WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 6.805
Volume 5, Issue 10, 1123-1129. Research Article ISSN 2277– 7105

SYNERGISTIC ANTIBACTERIAL AND ANTIBIOFILM ACTIVITY OF *T. AMMI*, BLACK SALT AND COPPER METAL

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Article Received on
17 August 2016,
Revised on 06 Sept. 2016,
Accepted on 26 Sept. 2016
DOI: 10.20959/wjpr201610-7179

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ABSTRACT

An *in-vitro* synergistic effect of antibacterial activities of different concentration of *T. ammi* aqueous extract with rock salt in presence and absence of copper ions on bacteria causing respiratory tract infection is compared. The synergistic effects of these combinations are also extended for analyzing the destruction of biofilm formation which limits the accessibility of drug/natural product and is a major concern with respiratory tract infections.

KEYWORD: antibacterial, antibiofilm, rock salt, plant extracts, *Trachyspermum ammi*, Copper

INTRODUCTION

In recent times, preparations based on different plants and salts are generally employed to prevent common bacterial infections because of growing antibiotic resistance strains. Among the organisms that causes urinary tract, respiratory tract and skin infections, *Pseudomonas aeuroginosa* and *Staphylococcus aureus* have been encountered very frequently. Further the extensive uncontrolled use of antibiotics has led to the emergence of drug resistant strains which shows failure to growth inhibition with combinational therapy. This stimulates the incorporation of various extracts of plants as antibacterial agents. Of the various herbal plants, *spices* have been the choice of substance to extract the active ingredients which alone or in combination have shown to exhibit synergistic antibacterial activity and have also overcome the antibiotic resistance dilemma among multiple drug resistant and medically important organisms.

The plant *Trachyspermum ammi*. Linn. (*T. ammi*) is a grassy, aromatic herb belonging to *Umbelliferae* family finds application in traditional ayurvedic, Sidhha, Homeopathy, Naturopathy and Unani practices. Several studies have reported the use of *T ammi* as antiseptic, antibacterial, anti-inflammatory, antifungal, antioxidant, carmative along with its expectorant, diuretic and antitusssive properties (Kamaljeet et al 2012, Saini et al 2014). Since ancient times report on storage of drinking water in copper vessels have been practiced (Preeti et al 2012). Recent studies have also reported antibacterial effect of copper nanoparticles or copper rods as antibiofilm formers (Marin et al 2016). Ayurvedic preparations reports the incorporation of black salt and rock salt in various herbal vati and formulations, however its synergistic effect with copper and *T. ammi* extracts have not been investigated. This research is an attempt to evaluate the combinatorial effect of plant extracts with salt and metal as antibacterial agents.The study also deals with comparison of the antibacterial effect of aqueous fresh and overnight extracts of *T. ammi* extracts with and without black salt and copper ions. Simple agar diffusion assay, coverslip assay and microtiter assay have been employed for studying the antibiofilm activity of these extracts.

MATERIALS AND METHODS

Collection of materials: *T. ammi* seeds and rock salt were purchased from commercial sources, while copper beakers of 500ml were used for the study. The biofilm formers viz. *P. aeruginosa* (ATCC 27853) and *S. aureus* (ATCC 25923) were procured from National Chemical Laboratory (NCL), Pune. The cultures were prepared for antibacterial activity as mentioned by Kinjal et al (2015). Log phase cultures of overnight grown cultures were used for all screening of antibacterial activity.

Preparation of Extracts: *T. ammi* seeds were washed twice with sterile distilled water and excess water drained on water absorbent paper. The seeds were dried in shade and were grinded using sterile motor and pestle into fine powder. The powder was stored in air tight container for further use.

Preliminary screening: For antimicrobial activity, fresh and overnight aqueous extracts of *T. ammi* powder at room temperature (at concentration in the range of 5 - 10 %) in presence or absence of black salt (5%) and copper beaker were prepared as follows:

a) Fresh extract in glass beaker
b) Fresh extract with black salt in glass beaker
c) Fresh extract in copper beaker

d) Fresh extract with black salt in copper beaker
e) overnight extract in glass beaker
f) overnight extract with black salt in glass beaker
g) overnight extract in copper beaker
h) overnight extract with black salt in copper beaker

All the extracts were filtered through sterile Whatman No 1 filter paper and centrifuged at 1000 rpm for 2 min. The clear supernatant (20 µl) was used for determining of antibacterial activity by the agar diffusion method (Kinjal et al 2015). Controls like Positive control with sterile distilled water, salt control (5%) and copper water control (o/n stored water in copper beaker) were also used.

Antibacterial and antibiofilm screening: The antibacterial activity of the above mentioned combinations were further performed in the range of 5-10 % of *T. ammi* extracts using the undiluted, 1:2, 1: 5 and 1: 10 dilution for both the test cultures. Based on the MIC value obtained the antibiofilm activity was carried by the coverslip assay (Walker et al 2012) and microtiter method at MIC and sub MIC values (Kinjal et al 2015). All the assays were repeated thrice.

RESULT AND DISCUSSION

Plant extracts, copper metal ions and components of black salt are known to contain bioactive compound at diminutive concentration, which alone may not reveal antibacterial activity for some of the pathogens but may exhibit when combined (Preeti et al 2012). The present study focused on the antibacterial and antibiofilm activity of fresh and overnight aqueous extracts of *T. ammi* synergistically in presence of black salt and copper ions. Fresh, aqueous *T. ammi* fruit extract with and without black salt and overnight aqueous extracts in copper vessel without black salt gave no antimicrobial activity against the two organisms. Overnight aqueous *T. ammi* fruit extract with black salt in copper beaker showed antimicrobial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus* at both 5 and 10 % concentration.

Although previous studies (Aggarwal and Goyal 2012, Tariq et al 2012, Javed et al 2012, Masiq et al 2012) have demonstrated the role of solvent, temperature of extraction, extraction methods, nanoparticle involvement, this study is unique in employing a simple aqueous extraction and synergistically studying the antimicrobial activity of *T. ammi* extracts with black salt and non- nanoparticles of copper. The aqueous overnight extract showed more

antimicrobial activity against gram positive than gram negative bacteria. Ergo maximum antibacterial activity was seen in hexane, acetone, methanol, ethanol and petroleum ether of *T. ammi* fruit extract (Aggarwal and Goyal 2012), however, synergistic activity of metals with black salt has not been reported. The MIC of the *T. ammi* fruit extract by agar well diffusion assay was maximum at 8 % and nil at 3% for *S. aureus*, while was observed only at 8% for *P. aeruginosa* (Fig 1 and 2). Tariq et al have demonstrated the effectiveness of ethanolic extract in the range of 1-10%, while Javed et al (2012) demonstrated 20% concentration of *T. ammi* aqueous extracts to give a higher zone of inhibition for *S. aureus* than for *P. aeuroginosa*. Also maximum AMA was seen in the hexane extracts of essential oils for both the test organism.

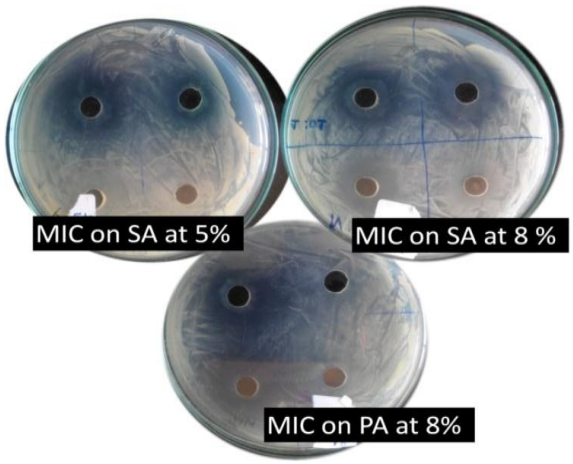

Fig 1: Zone of inhibition of *S. aureus* and *P. aeuroginosa* using different dilutions of *T. ammi* with copper and black salt.

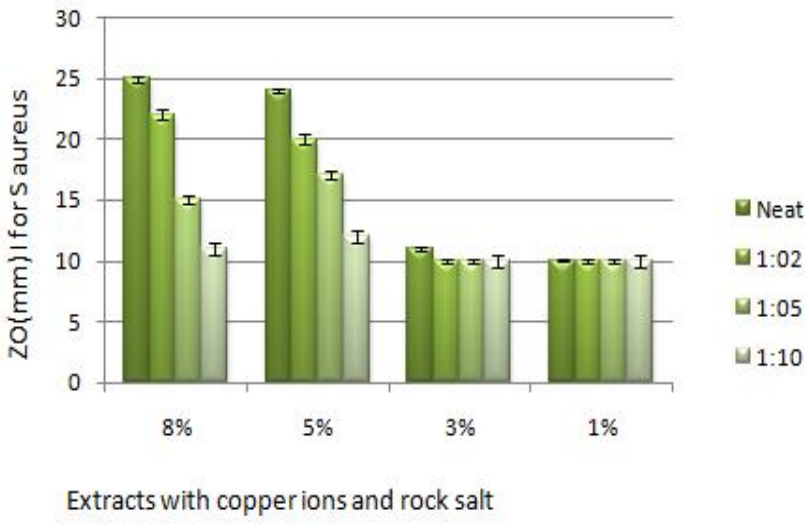

Fig 2: MIC of *T. ammi* extracts with copper and black salt on *S. aureus*

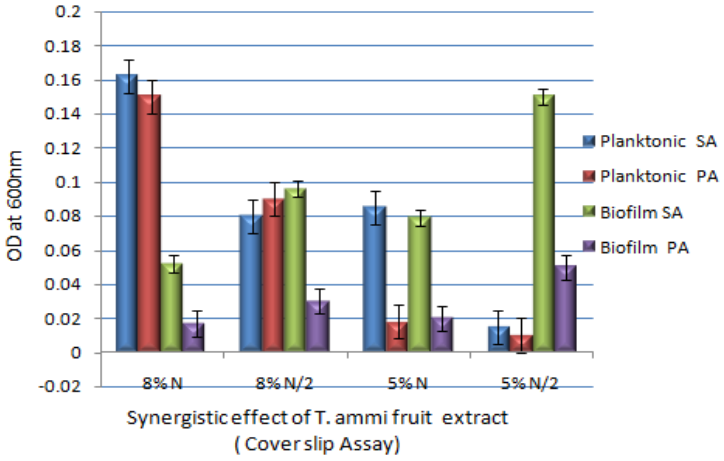

Fig 3: Effect of synergistic activity of *T. ammi* extracts with black salt and copper on the planktonic and biofilm formation by the coverslip assay on *S. aureus* and *P. aeuroginosa*

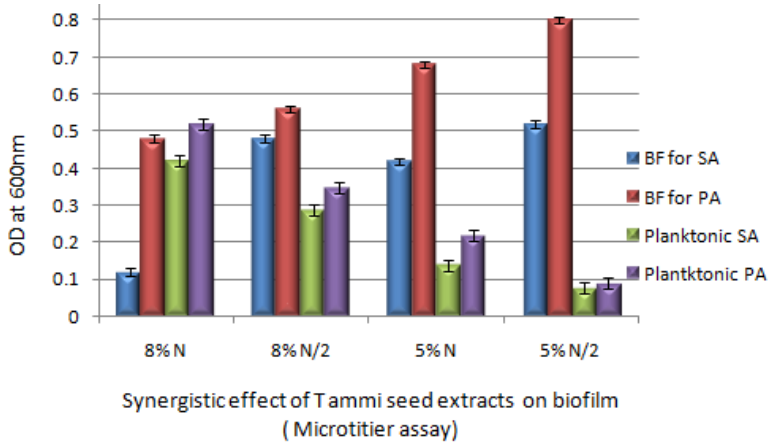

Fig 4: Effect of synergistic activity of *T. ammi* extracts with black salt and copper on the planktonic and biofilm formation by the microtiter assay.

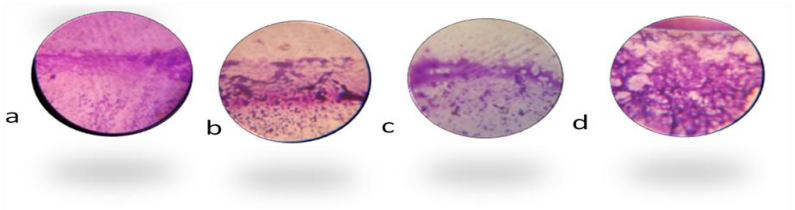

Fig 5: A view of a microscopic field for *S. aureus* Biofilm formed on coverslip (a) MIC and (b) sub MIC level. For *P. aeruginosa* (c) MIC and (d) sub MIC level of the extracts

By both coverslip assay (Fig 3 and 5) and microtiter plate assay (Fig 4) the number of planktonic cells were found to be inversely proportional to the dilutions of extracts, while number of biofilm cells was directly proportional to dilutions of the extracts. *S. aureus* showed 5 times reduced biofilm formation than *P. aeuroginosa* by the microtiter assay.

Researchers have suggested various mechanisms for exhibiting reduction in biofilm formation in presence of bioactive compounds (Rahman et al 2015). It is also known that many extracts with poor antimicrobial activity can effectively reduce biofilm formation (Upadhayay et al 2014). In the present study biofilm formation was seen to be inhibited at the sub MIC level which may be partially attributed to oligodynamic action of copper ions in synergism with black salt and bioactive phytonutrients from *T. ammi*.

Cover slip assay and microtiter assay was attempted as a miniaturized way of testing the formation of biofilm optically and microscopically which is less time consuming and laborious. Although the study includes mimicking system under static condition for antibiofilm activity, these phytochemicals and its synergistic effect with other metal ions needs to be assessed further by steady flow system to be use in medicine.

CONCLUSION

Further studies are mandatory to unveil the detailed mechanisms of synergistic action of copper with black salt and different plant extracts utilizing different organisms. This study can be used as a preliminary step to evaluate the synergistic effect of the three combination which could drastically reduce the biofilm formation.

ACKNOWLEDGEMENTS

The author acknowledge the School of Biotechnology and Bioinformatics, D. Y. Patil University in providing the infrastructure to carry out the experiments and Dr N. Kushwah, Chemistry Division, BARC for her help in planning the experiment.

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