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Original Article

ANTIBACTERIAL POTENTIAL OF NIMBOLIDE FROM *AZADIRACHTA INDICA*

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ABSTRACT

Objective: The present study was designed to evaluate the antibacterial activity of the isolated Nimbolide compound from *Azadirachta indica*.

Methods: Antibacterial potential of the isolated nimbolide compound of *Azadirachta indica* plants was screened by disc diffusion assay against *Bacillus subtilis*, *Enterococcus faecalis*, *Streptococcus epidermis*, *Enterobacter aerogene*, *Enterobacter cloacae*, and *Salmonella typhimurium*. Ciprofloxacin is used as standards for bacteria. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of nimbolide compound was determined using the macro dilution method.

Results: The antibacterial potency of nimbolide compound from *Azadirachta indica* was assessed by their zone of inhibition values. Nimbolide showed satisfactory results against almost all the organisms, among them against *Salmonella typhimurium* and *Bacillus subtilis* showed highest zone of inhibition 18.1 mm and 17.3 mm (0.4mg/ml) with the MIC values of 0.078 mg/ml and MBC values of 0.156 mg/ml. Other pathogens like *Streptococcus epidermis, Enterococcus faecalis* showed good zone of inhibition 13.2 mm and 11.8 mm (0.4mg/ml). But *Enterobacter cloacae* was found to be resistant with more MIC, more MBC value and with a very less zone of inhibition of 8.5 mm (0.4mg/ml) when compared with the standards (Ciprofloxacin).

Conclusion: The present investigations revealed that nimbolide have significant antibacterial activity. So, the potent antibacterial agent nimbolide is preferred for infectious disease.

Keywords: Nimbolide, *Azadirachta indica*, Antibacterial activity, MIC, MBC.

INTRODUCTION

The development of bacterial resistance to presently available antibiotics has necessitated the search for new antibacterial agents [1]. Natural products especially, those used in ethno-medicine provide a major source of innovative therapeutic agents for various conditions including infectious diseases [2,3]. Ayurveda and siddha medicines were alternative systems for medicine which were become popular in recent days [4]. History of medicine dates back practically to the existence of human civilization. The current accepted modern medicine or allopathy has gradually developed over the years by scientific and observational efforts of scientists [5]. *Azadirachta indica* (Family-Meliaceae) is an important medicinal plant and aqueous extract of it is widely used as a tonic, stimulant and against various ailments [6]. Biological activities of *A. indica* extracts have been investigated intensively. In general, extracts of neem fruit, seeds, seed kernels, twigs, stem bark and root bark have been shown to possess anti-tumour, anti inflammatory and immune-stimulating activities [7-9]. Previous phytochemical investigations with *Azadirachta indica* led to the isolation of triterpenoid bitter principles (nimbidin, nimbin, nimbinine, 6-desacetylnimbinine, nimbidol, nimbolide and bakayanin), saponins, flavonoids, tannins and alkaloids. In addition to these, the leaves contain azadirachtin, salanin, meliantriol, margosopicrin, paraisine, azadinine [10], nimbinene, nimbolide, quercetin and its glycosides, beta-sitosterol, n-hexacosanol, nonacosane, ascorbic acid and amino acids. Barks contain nimbolins A, B, organic acids, tannin, margosin and azadarin. Flowers contain essential oil, kaempferol, kaempferol glucoside, nimbosterin and N-nonacosane. Fruits contain resins, tannins, triterpenoids, salanin and azadriachtin, melianone, oil and organic acids [11]. So, our approach involved to explore the antibacterial activity of nimbolide from *Azadirachta indica* (neem).

MATERIALS AND METHODS

Collection of nimbolide compound

The isolated compound nimbolide of *Azadirachta indica* was purchased from Asthagiri herbal research foundation, Chennai, Tamil Nadu, India.

Anti-bacterial activity

Antibacterial sensitivity testing using disc diffusion method

Circular disc of 6 mm diameter were made from the whatman No 1 filter paper. Discs were impregnated with equal volume (50 µl) of nimbolide at four different concentrations (0.05 mg/ml, 0.1mg/ml, 0.2mg/ml & 0.4mg/ml). The discs were aseptically placed over plates of Muller Hinton Agar (MHA, Difco) seeded with each of test pathogens, and the inoculums were adjusted to 0.5 Mc Farland turbidimetry [12]. The nimbolide compound under study was screened for the antibacterial activity with several human pathogenic bacteria such as Gram positive bacteria: *Bacillus subtilis*, *Enterococcus faecalis*, and *Streptococcus epidermis*, Gram-negative bacteria: *Enterobacter aerogene*, *Enterobacter cloacae*, and *Salmonella typhimurium*. The plates were incubated in an upright position at 37^{0}C for 24 hours and the zone of inhibition was measured (in mm diameter). Inhibition zones with diameter less than 10 mm were considered as having low antibacterial activity. Diameters between 11 and 15 mm were considered moderately active, and these with >16mm were considered highly active. The clinical strains were also tested for their sensitivity against the standard antibiotics, ciprofloxacin (5 mcg) by the disk diffusion method.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

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did not show any visible growth after macroscopic evaluation was considered as MIC. After the determination of MIC, the tubes which did not show any visible growth were diluted 100-fold with drug-free Mueller–Hinton broth and incubated at 37 °C for 48 h. The lowest concentration of the tube that did not show any visible growth was considered as the minimum bactericidal concentration (MBC). The assays were performed in triplicate.

RESULTS AND DISCUSSION

The isolated nimbolide compound tested for antibacterial activity on six human pathogenic bacteria was presented on (Table 1 and Figure. 1). The result showed that the antibacterial activity of the nimbolide was increased with increasing the concentration of isolated nimbolide compound. The nimbolide showed prominent activity on almost all the pathogens especially on *Bacillus subtilis*, *Enterococcus faecalis*, *Streptococcus epidermis*, *Salmonella typhimurium S. typhimurium* but only *Enterobacter cloacae* appears to be resistance with less zone of inhibition. The nimbolide isolated compound from *Azadirachta indica* showed highest activity against *Salmonella typhimurium* (18.1 mm for 0.4mg/ml) and *Bacillus subtilis* (17.3 mm for 0.4mg/ml) but on the other hand *Enterobacter aerogene* which is considered as resistant showed only 8.5 mm inhibition zone at same concentration as above.

Table 1: Antibacterial activity (Zone of inhibition) of Nimbolide compound

Extract	Conc. (µg/ml)	Disc diffusion method (inhibition zone, mm)					
		E.A.	E.C.	S.T.	B.S.	E.F.	S.E.
Nim-	50	7.4±0.15	--	10.3±0.18	9.8±0.34	7.5±0.24	7.3±0.13
bolide comp-	100	8.2±0.32	6.5±0.30	12.6±0.45	12.1±0.39	8.6±0.22	8.8±0.14
ound	200	9.5±0.19	7.1±0.20	15.8±0.20	14.5±0.10	10.1±0.80	11.5±0.19
	400	10.8±0.26	8.5±0.25	18.1±0.07	17.3±0.49	11.8±0.44	13.2±0.28
Cipro-	5.0	23.0±0.23	22.2±0.32	25.8±0.25	26.5±0.28	24.5±0.04	27.3±0.23
floxacin							

Results calculated from triplicate data(n=3) were expressed as means±S.D. -- indicates no zone of inhibition. E.A.: *Enterobacter aerogene*, E.C.: *Enterobacter cloacae*, S.T.: *Salmonella typhimurium*, B.S.: *Bacillus subtilis*, E.F.: *Enterococcus faecalis*, S.E.: *Streptococcus epidermis*.

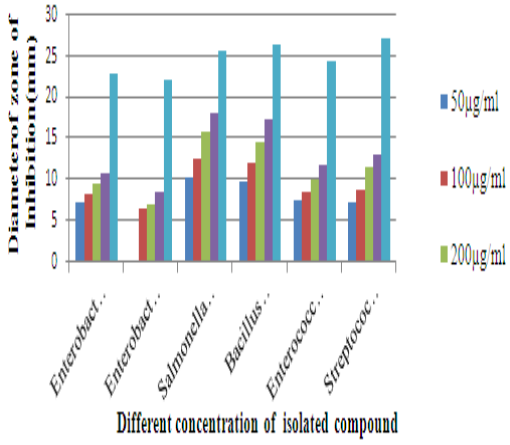

Fig. 1: Antibacterial activity (Zone of inhibition) of Nimbolide isolated compound.

The MIC and MBC analysis of the nimbolide compound showed satisfactory bacteriostatic and bcteriocidal concentration. The MIC of the nimbolide compound was studied from the range of 0.078 to 1.250 mg/ml. Gram negative *Salmonella typhimurium* and Gram positive *Bacillus subtilis* were highly sensitive with MIC values of 0.078 mg/ml and MBC values of 0.156 mg/ml. Nimbolide compound for the bacterial strain *Streptococcus epidermis* (Gram positive) appears to be moderately sensitive with MIC values of 0.156 mg/ml and MBC values of 0.312 mg/ml. Gram positive *Enterococcus faecalis* and Gram negative *Enterobacter aerogene* were less sensitive with MIC values of 0.156 mg/ml and MBC values of 0.625 mg/ml. Other Gram negative species like *Enterobacter cloacae* was also found to be sensitive with MIC values of 0.625 mg/ml and MBC values of 1.250 mg/ml. The nimbolide compound showed less MIC and less MBC against *Enterobacter cloacae*. The results are cited in (**Table 2**). This shows satisfactory MIC, MBC as well as zone of inhibition against almost all pathogens except *Enterobacter cloacae* which indicates its possible application against common human pathogenic bacterial infection.

Table 2: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Nimbolide compound

Test Drug	Gram positive bacteria					
	Enterobacter aerogene		*Enterobacter cloacae*		*Salmonella typhimurium*	
	MIC	MBC	MIC	MBC	MIC	MBC
Nimbolide	0.156	0.625	0.625	1.250	0.078	0.156
	Gram negative bacteria					
	Bacillus subtilis		***Enterococcus faecalis***		***Streptococcus epidermis***	
Nimbolide	MIC	MBC	MIC	MBC	MIC	MBC
	0.078	0.156	0.156	0.625	0.156	0.312

CONCLUSION

Based on the results, it can be concluded that the nimolide from *Azadirachta indica* plant has great potential antimicrobial component against micro-organisms and it can be used in the treatment of infectious disease caused by resistant microorganism. A detailed literature review on the *Azadirachta indica* plant in investigation has shown that so far there are some published reports worldwide, related to the possible anti-bacterial activities but no reports published related to the antibacterial potential of isolated nimbolide compound from *Azadirachta indica*. The present study is providing an isolated bioactive compound (nimbolide) of *Azadirachta indica* plant for antibacterial drug formulation.

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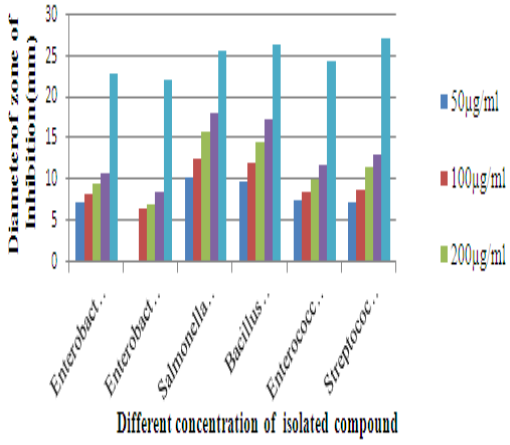

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Original Article

ANTIBACTERIAL POTENTIAL OF NIMBOLIDE FROM *AZADIRACHTA INDICA*

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ABSTRACT

Objective: The present study was designed to evaluate the antibacterial activity of the isolated Nimbolide compound from *Azadirachta indica*.

Methods: Antibacterial potential of the isolated nimbolide compound of *Azadirachta indica* plants was screened by disc diffusion assay against *Bacillus subtilis*, *Enterococcus faecalis*, *Streptococcus epidermis*, *Enterobacter aerogene*, *Enterobacter cloacae*, and *Salmonella typhimurium*. Ciprofloxacin is used as standards for bacteria. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of nimbolide compound was determined using the macro dilution method.

Results: The antibacterial potency of nimbolide compound from *Azadirachta indica* was assessed by their zone of inhibition values. Nimbolide showed satisfactory results against almost all the organisms, among them against *Salmonella typhimurium* and *Bacillus subtilis* showed highest zone of inhibition 18.1 mm and 17.3 mm (0.4mg/ml) with the MIC values of 0.078 mg/ml and MBC values of 0.156 mg/ml. Other pathogens like *Streptococcus epidermis, Enterococcus faecalis* showed good zone of inhibition 13.2 mm and 11.8 mm (0.4mg/ml). But *Enterobacter cloacae* was found to be resistant with more MIC, more MBC value and with a very less zone of inhibition of 8.5 mm (0.4mg/ml) when compared with the standards (Ciprofloxacin).

Conclusion: The present investigations revealed that nimbolide have significant antibacterial activity. So, the potent antibacterial agent nimbolide is preferred for infectious disease.

Keywords: Nimbolide, *Azadirachta indica*, Antibacterial activity, MIC, MBC.

INTRODUCTION

The development of bacterial resistance to presently available antibiotics has necessitated the search for new antibacterial agents [1]. Natural products especially, those used in ethno-medicine provide a major source of innovative therapeutic agents for various conditions including infectious diseases [2,3]. Ayurveda and siddha medicines were alternative systems for medicine which were become popular in recent days [4]. History of medicine dates back practically to the existence of human civilization. The current accepted modern medicine or allopathy has gradually developed over the years by scientific and observational efforts of scientists [5]. *Azadirachta indica* (Family-Meliaceae) is an important medicinal plant and aqueous extract of it is widely used as a tonic, stimulant and against various ailments [6]. Biological activities of *A. indica* extracts have been investigated intensively. In general, extracts of neem fruit, seeds, seed kernels, twigs, stem bark and root bark have been shown to possess anti-tumour, anti inflammatory and immune-stimulating activities [7-9]. Previous phytochemical investigations with *Azadirachta indica* led to the isolation of triterpenoid bitter principles (nimbidin, nimbin, nimbinine, 6-desacetylnimbinine, nimbidol, nimbolide and bakayanin), saponins, flavonoids, tannins and alkaloids. In addition to these, the leaves contain azadirachtin, salanin, meliantriol, margosopicrin, paraisine, azadinine [10], nimbinene, nimbolide, quercetin and its glycosides, beta-sitosterol, n-hexacosanol, nonacosane, ascorbic acid and amino acids. Barks contain nimbolins A, B, organic acids, tannin, margosin and azadarin. Flowers contain essential oil, kaempferol, kaempferol glucoside, nimbosterin and N-nonacosane. Fruits contain resins, tannins, triterpenoids, salanin and azadriachtin, melianone, oil and organic acids [11]. So, our approach involved to explore the antibacterial activity of nimbolide from *Azadirachta indica* (neem).

MATERIALS AND METHODS

Collection of nimbolide compound

The isolated compound nimbolide of *Azadirachta indica* was purchased from Asthagiri herbal research foundation, Chennai, Tamil Nadu, India.

Anti-bacterial activity

Antibacterial sensitivity testing using disc diffusion method

Circular disc of 6 mm diameter were made from the whatman No 1 filter paper. Discs were impregnated with equal volume (50 µl) of nimbolide at four different concentrations (0.05 mg/ml, 0.1mg/ml, 0.2mg/ml & 0.4mg/ml). The discs were aseptically placed over plates of Muller Hinton Agar (MHA, Difco) seeded with each of test pathogens, and the inoculums were adjusted to 0.5 Mc Farland turbidimetry [12]. The nimbolide compound under study was screened for the antibacterial activity with several human pathogenic bacteria such as Gram positive bacteria: *Bacillus subtilis*, *Enterococcus faecalis*, and *Streptococcus epidermis*, Gram-negative bacteria: *Enterobacter aerogene*, *Enterobacter cloacae*, and *Salmonella typhimurium*. The plates were incubated in an upright position at 37^{0}C for 24 hours and the zone of inhibition was measured (in mm diameter). Inhibition zones with diameter less than 10 mm were considered as having low antibacterial activity. Diameters between 11 and 15 mm were considered moderately active, and these with >16mm were considered highly active. The clinical strains were also tested for their sensitivity against the standard antibiotics, ciprofloxacin (5 mcg) by the disk diffusion method.

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did not show any visible growth after macroscopic evaluation was considered as MIC. After the determination of MIC, the tubes which did not show any visible growth were diluted 100-fold with drug-free Mueller–Hinton broth and incubated at 37 °C for 48 h. The lowest concentration of the tube that did not show any visible growth was considered as the minimum bactericidal concentration (MBC). The assays were performed in triplicate.

RESULTS AND DISCUSSION

The isolated nimbolide compound tested for antibacterial activity on six human pathogenic bacteria was presented on (Table 1 and Figure. 1). The result showed that the antibacterial activity of the nimbolide was increased with increasing the concentration of isolated nimbolide compound. The nimbolide showed prominent activity on almost all the pathogens especially on *Bacillus subtilis*, *Enterococcus faecalis*, *Streptococcus epidermis*, *Salmonella typhimurium S. typhimurium* but only *Enterobacter cloacae* appears to be resistance with less zone of inhibition. The nimbolide isolated compound from *Azadirachta indica* showed highest activity against *Salmonella typhimurium* (18.1 mm for 0.4mg/ml) and *Bacillus subtilis* (17.3 mm for 0.4mg/ml) but on the other hand *Enterobacter aerogene* which is considered as resistant showed only 8.5 mm inhibition zone at same concentration as above.

Table 1: Antibacterial activity (Zone of inhibition) of Nimbolide compound

Extract	Conc. (µg/ml)	Disc diffusion method (inhibition zone, mm)					
		E.A.	E.C.	S.T.	B.S.	E.F.	S.E.
Nim-	50	7.4±0.15	--	10.3±0.18	9.8±0.34	7.5±0.24	7.3±0.13
bolide comp-	100	8.2±0.32	6.5±0.30	12.6±0.45	12.1±0.39	8.6±0.22	8.8±0.14
ound	200	9.5±0.19	7.1±0.20	15.8±0.20	14.5±0.10	10.1±0.80	11.5±0.19
	400	10.8±0.26	8.5±0.25	18.1±0.07	17.3±0.49	11.8±0.44	13.2±0.28
Cipro-	5.0	23.0±0.23	22.2±0.32	25.8±0.25	26.5±0.28	24.5±0.04	27.3±0.23
floxacin							

Results calculated from triplicate data(n=3) were expressed as means±S.D. -- indicates no zone of inhibition. E.A.: *Enterobacter aerogene*, E.C.: *Enterobacter cloacae*, S.T.: *Salmonella typhimurium*, B.S.: *Bacillus subtilis*, E.F.: *Enterococcus faecalis*, S.E.: *Streptococcus epidermis*.

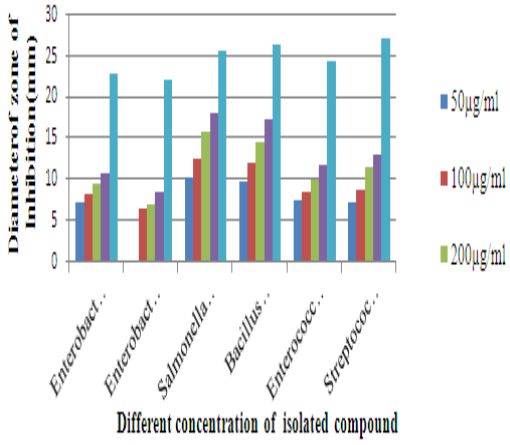

Fig. 1: Antibacterial activity (Zone of inhibition) of Nimbolide isolated compound.

The MIC and MBC analysis of the nimbolide compound showed satisfactory bacteriostatic and bcteriocidal concentration. The MIC of the nimbolide compound was studied from the range of 0.078 to 1.250 mg/ml. Gram negative *Salmonella typhimurium* and Gram positive *Bacillus subtilis* were highly sensitive with MIC values of 0.078 mg/ml and MBC values of 0.156 mg/ml. Nimbolide compound for the bacterial strain *Streptococcus epidermis* (Gram positive) appears to be moderately sensitive with MIC values of 0.156 mg/ml and MBC values of 0.312 mg/ml. Gram positive *Enterococcus faecalis* and Gram negative *Enterobacter aerogene* were less sensitive with MIC values of 0.156 mg/ml and MBC values of 0.625 mg/ml. Other Gram negative species like *Enterobacter cloacae* was also found to be sensitive with MIC values of 0.625 mg/ml and MBC values of 1.250 mg/ml. The nimbolide compound showed less MIC and less MBC against *Enterobacter cloacae*. The results are cited in (**Table 2**). This shows satisfactory MIC, MBC as well as zone of inhibition against almost all pathogens except *Enterobacter cloacae* which indicates its possible application against common human pathogenic bacterial infection.

Table 2: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Nimbolide compound

Test Drug	Gram positive bacteria					
	Enterobacter aerogene		*Enterobacter cloacae*		*Salmonella typhimurium*	
	MIC	MBC	MIC	MBC	MIC	MBC
Nimbolide	0.156	0.625	0.625	1.250	0.078	0.156
	Gram negative bacteria					
	Bacillus subtilis		***Enterococcus faecalis***		***Streptococcus epidermis***	
Nimbolide	MIC	MBC	MIC	MBC	MIC	MBC
	0.078	0.156	0.156	0.625	0.156	0.312

CONCLUSION

Based on the results, it can be concluded that the nimolide from *Azadirachta indica* plant has great potential antimicrobial component against micro-organisms and it can be used in the treatment of infectious disease caused by resistant microorganism. A detailed literature review on the *Azadirachta indica* plant in investigation has shown that so far there are some published reports worldwide, related to the possible anti-bacterial activities but no reports published related to the antibacterial potential of isolated nimbolide compound from *Azadirachta indica*. The present study is providing an isolated bioactive compound (nimbolide) of *Azadirachta indica* plant for antibacterial drug formulation.

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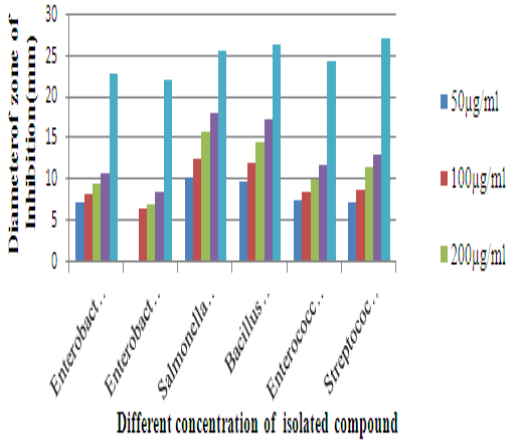

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