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PRELIMINARY STUDIES ON COMBINATIONAL ANTIBACTERIAL EFFECTS OF ROOT EXTRACTS OF *WITHANIA SOMNIFERA* AND *CARICA PAPAYA*

Aishwarya Dey[1], Nibedita Dey[2] and Shaleesha Stanley*

[1]*Department of Biotechnology, Jeppiaar Engineering College, Chennai, Tamil Nadu, India.

[2]Centre for Nanoscience and Technology, Anna University, Chennai, Tamil Nadu, India.

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***Corresponding Author**
Dr. Shaleesha Stanley
Department of Biotechnology, Jeppiaar Engineering College, Chennai, Tamil Nadu, India.

ABSTRACT

The ethanolic root extract of Ashwagandha (*Withania somnifera*) and Papaya (*Carica Papaya*) is said to have anti-microbial activity. This project was undertaken to analyze the anti-bacterial activity of these plant extracts against three Gram Negative and two Gram Positive isolates in Chennai. Combinational dosages of both were next studied on the most prevalent gram negative and gram positive strain. The present study suggests that 1:1 ratio of both the flavonoids have a synergistic effect by increasing the antibiotic activity of papaya.

KEYWORDS: *Withania Somnifera*, *Carica Papaya*, *Escherichia coli*, *Staphylococcus aureus*.

1. INTRODUCTION

One of the emerging severe global problems is antimicrobial resistance. This leads to increasing in time for controlling bacterial diseases and cost for the production of medicines. There is a concern worldwide that antibiotics are being overused contributing toward the growing number of infections that are becoming resistant due to mutation due to increased exposure to medications. Resistance to antimicrobial agents (AMR) has resulted in morbidity and mortality from treatment failures and increased health care costs. With the fast pace world people in search of instant cure consume bacteriostats and bateriocidals in huge numbers causing high resistance in pathogenic strains and also bloom in the pharmaceutical industry to come up with more medications of these types.[1] The side for the same ranges from kidney stones formations, Blood disorders, Deafness, inflamed bowels, immediate or delayed hypersensitivity reactions, etc. The Centre for Disease Control and Prevention (CDC)

and European Centre for Disease Prevention and Control (ECDC) monitors the outpatient antibiotic overuse in the United States and European countries. They gave a statement in 2012, stating around 25,000 people die each year in the European Union from antibiotic-resistant bacterial infections. Dey et al in 2015 gave report on plasmid mediated resistance in gram negative bacteria in Chennai had grown to 100 percent for Cephalosporin (a well known antibiotic).[2] As the world is going back to its roots to nature, use of natural therapeutics as medications is drawing the limelight. The increasing failure of chemotherapeutics and antibiotic resistance exhibited by microbial pathogens has led to the screening of medicinal plants for their potential antimicrobial activity.[3] The tradition of using plants as a potent antimicrobial, antifungal, antiviral agent dates back to centuries. Their use in treating many diseases has still been in use in rural regions of India, China, Japan, Korea, Africa, etc.[4] Among the lakhs of available species of higher plants, only 5-10% has been studied phytochemically and a fraction of it was submitted to biological or pharmacological screenings.[5]

Carica papaya Linn or Papaya or L. Caricaceae is valuable as food and as traditional medicine commonly known for its food and nutritional values worldwide. Each part of this plant possesses economic value when it is grown on a commercial scale.[6] The active components are extracted from all parts of this plant, but the concentration of bioactive compounds may vary from structure to structure. However, the highest concentration for therapeutic purposes can extracted from the leaves, barks, roots, rhizomes, woods, flowers, fruits and the seeds.[7] The plant parts possess some properties like analgesic, antimodulatory, antibacterial, cardiotonic, febrifuge, digestive, amebicide, hypotensive, pectoral, laxative, anti- tumor.[8,9]

Withania Somnifera also known as Winter Cherry, Ashwagandha, Indian ginseng, etc is a small medium shrub belonging to the Solanaceae family. The plant has been found useful in the treatment for dermatological disorders, gastrointestinal diseases, etc.[10-12] It has hemopoetic and properties besides positively influencing the endocrine, arthritis, cardiopulmonary, antiserotogenic, central nervous systems and many more.[13-18]

The present studies focuses on the combinational effects of both root extracts on prominent bacterial pathogens, to suggest potential herbal medications in future.

2. MATERIALS AND METHODS

2.1. Collection of Medicinal leaves

Fresh, disease free, young and green leaves were collected locally from the farmers of AVG Reddy Herbal Shop, Mylapore. The leaves were washed thoroughly10 times in sterile distilled water. Then, they were air-dried under shade at room temperature for 14 days and finely powdered using a blender.[19]

Withania somnifera roots were collected locally from the farmers of AVG Reddy Herbal Shop, Mylapore and washed under running tap water, dried in shade and powdered using blender. The powdered form of plant material was stored in air tight glass bottles protected from sunlight until required for testing.[20]

2.2. Pathogen Used in Study

The test pathogens were procured from Military hospital Chennai in stock cultures and they were reconfirmed by morphological, cultural and biochemical characteristics. The most common virulent species present in the region were rounded to *Escherichia coli [E. coli], Klebsiella pneumonia, Proteus mirabilis, Staphylococcus aureus and Staphylococcus epidermidis.* The cultures were emulsified in 5ml of Nutrient Broth (NA) and incubated for 24 hrs. They grown in nutrient agar plates and stored in refrigerators in 4°C until use. They were revived every two day to maintain their virulent property. Fresh cultures were employed for assessing antibacterial activity of the papaya root-extracts.

2.3. Extract Preparation

The crude extract from the root of papaya was prepared according to the method proposed by.[21] The ethanol extract was prepared by suspending 25grams of the finely blended dried root powder was mixed with in 100ml of 95% ethanol. The mixture was then treated by agitating it in the blender and heated till evaporation at 60°C. The crude extracts left after evaporation were scratched against the walls of the beaker and weighed. This extract was then stored in the refrigerator at 4°C until use.

2.4. Preparation of Stocks

The stock was prepared by reconstituting 1g of each of the extracts in 1ml of DMSO. Different concentrations (100mg/ml, 80mg/ml, 60mg/ml, 40mg/ml and 20mg/ml) of each of the extracts were then prepared from their stock.[22]

2.5. Test for Antibacterial Activity

Antibacterial activity by disc diffusion method was performed against two gram positive (*Staphylococcus epidermidis* and *Staphylococcus aureus*) and three gram negative (*Escherichia coli*, *Proteus mirabilis* and *Klebsilla pneumoniae)* bacterial sample and was inoculated using spread plate technique for uniform distribution of bacterial test sample over the agar plates. Sterilized Whatman filter paper No.1 discs (6 mm diameter) contained the plant sample with the 100mg/ml, 80mg/ml, 60mg/ml, 40mg/ml and 20mg/ml concentrations were mounted over the culture plate. Gentamycin (GEN10) and sterilized water were used as positive and negative control respectively. The zone of inhibition was expressed in millimetre.[23]

2.6. Phytochemical Test

The following tests were done in order to know the phytochemical properties of the two plant extracts as previously reported by Abdul Wadood et al.[24]

3. RESULTS AND DISCUSSION

Initially, antimicrobial susceptibility test were performed for 3 of Gram negative and 2 Gram positive virulent isolates. The bacterial isolates were screened by using 9 antibiotics shown in table 1. These 5 strains were selected based on their occurrence as the most frequently infecting bacterial species in that area.

Table 1. Antibacterial Discs used for Antibacterial susceptibility test

S.No	Antibiotics	Symbol/ concentration	Mode of action
1.	Tobramycin	TOB 10mcg/disc	Inhibits bacterial protein synthesis
2.	Cefoxitin	Cx 30mcg/disc	Inhibits one of the enzymes involved in the synthesis of the bacterial cell wall.
3.	Nitrofurantoin	NIT 30mcg/disc	alter bacterial ribosomal proteins and other macromolecules
4.	Azithromycin	AT 30mcg/disc	Inhibits translation of mRNA.
5.	Amoxyclav	Ac 30mcg/disc	Class A ESBL enzyme inhibitor.
6.	Cefepime	Cpm 30mcg/disc	Inhibits one of the enzymes involved in the synthesis of the bacterial cell wall.
7.	Imipenem	IPM 10mcg/disc	Inhibits bacterial cell wall synthesis.
8.	Gentamicin	GEN 10mcg/disc	irreversibly binding the 30S subunit of the bacterial ribosome, interrupting protein synthesis
9.	Meropenem	MRP 10mcg/disc	inhibition of cell wall synthesis

All the 5 strains showed sensitivity to commercial antibiotics. These strains were screened for sensitivity based on zone diameter as per CLSI guidelines.[25] Figure 1 shows the antimicrobial susceptibility test for all the 5 isolates.

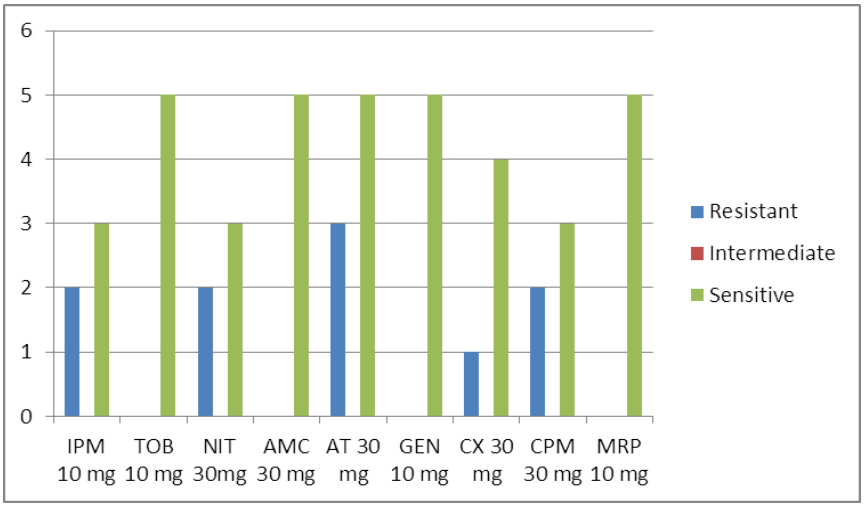

Figure 1 Antibacterial susceptibility test for all 5 strains with commercial antibiotics discs

These strains were next subjected to sensitivity test for papaya and Ashwagandha extract in variable concentrations ranging from 100mg/ml - 20mg/ml. A triplicate for each extract was noted for better reproducibility. Figure 2 shows the three trials for papaya ethanolic extract.

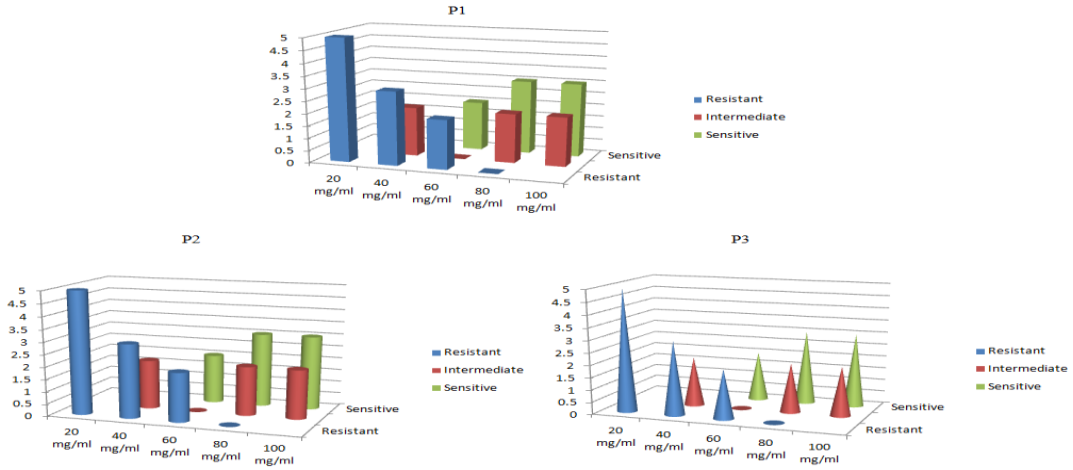

Figure 2 Antibacterial susceptibility test triplicates for Papaya extracts- P1 (trial 1), P2 (trial 2) and P3 (trial 3).

The plant extracts were made in different concentrations starting from 100mg/ml, 80mg/ml, 60mg/ml, 40mg/ml and 20mg/ml. It's been demonstrated earlier that organic extracts were

more effective than aqueous extracts due to better solubility of the active components in organic solvents.[26] Different solvents have different polarities and therefore different degrees of solubility for the phyto constituents.[27, 28] Based on the previous literature we chose ethanol as our solvent over others because of the unlimited spectrum of activity of ethanol that active component are more soluble in them.

Prot. Mirabilis and *Staphylococcus Epidermidis* were highly resistant to papaya ethanolic extract till 60mg/ml. 60mg/ml is the average minimum concentration that shows the initialization of sensitivity in *E.Coli, Klebshiella pnuemoniae and Staph. Aureus.* As the concentration increases the by 100mg/ml all the five strains are highly sensitive to the extract. Chymenopapain and papain are the two important bioactive compounds present in papaya.[29] They also have therapeutic compounds, such as, p-coumaric acid, caffeic acid, 5, 7-dimethoxycoumarin, kaempferol, quercetin, protocatechuic acid and chlorogenic acid.[30,31] The zones of inhibition values indicated extent of effectiveness of the extract with increase in concentration. It has been suggested in literature that the mode of action may be against the bacteria and fungi cell wall formation, resulting in a leakage of cytoplasmic constituents.[32,33] Hence, we were able to justify the sensitivity produced by papaya ethanolic extract towards these virulent strains.

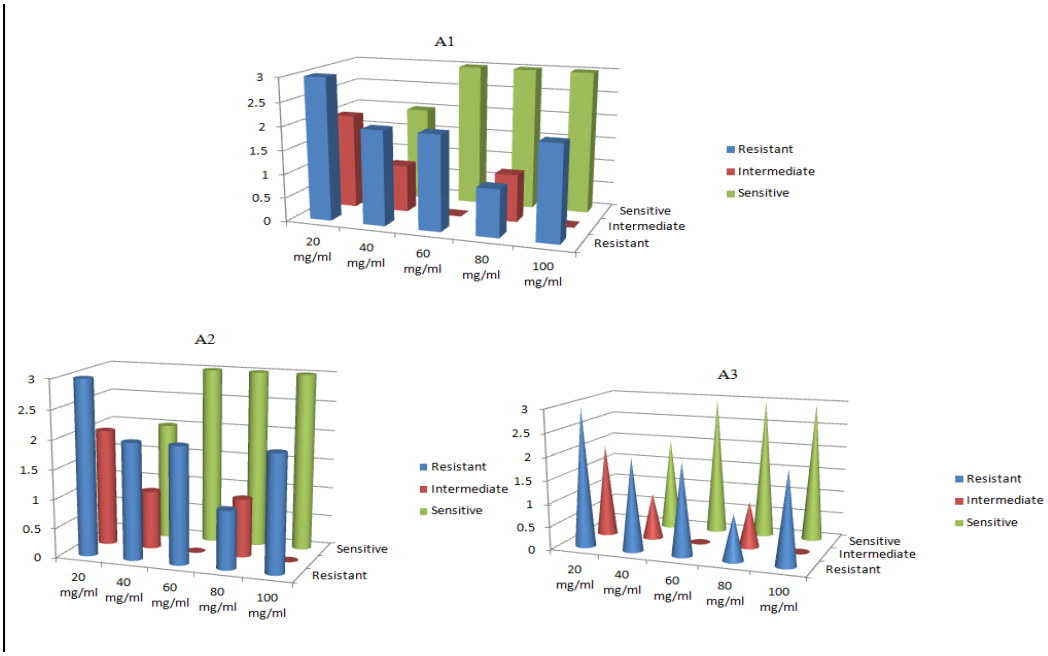

Figure 3 Antibacterial susceptibility test triplicates for Ashwagandha extracts- A1 (trial 1), A2 (trial 2) and A3 (trial 3).

Figure 3 exhibits the triplicate for Ashwagandha root ethanolic extract from 20mg/ml to 100mg/ml. *Prot. Mirabilis* and *Staphylococcus Epidermidis* were highly resistant to papaya ethanolic extract till 40mg/ml. Sensitivity of the 3 other virulent species were high in aswagandha as compared to the papaya extract. Awagandha seems to be more potent antibiotic in comparison to papaya. It's been reported that the major biochemical constituents are steroidal alkaloids and steroidal lactones. They belong to a class of constituents called withanolide. Of which Withaferin A and Withanolide D are the main constituents that contribute to most of the biological activity. Indian root alkaloid constitution varies in between 0.13% to 0.31% [34]. Since literature reports that this drug has potency to standard commercial antibiotic drugs hence proposed this study to check for combinational effects of this extract with papaya to come up with possible natural medication dosages. The triplicate of each extract was tabulated to standard deviation for each root extract with the 5 strains. The data in table 2 and 3 standard deviation values for the papaya and ashwaganda extracts respectively.

Table 2 Standard deviation Chart for Antibacterial susceptibility test with papaya root extract

S.No.	Bacteria	20 mg/ml	40 mg/ml	60 mg/ml	80 mg/ml	100 mg/ml
Unit		(mm)	(mm)	(mm)	(mm)	(mm)
1	*E.coli*	-	-	8.33 ± 1.04	12.33 ± 1.25	15.5 ± 0.5
2	*Prot. Mirabilis*	-	-	0 ± 1.03	8.167 ± 1.82	9.833 ± 0.286
3	*Klebsilla pneumoniae*	-	-	9 ± 0.5	12 ± 0.50	14.5 ± 0.5
4	*Staph. Aureus*	-	8 ± 0.10	12.43 ± 0.763	15.167 ± 1.258	19.833 ± 0.286
5	*staph. Epidermidis*	-	-	0 ± 4.30	7.833 ± 1.04	10.5 ± 0.5

Table 3 Standard deviation Chart for Antibacterial susceptibility test with Ashwagandha root extract

S.No.	Bacteria	20 mg/ml	40 mg/ml	60 mg/ml	80 mg/ml	100 mg/ml
Unit		(mm)	(mm)	(mm)	(mm)	(mm)
1	*E.coli*	7.33 ± 0.573	10.167 ± 0.763	12.1667 ± 0.726	14.833 ± 0.288	17.667 ± 0.573
2	*Prot. Mirabilis*	-	-	-	-	7.5 ± 0.5
3	*Klebsilla pneumonia*	8.83 ± 0.2867	11.833 ± 0.637	14.176 ± 0.2813	18.5 ± 0.8660	21.676 ± 0.5350
4	*Staph. Aureus*	7.5 ± 0.5	12.5 ± 0.5	15.187 ± 0.465	18.67 ± 0.51316	20.833 ± 0.7637
5	*Staph. Epidermidis*	-	-	-	7.33 ± 0.5773	9.5 ± 0.5

3.1 Phytochemical activity

The qualitative test for the phytochemical activity of the two extracts is depicted in table 4. These tests reveal that both the extracts consist of considerable quantity of alkaloids, tannins, flavonoids and glycosides. Only papaya extracts have saponins in them.

Table 4 Phytochemical tests for qualitative determination of both the root extracts

Sno	Material	Test for Phytochemical	Reagent	Color change	Confirmation
1.	Papaya Ashwagandha	Alkaloid	Mayer test	Cream yellow precipitate	Positive Positive
2.	Papaya Ashwagadha	Tannin	$FeCl_3$	Green color	Positive Positive
3.	Papaya Ashwagadha	Flavonoids	NaOH $AlCl_3$ H_2SO_4	Yellow Precipitate	Positive Positive
4.	Papaya Ashwagadha	Saponin	Olive oil test	Formed an Stable emulsion	Positive Negative
5.	Papaya Ashwagadha	Glycoside	Distilled water H_2SO_4 KOH Fehling's solutions	Brick red Precipitate	Positive Positive

3.2 Combinational effect

As discussed before from antibacterial susceptibility test shows that *Klebsiella pneumonia*, *Proteus mirabilis* and *Staphylococcus epidermidis* do not respond well to the plant extracts being studied. From the 5 types of strains evaluated, one gram negative and one gram positive strain are chosen based on the most prevalent strain in the region and also that shows some sensitivity to the drug being analysed. Hence, *E.Coli* and *Staphylococcus Aureus* were taken up further for combinational studies of both the ethanolic root extracts.

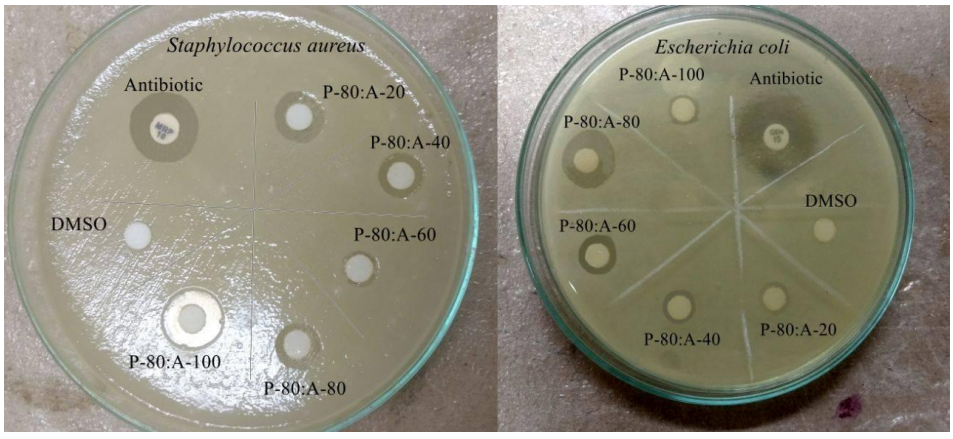

Figure 4 Combinational Antibacterial effects of both root extracts on *Staphylococcus Aureus* (left) and *E.Coli* (right).

To see the combinational effect of both the root extracts, we made filter paper discs of various combinations of papaya and ashwagandha extracts. The papaya concentrations were given the letter 'P' followed by the concentration, for e.g. P20 (papaya extract 20mg/ml), P40 (papaya extract 40mg/ml), P60 (papaya extract 60mg/ml), P80 (papaya extract 80mg/ml) and P100 (papaya extract 100mg/ml). The same nomenclature was used for ashwagandha also (A20, A40, A60, A80 and A100). The combinations of both the extracts were labelled based on the amount of each extract used in the combination. For e.g. a combination of 20mg/ml of papaya extract with 20 mg/ml of ashwagandha extract was labelled as P20 A20. Hence the different combinations of extracts used to study anti bacterial activity are depicted in table 5 along with the zone of inhibition produced in millimetre.

Table 5 Combinational effect of papaya and aswagandha extracts

Dosage / Isolates	**P20 A20 A40 A60 A80 A100 (mg/ml)**	**P40 A20 A40 A60 A80 A100 (mg/ml)**	**P60 A20 A40 A60 A80 A100 (mg/ml)**	**P80 A20 A40 A60 A80 A100 (mg/ml)**	**P100 A20 A40 A60 A80 A100 (mg/ml)**
Unit	**(mm)**	**(mm)**	**(mm)**	**(mm)**	**(mm)**
Ecoli	8 10 12 8 8	14 19 10 12 12	14 7 18 16 14	9 9 10 20 10	8 14 16 12 19
Staph Aureus	- 8 7 - -	8 7 8 7 8	16 14 12 10 10	12 10 8 11 20	12 10 16 12 10

We have noticed that lower concentration of papaya itself gets the synergistic effect by Aswagandha ethanolic extract. For the gram negative *E.Coli*, due to the combinational effect of plant extracts, papaya shows higher antibacterial activity from 40mg/ml itself. As we have shown in figure 2 previously, that papaya extract alone showed activity only from 80mg/ml for the strains. This rise in antibacterial property might be due to its combination with ashwagandha. Especially the 80mg/ml of papaya extract and 80mg/ml of ashwagandha extract shows the maximum zone of inhibition. In both the strains 1:1 ratio combination seems to show better results. For the gram positive strain, we inferred that the combinational effect of the flavonoids increased the sensitivity of papaya extract that it showed large zone of inhibitions from 60mg/ml. The zones are depicted in figure 4. The mechanism of the antibacterial activity of these combinational dosages could be due to the phytochemical compounds present in them. Tannins coagulate the wall proteins; entry of toxic material gets facilitated by saponins which further cause leakage of vital constituents.[35] Activity of enzymes gets inhibited, by forming complexes with cell walls and soluble proteins, by flavonoids, hence disrupt cell wall and membrane integrity.[36-38]

Therefore, we suggest that the root sample extracts were active against both gram-negative and gram-positive bacteria. Hence, this may indicate a broad spectrum of activity. These results are very significant as they pave way for the possibilities for developing therapeutic substances that can be active against multidrug-resistant organisms and still have a natural origin.

4. CONCLUSION

The present study was done to check the combinational effect of root extracts of plant *Withania somnifera* and *Carica Papaya.* The individual and combinational dosages of both were studied on gram negative and gram positive bacteria. Based on the results obtained, we would like to conclude that ashwagandha extract being more potent antibacterial agent influences the activity of papaya extract, by increasing papaya extract's antibacterial activity. This increase in activity may be due to the combinational effect of the phytochemicals from both the extracts. Quantification of these combinational forms needs to be studied in detail in future.

CONFLICT OF INTEREST

The authors declare that the content submitted to the journal is not submitted for consideration or published elsewhere. There is no conflict of interest between the authors on any financial or personal interests.

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