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IN VITRO SYNERGISTIC EFFECT OF ANTIBACTERIAL ACTIVITY OF *PIPER NIGRUM* AND *TRIBULUS TERRESTRIS* AGAINST CERTAIN PATHOGENS

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ABSTRACT

The synergistic effect of medicinal plants, *Piper nigrum* and *Tribulus terrestris* was tested for their antibacterial activity against six characterized pathogens *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus warneri*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Shigella dysentriae*. Preparation of aqueous, ethanol and chloroform extracts of fruit of *Piper nigrum* and *Tribulus terrestris* were done by standard methods. The mix of three solvent extracts of these two plants was prepared in a ratio of 50:50 (20µ/ml of each). Total of three treatments (T1a, T1b, T1c) were prepared of three different solvent extracts. Results indicated that the second treatment (T1b) i.e. mix of ethanol extract of the two plants gave the largest inhibition zone as compared two other two solvent mix against *Staphylococcus warneri* (18.9mm), followed by aqueous extract mix (first treatment/ T1a) which again gave largest inhibition zone against *Staphylococcus warneri* (18.7mm) and then treatment three (chloroform extract mix/ T1c) which are larger than the used standard antibiotic Norfloxacin. The smallest inhibition zone was against *Escherichia coli* (17.0mm) by treatment two (Ethanol extract mix).

Key words: *Piper nigrum*, *Tribulus terrestris*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus warneri*, S*almonella typhi*, *Klebsiella pneumoniae* Norfloxacin, Synergism, Antimicrobial activity.

INTRODUCTION

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources, many of which based on their use in traditional medicine. It has been noted that the original source of many important pharmaceuticals currently in use have been plants used by indigenous people [1]. Herbal medicine or phyto-medicine refers to the use of any plant's seeds, berries, roots, leaves, bark, or flowers for medicinal purposes [2-6].

Piper nigrum is a much branched climbing shrub, rooting at the nodes. The Leaves are simple alternate, cordate, broadly ovate, 5-9 nerved, and dark green. Five Phenolic amides have been identified from *Piper nigrum* in a study carried out by [7]. Piperine is the alkaloid responsible for the pungency of black pepper along with chavicine (an isomer of piperine) [8,9]. It has been reported that piperine is widely used in various herbal cough syrups for its potent anti-tussive and bronchodilator properties. It is used in anti-inflammatory, anti-malarial, anti-leukemia treatment. The antioxidant and radical scavenging activities of black pepper (*Piper nigrum* Linn.) seeds have been well reported [10]. The advantage of utilizing black pepper (as opposed to the standard quinine) in the treatment of refractory intermittent fevers, which are symptomatic of malarial infections, was reported by Taylor [11]. In traditional Chinese medicine, black pepper has been used for the treatment of Epilepsy [12]. Peppers have been traditionally used as local anesthetics, but the

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mechanism of this analgesic (pain-relieving) action has only been recently described [13]. Medicinally black pepper can be used for digestive disorders like large intestine toxins, different gastric problems, diarrhea and indigestion and also can be used against respiratory disorders including cold, fever, and asthma [14-16]. It has anti-inflammatory activity, thermogenic action, growth stimulatory activity, anti-thyroid activity and is chemopreventive [17]. In a study, the antibacterial activity of Alcoholic extracts of ten South Indian spices against Multi-resistant gram positive and Gram-negative Bacteria was done. Out of the various spices tested, Black pepper (*Piper nigrum*) revealed good activity against *Staphylococcus aureus* [18].

Tribulus terrestris is a flowering plant in the family Zygophyllaceae, native to warm temperate and tropical regions of the Old World in southern Europe, southern Asia, throughout Africa, and in northern Australia. They are usually prostrate, forming flat patches, though they may grow more upwards in shade or among taller plants. *Tribulus terrestris* L. has been found to contain biologically active substances as saponins, flavonoids [19], glycosides, phytosterols [20], alkaloids and other constituents [21]. Its main active components are saponins of the furostanol type, termed protodioscin [22]. Flavonoids have anti-inflammatory effect and improve the overall physiological status of the animals. Tannins influence gastrointestinal microflora and exert astringent effect due to their antibacterial properties [23]. Furostanol and Spirostanol saponins, flavonoid glycosides, alkaloids and some amides have been reported to be found in *Tribulus terrestris* [24-28]. The major constituents of this plant are steroidal saponins [29]. *Tribulus terrestris* is used in folk medicine as a tonic, aphrodisiac, palliative, astringent, stomachic, anti-hypertensive, diuretic, lithon-triptic and urinary anti-infectives [30,31]. Crude saponin fraction of the whole plant has been used as a cordial drug [32]. The ash of the whole plant is good for external application in rheumatic-arthritis [33]. It has been reported that *Tribulus terrestris* stimulates spermatogenesis, increases the activity of sertole cells, diminishes urinary oxalate excretion and decreases the activity of liver enzymes such as GAO (Glycolate oxidase) and GAD (Glycolate dehydrogenase) [34].

The rate of disease incidences are increasing. In humans, *E. coli* is the most common cause of urinary tract infection (UTI). Approximately 85% of urethro cystitis is caused by *E. coli* [35]. *Pseudomonas aeruginosa* is an opportunistic pathogen and a major cause of nosocomial infections [36]. *S. warneri* is coagulase-negative *Staphylococci* and represents just less than 1% of total Staphylococcal population, still it is found in 50% of the population and is commensal of skin [37]. *Salmonella typhi* is the gram-negative bacteria which is responsible for causing the debilitating condition of typhoid fever [38]. *Klebsiella pneumoniae* can cause UTI [39]. *Shigella dysentriae* invade a variety of host intestinal cells, including the enterocytes, macrophages and dendritic cells, which leads to severe inflammatory responses in intestinal tissues [40].

In recent years, because of the development of multiple antimicrobial resistant strains it has become a public health concern, emphasizing the importance of continuous monitoring of the pathogen. Therefore, the present study aimed to investigate *in vitro* synergistic effect of antibacterial activity of *Piper nigrum* (Black piper) and *Tribulus terrestris* (Gokharu) against certain pathogens.

MATERIAL AND METHODS

1. Characterized Pathogens: Characterized *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus warneri*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Shigella dysentriae* were selected for present study.

2. **Extraction method:** Three extract were prepared i.e. aqueous extract, ethanolic extract and chloroform extract.

(a) Preparation of aqueous extract

Approx. 30 grams of dried powder of medicinal plant were transferred into soxhlet unit. Extraction was done at 95^{o}C for 24 hours. Bottom of soxhlet extraction unit contains plant extract which was filtered through 8 layers of muslin cloth. The filtrate was evaporated and dried using rotary evaporator at 60°C. The powder was stored at 4ºC.

(b) Preparation of ethanol extract

Approx. 30 grams of dried powder of medicinal plant were transferred into soxhlet unit. Extraction was done at 45^{o}C for 72 hours. Bottom of soxhlet extraction unit contains plant extract which was filtered through 8 layers of muslin cloth. The filtrate was evaporated and dried using rotary evaporator. The powder was stored at 4ºC.

(c) Preparation of chloroform extract

Powdered sample (100 g) of seeds were extracted with chloroform using a soxhlet extractor for continuously 10 h or until the used solvent turned pure and colorless. The solvent was removed by evaporator at 40°C to give a concentrated extract, which was then frozen and freeze-dried until further used. The powder was stored at 4 ºC.

(d) Sterilization of extract

The dried extracts were exposed to ultra violet light (UV rays for 24 h to sterilize [40]. Liquid extracts were sterilized using a membrane filter (0.45-micron sterile filter).

(e) Sterility Test

The sterility was checked by streaking the extracts on nutrient agar plate and incubated at 37° C for 24 h. It was confirmed that there were no artifacts to contaminate the sensitivity testing.

3. Activation of test organisms: The microorganism was activated by inoculating a loopful of the strain in the nutrient broth (50 ml) and incubated on a rotary shaker. Then 0.2 ml of inoculum (inoculum size was 10^8 cells/ml as

per McFarland standard) was inoculated into the molten Muller Hinton agar media and after proper homogenization it was poured into the Petri plate [41,42].

4. Antibacterial Activity by agar well diffusion method: The microorganism was activated by inoculating a loopful of the strain in the nutrient broth (30 ml) and incubated on a rotary shaker. Then 0.2 ml of inoculum (inoculum size was 10^8 cells/ml as per McFarland standard) was inoculated into the molten Muller Hinton agar media and after proper homogenization it was poured into the Petri plate. For agar well diffusion method, a well was made in the seeded plates with the help of a cup-borer. The test compound at four different concentrations i.e. 15 µg/ml, 20 µg/ml 25 µg/ml 30 µg/ml, was introduced into the well and the plates were incubated at 37 ºC for 24 h. Microbial growth was determined by measuring the diameter of zone of inhibition. For each bacterial strain, controls were maintained in which pure solvents were used instead of the extract. The control zones were subtracted from the test zones and the resulting zone diameter is shown in the graph. The experiment was done three times and the mean values are presented.

Table 1. Antimicrobial activity of aqueous extract of *Piper nigrum* and *Tribulus terrestris* against certain pathogens

Pathogens	Inhibition zone (mm)									
	Piper nigrum				Norfloxacin	*Tribulus terrestris*				(T1a) Mix#
	15 µg/ml	20 µg/ml	25 µg/ml	30 µg/ml	15 µg/ml	15 µg/ml	20 µg/ml	25 µg/ml	30 µg/ml	20 µg/ml
Escherichia coli	13.4ns± 0.3	15.1 ns ±0.2	18.8 ns ±0.3	23.1 ns ±0.3	12.7 ns ±0.2	17.8 ns ±0.2	21.2 ns ±0.3	23.9*± 0.1	27.3 ns ±0.3	18.2*±0.2
Pseudomonas aeruginosa	13.6ns± 0.2	15.3 ns ±0.3	19.1 ns ±0.3	23.4 ns ±0.2	13.2 ns ±0.3	15.6 ns ±0.1	18.9 ns ±0.2	22.4 ns ±0.2	28.0*± 0.2	15.2 ns ±0.2
Staphylococcus warneri	18.2** ±0.2	20.9*± 0.3	24.2** ±0.3	26.7 * ±0.3	17.8*±0.3	19.4*±0.3	20.6 ns ±0.2	22.5 ns ±0.2	24.9 ns ±0.3	18.7*±0.2
Salmonella typhi	13.6 ns ±0.3	15.4 ns ±0.3	18.9 ns ±0.2	23.5 ns ±0.3	13.9 ns ±0.4	16.3 ns ±0.2	18.1 ns ±0.2	21.3 ns ±0.3	26.4 ns ±0.2	16.2 ns ±0.3
Klebsiella pneumoniae	13.8 ns ±0.2	15.7 ns ±0.2	19.2 ns ±0.2	23.9 ns ±0.2	13.8 ns ±0.3	16.0 ns ±0.2	18.5 ns ±0.2	21.7 ns ±0.2	26.8 ns ±0.2	15.6 ns ±0.2
Shigella dysentriae	14.2 ±0.4	17.1±0.3	19.5±0.2	23.3±0.3	13.9 ns ±0.4	17.2±0.1	20.3 ±0.3	22.4±0.2	26.5±0.1	16.8±0.3

Values are mean of five replicates. # = 20 µg/ml of both *Piper nigrum* and *Tribulus terrestris*.; **, Significant at 0.01 level of LSD compared to *Shigella dysentriae* ; *Significant at 0.05 level of LSD compared to *Shigella dysentriae*; ns= non-significant .

Table 2. Antimicrobial activity of ethanolic extract of *Piper nigrum* and *Tribulus terrestris* against certain pathogens

Pathogens	Inhibition zone (mm)									
	Piper nigrum				Norfloxacin	*Tribulus terrestris*				T1b Mix#
	15 µg/ml	20 µg/ml	25 µg/ml	30 µg/ml	15 µg/ml	15 µg/ml	20 µg/ml	25 µg/ml	30 µg/ml	20 µg/ml
Escherichia coli	13.1 ns ±0.3	14.7 ns ±0.2	18.4 ns ±0.3	22.6 ns ±0.3	12.7 ns ±0.2	17.4 ns ±0.2	20.5 ns ±0.3	23.4 ns ±0.4	27.0 ns ±0.2	17.0 ns ±0.2
Pseudomonas aeruginosa	13.2 ns ±0.3	14.8 ns ±0.3	18.8 ns ±0.4	23.0 ns ±0.2	13.2 ns ±0.3	15.3 ns ±0.1	18.4 ns ±0.2	22.1 ns ±0.2	27.5 ns ±0.2	15.1 ns ±0.3
Staphylococcus warneri	17.9** ±0.2	20.5** ±0.4	23.8** ±0.3	26.3*± 0.3	17.8**±0.3	19.0*±0.3	20.3 ns ±0.3	22.1 ns ±0.3	24.4 ns ±0.3	18.9*±0.2
Salmonella typhi	13.3 ns ±0.3	15.1 ns ±0.3	18.6 ns ±0.2	23.1 ns ±0.3	13.9 ns ±0.4	15.9 ns ±0.4	17.8 ns ±0.2	20.8 ns ±0.2	26.0 ns ±0.3	16.5 ns ±0.3
Klebsiella pneumoniae	13.3 ns ±0.4	15.2 ns ±0.2	18.8 ns ±0.1	23.6 ns ±0.1	13.8 ns ±0.3	15.6 ns ±0.2	18.1 ns ±0.2	21.3 ns ±0.2	26.5 ns ±0.2	15.8 ns ±0.2
Shigella dysentriae	13.8±0.1	16.7±0.2	19.1±0.2	22.9±0.3	13.9±0.4	16.8±0.3	20.0 ns ±0.1	22.0 ns ±0.2	26.1 ns ±0.2	16.4±0.4

Values are mean of five replicates. # = 20 µg/ml of both *Piper nigrum* and *Tribulus terrestris* ; **, Significant at 0.01 level of LSD compared to *Shigella dysentriae* ; *Significant at 0.05 level of LSD compared to *Shigella dysentriae*; ns= non-significant .

Table 3. Antimicrobial activity of chloroform extract of *Piper nigrum* and *Tribulus terrestris* against certain pathogens

Pathogens	Inhibition zone (mm)									
	Piper nigrum				Norfloxacin	*Tribulus terrestris*				T1c Mix[#]
	15 µg/ml	20 µg/ml	25 µg/ml	30 µg/ml	15 µg/ml	15 µg/ml	20 µg/ml	25 µg/ml	30 µg/ml	20 µg/ml
Escherichia coli	13.1[ns] ±0.3	14.6[ns] ±0.1	18.2[ns] ±0.3	22.6[ns] ±0.3	12.7[ns] ±0.2	17.4[ns] ±0.2	20.6[ns] ±0.1	23.3[ns] ±0.1	26.7[ns] ±0.3	17.6[ns] ±0.2
Pseudomonas aeruginosa	13.5[ns] ±0.1	14.6[ns] ±0.3	18.2[ns] ±0.3	23.0[ns] ±0.2	13.2[ns] ±0.2	15.2[ns] ±0.2	18.3[ns] ±0.2	21.6[ns] ±0.3	27.4[ns] ±0.3	14.6[ns] ±0.3
Staphylococcus warneri	18.0**± 0.2	20.3** ±0.3	23.5** ±0.3	26.2*± 0.2	17.8*±0.2	19.0*±0.3	20.2[ns] ±0.3	22.1[ns] ±0.2	24.4[ns] ±0.3	18.2*±0.2
Salmonella typhi	13.3[ns] ±0.2	15.0[ns] ±0.3	18.2[ns] ±0.1	23.1[ns] ±0.3	13.9[ns] ±0.1	16.1[ns] ±0.3	17.5[ns] ±0.2	20.8[ns] ±0.4	26.0[ns] ±0.4	15.8[ns] ±0.4
Klebsiella pneumoniae	13.2[ns] ±0.2	15.1[ns] ±0.2	18.6[ns] ±0.2	23.3[ns] ±0.2	13.8[ns] ±0.3	15.7[ns] ±0.2	18.1[ns] ±0.2	21.3[ns] ±0.2	26.4[ns] ±0.2	16.3[ns] ±0.2
Shigella dysentriae	13.7±0.3	16.4±0.2	19.0±0.3	22.8±0.4	13.9±0.2	16.6±0.1	19.7±0.3	22.0±0.2	26.1±0.2	17.8±0.2

Values are mean of five replicates. # = 20 µg/ml of both *Piper nigrum* and *Tribulus terrestris*.

RESULT AND DISCUSSION

The aim of present study was to evaluate the synergistic effect of three different solvent extracts mix of two medically important plants *Piper nigrum* and *Tribulus terrestris* as they were tested for their antibacterial activity against six characterized pathogens *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus warneri*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Shigella dysentriae*. Total of three treatments were prepared namely T1a (20µg/ml each aqueous extract of *Piper nigrum* + *Tribulus terrestris*), T1b (20µg/ml each ethanol extract of *Piper nigrum* + *Tribulus terrestris*), T1c (20µg/ml each chloroform extract of *Piper nigrum* + *Tribulus terrestris*). Although all the three mix treatments were effective against all six pathogens but the best results were obtained against *Staphylococcus warneri* and *Escherichia coli*. The treatment T1b gave the largest inhibition zone against *Staphylococcus warneri* (18.9mm) and treatment T1a gave the largest inhibition zone against *Escherichia coli* (18.2mm). Treatment T1c was also found to be highly effective against *Staphylococcus warneri* (18.2mm) as compared to standard antibiotic used i.e. Norfloxacin which gave a 17.8mm inhibition zone against *Staphylococcus warneri* and 12.7mm against *Escherichia coli* at same concentration. Similar types of observations have been found in the past researches. In a study [2], antibacterial activity of Black pepper (*Piper nigrum*) was tested against a number of Gram-positive and Gram-negative bacteria. The results indicated excellent inhibition on the growth of Gram-positive bacteria like *S. aureus*, followed by *B. cereus* and *Streptococcus faecalis*. Among the Gram-negative bacteria *P. aeruginosa* was more susceptible followed by *S. typhi* and *E. coli*.

The antibacterial activity of *Piper nigrum* was measured against various pathogenic bacteria and fungus [3]. Aqueous, ethanolic and methanolic extracts of black pepper exhibited activity against *S. aureus* and *E. coli*. *Tribulus terrestris* was evaluated for antimicrobial activity against Gram-positive organisms like *Bacillus subtilis*; *Staphylococcus aureus* and Gram–negative organisms like *E. coli*, *Proteus vulgaris*. The antimicrobial activity was found to be highest against *Staphylococcus aureus* in case of Gram-positive bacteria and *E. coli* in case of Gram-negative bacteria [4]. Antimicrobial activity of organic and aqueous extracts from fruits, leaves and roots of *Tribulus terrestris* L., was examined against 11 species of pathogenic and non-pathogenic microorganisms: *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Corynebacterium diphtheriae*, *Escherichia coli*, *Proteus vulgaris*, *Serratia marcescens*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Candida albicans* [5].

CONCLUSION

The antimicrobial study revealed that the mix of equal proportion of ethanol extract of fruit of *Piper nigrum* and *Tribulus terrestris* is highly effective against Gram-positive *Staphylococcus warneri* and Gram-negative *Escherichia coli*. Also the mix extract of aqueous extract of fruit of *Piper nigrum* and *Tribulus terrestris* shows good inhibitory effect against *Staphylococcus warneri*. The results of the *In vitro* synergistic effect of antibacterial activity of *Piper nigrum* and *Tribulus terrestris* against certain pathogens indicates that they can be used against these pathogens.

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