and leaves of *V. nilotica*, respectively against *S. aureus*, *B. subtilis*, *E. coli* and *P. aeruginosa*. This suggests the potential of *V. nilotica* is shown in all parts of the plant extract.

Our study also showed conflicting results when compared to other studies (Mahesh and Satish, 2008; Bansa, 2009) were reported that methanolic extracts of *V. nilotica* leaves and bark showed low activity against *B. subtilis* and *E. coli*. Those are probably due to using low concentration of the extracts.

In the MICs results, *T. ammi* showed different MICs value but better more than the result showed by Doughari *et al.* (2008) this conflicting in the results I think because they used clinical isolates.

Senna alexandrina extracts were found to have lowest MICs value when we compared with the result reported by El-Morsy (2013) this might be due to the difference in the method of extraction.

A promotion result of MICs showed in *V. nilotica* extract and this result is lowest than the result found in 2009 by Bansa and Khan, they showed that MIC of ethanolic extract ranged between (4.9-50 mg mL⁻¹) against *B. subtilis*, *S. aureus* and *E. coli*.

This variation of the MICs results were found because strains are genetically different from each other and this is probably due to the different chemical composition and structure of cell wall of types of microorganisms.

Synergistic activity resulting from the interaction of antibiotics with different plant extracts has been studied and experimented by a limited number of scientists. The methanolic extract of *T. ammi* showed synergistic effect when

were mixed with tetracycline only against *B. subtilis* this results disagree with Tariq *et al.* (2014) they showed synergistic activity when they mixed *T. ammi* with ampicillin this different in result due to they used different parts of plant. So, the combination of methanolic extract of *T. ammi* is not useful in the treatment of infectious disease.

Senna alexandrina were found to have good synergistic activity with all antibiotics against *B. subtilis* and with ampicillin only against *S. aureus*, no synergistic activity showed against gram-negative bacteria. That means *S. alexandrina* extract is better to used alone against gram-negative bacteria.

The synergy between *V. nilotica* and antibiotics showed a high potential and a strong bactericidal activity thus proving the synergy achieved by this combination. These results indicated that combination between *V. nilotica* extract and antibiotics could be useful in treatment of bacterial infection except against *S. aureus* were found no synergistic activity with all antibiotics used this result is a type of antagonism.

The synergistic interaction between antibiotics and extracts was highly dependent on the species of bacteria against which the combination was tested. This result is in agreement with what has been reported by other studies (Sefanovic and Comic, 2012).

The use of antimicrobial agents presenting synergy is one of the well established indications for combination antimicrobial therapy.

CONCLUSION

This study confirms the antibacterial activity of *V. nilotica* extract and shows their potential use as agents which enhance antibiotic activity. Our future study will be isolation and identification of active compounds, responsible for these interesting activities and closer investigation of a mechanism of action between phytochemicals and antibiotics.

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