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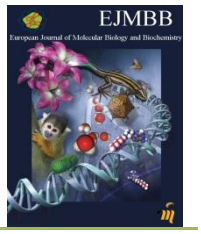

IN VITRO SYNERGISTIC EFFECT OF ANTIBACTERIAL ACTIVITY OF *PIPER NIGRUM* AND *RAUWOLFIA SERPENTINA* AGAINST CERTAIN PATHOGENS

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ABSTRACT

The medicinal plants, *Piper nigrum* (fruit) and *Rauwolfia serpentina* (Root) was tested for their synergistic effect as antibacterial agents against six characterized pathogens *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus warneri*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Shigella dysentriae*. The aqueous, ethanol and chloroform extracts of fruit of *Piper nigrum* and *Rauwolfia serpentina* were Preparation by standard methods. In a ratio of 50:50 (20µ/ml of each), the mix of three solvent extracts of these two plants was prepared. Of three different solvent extracts, total of three treatments (T2a, T2b, T2c) were prepared. The results showed that the first treatment (T2a) i.e. mix of aqueous extract of the two plants gave the largest inhibition zone as compared two other two solvent mix against *Staphylococcus warneri* (17.2mm), followed by chloroform mix (third treatment/ T2c) which again gave largest inhibition zone against *Staphylococcus warneri* (17.0mm). The first treatment (T2a) showed very good inhibition effect against *Shigella dysentriae* (16.2mm) which was seen to be more than individual plant extracts as well as standard antibiotic Norfloxacin (13.9mm). Then treatment two (ethanol extract mix/ T2b) gave largest inhibition zone against *Staphylococcus warneri* (16.8mm) and all were slightly less than the used standard antibiotic Norfloxacin (17.8mm). The smallest inhibition zone was against *Escherichia coli* (14.8mm) by treatment three (chloroform extract mix).

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].

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 [3]. Piperine is the alkaloid responsible for the pungency of black pepper along with chavicine (an isomer of piperine) [4]. It was 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 [5]. The antioxidant and radical scavenging activities of black

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pepper (*Piper nigrum* Linn.) seeds have been well reported [6]. 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 [7]. In traditional Chinese medicine, black pepper has been used for the treatment of Epilepsy [8]. Peppers have been traditionally used as local anesthetics, but the mechanism of this analgesic (pain-relieving) action has only been recently described [9]. 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 [10, 11, and 12]. It has anti-inflammatory activity, thermogenic action, growth stimulatory activity, anti-thyroid activity and is chemopreventive [13]. 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* [14].

Rauwalfia serpentina (Sarpagandha) has been used in India from century. The genus name was selected in honor of Dr. Leonhard Rauwolf, a 16th century German botanist, Physician & explorer [15]. *Rauwolfia serpentina* (Sarpagandha) is an important medicinal plant distributed in the foot-hills of Himalayan range, up to the elevation of 1300-1400 m. and almost all over the country. It is an erect, evergreen shrub, merely 15 to 45 cm high. It is perennial from a woody root stock. Leaves are lancealate or oblancealate, 13-18 X 6-8 cm, acute or acuminate, shining. The Calyx lobes are lanceolate. Corella is white, tube swollen above the middle, lobes elliptic-oblong, and drupes 0.5-0.7 cm across, purplish black [16]. In a study, *Rauwolfia serpentina* was analyzed for its chemical composition, vitamins and minerals. The results revealed the presence of bioactive constituents comprising alkaloids, saponins, flavonoids, phenols and tannins. The medicinal plants contained ascorbic acid, riboflavin, thiamine and niacin. These herbs are good sources of minerals such as Ca, P, K, Mg, Na, Fe and Zn [17]. The plant contains more than 50 different alkaloids which belong to the monoterpenoid indole alkaloid family. The major alkaloids are ajmaline, ajmalicine, ajmalimine, deserpidine, indobine, indobinine, reserpine, reserpiline, rescinnamine, rescinnamidine, serpentine, serpentinine and yohimbine [18]. Presence of high quantity of total polyphenolic compounds in *R. serpentina* shows significant antidiabetic and hypolipidemic properties [19, 20]. The major alkaloid present in root, stem and leaves of the plant is Reserpine varies from 1.7 to 3.0 %. The root barks has more than 90% of the total alkaloids in roots. The minor alkaloids present in the plant are Ajmalicine, ajmaline, isoajmaline, ajmalinine, chandrine, rauwolfinine, renoxidine, rescin-namine, reserpiline, reserpin, reserpinine, sarpagine, serpentine, serpentinine, tetraphyllicine, yohimbine, and 3-epi-a-yohimbine. The root contains ophioxylin, resin, starch and wax [21]. *Rauwolfia serpentina* has been used for centuries for the relief of various central nervous system disorders, both psychic and motor, including anxiety states, excitement, maniacal behaviour associated with psychosis, schizophrenia, insanity, insomnia and epilepsy. Long back [22] in their study confirmed the moderate hypotensive and symptomatically beneficial effects of *Rauwolfia serpentina* in hypertensive patients. In Ayurvedic medicines, the roots of *R. serpentina* is used as a remedy for curing hypertension, insomnia, mental agitation, gastrointestinal disorders, excitement, epilepsy, traumas, anxiety, excitement, schizophrenia, sedative insomnia and insanity [23, 24]. In Siddha medicine, *R. serpentina* roots are used for curing hypertension-associated headache, dizziness, amenorrhea, oligomenorrhea and dysmenorrhea like abnormalities. The root juices or extract is used to treat liver and abdomen pain, various gastrointestinal disorders and to expel intestinal worms from the children's [25, 26 and 27].Extracts of the roots is valued for the treatment of intestinal disorders, particularly diarrhoea and dysentery and also an anthelmintic. Mixed with other plant extracts, they have been used in the treatment of cholera, colic and fever.

In India and Nepal, it is a common treatment for hypertension and insomnia. The antimicrobial activity of *Rauvolfia serpentina* (roots) was tested against Acne-Inducing Bacteria namely *Propionibacterium acnes* and *Staphylococcus epidermidis* [28]. In a study, antibacterial activity of the methanol extracts of leaf, root of *Rauvolfia serpentina* was tested against Gram-positive and Gram-negative bacteria. As a result it was found that the extract showed a good antibacterial activity against Gram-negative organisms [29].

The rate of disease incidences are increase. In humans, *E. coli* is the most common cause of urinary tract infection (UTI). Approximately 85% of urethro cystitis is caused by *E. coli* [34]. *Pseudomonas aeruginosa* is an opportunistic pathogen and a major cause of nosocomial infections [35]. *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 [36]. *Salmonella typhi* is the gram-negative bacteria which is responsible for causing the debilitating condition of typhoid fever [37]. *Klebsiella pneumoniae* can cause UTI [38].

In recent years, it has become a public health concern because of the development of multiple antimicrobial resistant strains, 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 *Rauwolfia serpentina* 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°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°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 4 ºC.

(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 [39]. 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 loop full 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 [40].

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. Similarly, 20 µg/ml concentrations of *Piper nigrum* and *Rauwolfia serpentina* mix was prepared by mixing each of their solvent extracts at ratio of 50:50. The test compound mix 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 water extract of *Piper nigrum* and *Rauwolfia serpentina* against certain pathogens

Pathogens	Inhibition zone (mm)									
	Piper nigrum				Norfloxacin	*Rauwolfia serpentina*				T2a 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	13.4 ns ±0.3	15.1 ns ±0.2	18.8 ns ±0.3	23.1 ns ±0.3	12.7±0.2	15.9 ns ±0.3	19.1 ns ±0.2	22.2*± 0.2	24.9 ns ±0.2	15.3 ns ±0.3
Pseudomonas aeruginosa	13.6 ns ±0.2	15.3 ns ±0.3	19.1 ns ±0.3	23.4 ns ±0.2	13.2±0.2	13.7 ns ±0.3	16.7 ns ±0.3	20.6 ns ±0.3	26.2*± 0.2	13.2 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	17.3*±0 .3	18.5*± 0.2	20.3 ns ±0.2	22.6 ns ±0.3	17.2*±0 .3
Salmonella typhi	13.6 ns ±0.3	15.4 ns ±0.3	18.9 ns ±0.2	23.5 ns ±0.3	13.9±0.1	14.1 ns ±0 .2	15.9 ns ±0.3	19.4 ns ±0.2	24.1 ns ±0.3	13.7 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±0.2	14.2 ns ±0.3	16.1 ns ±0.2	19.9 ns ±0.3	24.5 ns ±0.2	13.8 ns ±0.2
Shigella dysentriae	14.2 ±0.4	17.1±0 .3	19.5±0 .2	23.3±0 .3	13.9±0.1	15.6±0. 3	18.2±0 .3	20.2±0 .3	24.1±0 .2	16.2±0. 2*

Values are mean of five replicates. # = 20 µg/ml of both *Piper nigrum* and *Rauwolfia serpentina* ; **, 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 *Rauwolfia serpentina* against certain pathogens

Pathogens	Inhibition zone (mm)									
	Piper nigrum				Norfloxacin	*Rauwolfia serpentina*				T2b 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±0.2	15.5 ns ±0.2	18.6 ns ±0.1	21.6*± 0.1	24.4 ns ±0.2	14.9 ns ±0.3
Pseudomonas aeruginosa	13.2 ns ±0.3	14.8 ns ±0.3	18.8 ns ±0.4	23.0 ns ±0.2	13.2±0.2	13.2 ns ±0.3	16.3 ns ±0.3	20.2 ns ±0.2	25.7*± 0.2	13.0 ns ±0.2
Staphylococcus warneri	17.9** ±0.2	20.5** ±0.4	23.8** ±0.3	26.3*± 0.3	17.8±0.3	16.7* ±0.1	18.2*± 0.2	19.7 ns ±0.2	22.2 ns ±0.3	16.8*±0 .3
Salmonella typhi	13.3 ns ±0.3	15.1 ns ±0.3	18.6 ns ±0.2	23.1 ns ±0.3	13.9±0.1	13.5 ns ±0.2	15.3 ns ±0.4	18.5 ns ±0.4	23.8 ns ±0.4	13.5 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±0.2	13.6 ns ±0.4	15.7 ns ±0.4	19.4 ns ±0.3	24.2 ns ±0.2	13.6 ns ±0.2
Shigella dysentriae	13.8±0 .1	16.7±0 .2	19.1±0 .2	22.9±0 .3	13.9±0.1	15.2±0. 3	17.7±0 .2	19.6±0 .4	23.4±0 .4	15.2±0. 2

Values are mean of five replicates. # = 20 µg/ml of both *Piper nigrum* and *Rauwolfia serpentina* ; **, 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 *Rauwolfia serpentina* against certain pathogens

Pathogens	Inhibition zone (mm)									
	Piper nigrum				Norfloxacin	*Rauwolfia serpentina*				T2c 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±0.2	15.5 ns ±0.3	18.6*± 0.2	21.6*± 0.2	24.5 ns ±0.2	14.8 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±0.2	13.3 ns ±0.1	16.3 ns ±0.3	20.2 ns ±0.1	25.7*± 0.2	12.9 ns ±0.4
Staphylococcus warneri	18.0** ±0.2	20.3** ±0.3	23.5** ±0.3	26.2*± 0.2	17.8±0.3	17.0*±0 .3	18.2 ns ±0.2	20.0 ns ±0.2	22.1 ns ±0.3	17.0*±0 .3
Salmonella typhi	13.3 ns ±0.2	15.0 ns ±0.3	18.2 ns ±0.1	23.1 ns ±0.3	13.9±0.1	13.8 ns ±0.2	15.7 ns ±0.3	19.1 ns ±0.2	23.7 ns ±0.4	13.3 ns ±0.3
Klebsiella pneumoniae	13.2 ns ±0.2	15.1 ns ±0.2	18.6 ns ±0.2	23.3 ns ±0.2	13.8±0.2	13.9 ns±0.2	15.8 ns ±0.2	19.8 ns ±0.3	24.2 ns ±0.2	13.2 ns ±0.2
Shigella dysentriae	13.7±0 .3	16.4±0 .2	19.0±0 .3	22.8±0 .4	13.9±0.1	15.2±0. 3	17.2±0 .1	19.9±0 .1	23.7±0 .1	16.0±0. 4

Values are mean of five replicates. # = 20 µg/ml of both *Piper nigrum* and *Rauwolfia serpentina* ; **, 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 .

RESULT AND DISCUSSION

Evaluation of the synergistic effect of three different solvent extracts mix of two medically important plants *Piper nigrum* (Fruit) and *Rauwolfia serpentina* (Root) was the aim of present study, 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 T2a (20µg/ml each aqueous extract of *Piper nigrum* + *Rauwolfia serpentina*), T2b (20µg/ml each ethanol extract of *Piper nigrum* + *Rauwolfia serpentina*), T2c (20µg/ml each chloroform extract of *Piper nigrum* + *Rauwolfia serpentina*). Although all the three mix treatments were effective against all six pathogens but the best results were obtained against *Staphylococcus warneri* and *Shigella dysentriae*. The treatment T2a gave the largest inhibition zone against *Staphylococcus warneri* (17.2mm) and treatment T2b also gave the largest inhibition zone against *Staphylococcus warneri* (16.8mm). Treatment T2c was also found to be highly effective against *Staphylococcus warneri* (17.0mm)

as compared to standard antibiotic used i.e. Norfloxacin which gave slightly larger, i.e. 17.8mm inhibition zone against *Staphylococcus warneri*. The first treatment (T2a) showed very good inhibition effect against *Shigella dysentriae* (16.2mm) which was seen to be more than individual plant extracts as well as standard antibiotic Norfloxacin (13.9mm). Similar types of observations have been found in the past researches. In a study carried, 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* [31]. The antibacterial activity of *Piper nigrum* was measured against various pathogenic bacteria and fungus [32]. 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 [33]. In a study, antibacterial activity of the methanol extracts of leaf, root of *Rauvolfia serpentina* was tested against Gram-positive and Gram-negative bacteria. As a result it was found that the extract showed a good antibacterial activity against Gram-negative organisms [29]. The antibacterial activity of methanol extracts of *R. serpentina* was tested against *Salmonella typhimurium*, *Escherichia coli*, *Citrobacter freundii*, *Proteus vulgaris*, *Enterococcus faecalis* and *Staphylococcus aureus* [30]. The study revealed that the highest zone of inhibition with lowest MIC was observed against *Staphylococcus aureus* and highest MIC was observed against *Escherichia coli*, whereas *Proteus vulgaris* was observed resistant to tested extracts.

CONCLUSION

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

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