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Bacteria Killing Kinetics of the Four Plant Hormones

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Abstract: The aim of the present study was to evaluate the antibacterial characteristic of the four synthetic plant hormones Indoleacetic acid (IAA), 2,4- dichlorophenoxyacetic acid (2,4-D), Naphthaleneacetic acid (NAA) and Gibberellic acid (GA). Antibacterial potency was assessed by measuring the zone of inhibitions on semi-solid nutrient agar bacterial inoculating petri dishes. NAA and IAA gave potent antibacterial activity at concentration of 150 μg $disc^{-1}$ giving zones at 26-35 mm. Bacteriostatic and bactericidal concentrations against the pathogens were determined by serial dilution technique. Bacteriocidal concentrations of the tested principles were found to be significantly higher than their respective bacteriostatic concentrations. Minimum inhibitory concentrations that are bacteriostatic concentrations of the tested compounds were found at 32-128 μg mL^{-1} whereas, bactericidal concentrations established at 256-512 μg mL^{-1} against eight pathogenic bacteria *Streptococcus β-haemolyticus*, *Staphylococcus aureus*, *Pseudomonas aeroginosa*, *Shigella dysenteriae*, *Shigella flexneri*, *E. coli*, *Salmonella typhi* and *Klebsiella pneumoniae*.

Key words: Plant hormones, bacteriostatic, bactericidal

INTRODUCTION

Plant hormones (auxin) play a major role in regulating growth. They are organic substances and produced in one tissue and transported to another, where they cause physiological responses. Auxin regulates the amount, type and direction of plant growth. They include both naturally occurring substances and related synthetic compounds that have similar effects. Auxins are found in all members of the plant kingdom. They are most abundantly produced in growth areas (meristem), e.g., root and shoot tips, but are also produced elsewhere, e.g., in the stems and leaves[1,2]. The method of dispersal throughout the plant body is not yet fully understood. Auxins affect numerous plant processes, e.g., cell division and elongation, autumnal loss of leaves and the formation of buds, roots, flowers and fruit[1]. Auxins are widely used commercially to produce more vigorous growth, to promote flowering and fruiting and also root formation in plants not easily propagated by stem cuttings, to retard fruit drop and to produce seedless varieties (e.g., of tomatoes) by parthenogenetic fruiting[1,2]. The principal natural auxin is Indoleacetic acid (IAA); other common but less frequent plant hormones include the gibberellins (includes Gibberellic acid), lactones and kinins. 2,4-Dichlorophenoxyacetic acid (2,4-D) is a synthetic auxin which is widely used as a herbicide[2] and Naphthaleneacetic acid (NAA) is also a synthetic auxin which is commonly employed to induce the formation of adventitious roots in cutting and to reduce fruit drop in commercial crops[2].

In the previous investigations IAA, NAA, 2-4-D and Gibberellic acid (GA) were found to have excellent role as plant growth promoters but they also reported to have various important uses in different commercial aspects like 2-4-D used as herbicide[2], NAA was reported to reduce fruit drop[2] and GA along with IAA were reported to produce larger fruits[2]. Other important bioactive properties have been studied with these compounds e.g., anticancer property has been reported for the compound indoleacetic acid (IAA) and its different derivatives[3-5]. Diverse mechanisms have been reported associated with their activities[4,6,7]. IAA has also been reported to have antifungal property against some plant fungi[8,9].

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In the present study it is examined that bacteriostatic as well as bactericidal properties of the four plant hormones IAA, NAA, 2,4-D and GA with the aim to assess their bio-diversity which may be helpful for to estimate their mode of actions.

MATERIALS AND METHODS

The plant hormones Indoleacetic acid (IAA), Naphthaleneacetic acid (NAA), 2,4-Dichlorophenoxyacetic acid (2,4-D) and Gibberellic acid (GA) in a pure grade were made of Fluka company, Germany. They were collected and maintained at 4°C.

Antibacterial screening: The experiments were carried out in the Microbiology and Biotechnology Laboratory in the Department of Pharmacy, University of Rajshahi, Rajshahi, Bangladesh, during October to December 2003.

Disc diffusion method[10,11] is generally applied for *in vitro* antibacterial screening. This method is highly effective for rapidly growing microorganisms and the activities of the test compounds are expressed by measuring the diameter of the zone of inhibition. Generally the more susceptible the organism the larger is the zone of inhibition. The method is essentially a qualitative or semi quantitative test indicating sensitivity or resistance of microorganisms to the test materials as well as bacteriostatic or bactericidal activity of a compound[12]. Eight pathogenic bacteria (two Gram-positive: *Streptococcus β-haemolyticus* and *Staphylococcus aureus* and six Gram-negative: *Pseudomonas aeroginosa*, *Shigella dysenteriae*, *Shigella flexneri*, *E. coli*, *Salmonella typhi* and *Klebsiella pneumoniae*) were selected for this present study. The media used in this respect were nutrient agar (DIFCO). The compounds IAA, NAA, 2,4-D and GA were dissolved in the neutral solvent dimethylsulfoxide (DMSO) to obtain a concentration of 10 μg mL^{-1}. The discs (6 mm in diameter) were prepared by sterile filter paper and dried in an oven at 120°C for about 60 min to remove water and to make sterile. The solution of the compounds were then applied on the dried filter paper discs to obtain discs containing 30 and 150 μg of the compounds in each disc. Kanamycin discs (30 μg $disc^{-1}$) were used as standard in this study for the comparison of antibacterial activity and the discs were collected from market (Hi-media Lab. Pvt. India). Blank and control discs were also used to minimize experimental errors. Two discs of different concentrations (30 and 150 μg $disc^{-1}$) for each compound were deposited on the surface of nutrient agar (DIFCO) of Petri dishes, which had been previously inoculated with the test organisms, gave a final concentration of 10^7 cells mL^{-1}. The Petri dishes were incubated at 37°C for about 18-24 h. The experiments were repeated three times to minimize experimental errors.

Collection of the bacterial species: In this experiment eight pathogenic bacterial species *Streptococcus β-haemolyticus*, *Staphylococcus aureus*, *Pseudomonas aeroginosa*, *Shigella dysenteriae*, *Shigella flexneri*, *E. coli*, *Salmonella typhi* and *Klebsiella pneumoniae* were used all of which were collected from the Institute of Nutrition and Food Sciences (INFS), Dhaka University, Bangladesh.

Minimum Inhibitory Concentration (MIC) determination: Minimum Inhibitory Concentration, MIC, is the lowest concentration which resulted in maintenance or reduction of inoculum viability[13]. The determination of the MIC involves a semi quantitative test procedure which gives an approximation to the least concentration of an antimicrobial needed to prevent microbial growth. The end result of the test was the minimum concentration of antimicrobial (test materials) which gave a clear solution, i.e., no visual growth[14,15] Serial dilution technique[3] was applied for the determination of minimum inhibitory concentration of the testing compounds against the eight tested pathogenic bacteria. The media used in this respect were nutrient broth (DIFCO). Dilution series were setup with 2, 4, 8, 16, 32, 64, 128, 256, 512 and 1024 μg mL^{-1} of nutrient broth medium. To each tube 100 μL of standardized suspension of the testing bacteria (10^7 cell mL^{-1}) were added and incubated at 30°C for 24 h.

Minimum Bactericidal Concentration (MBC) determination: Each bacteria has a level of antimicrobials which will inhibit growth but not kill the organisms. This is called the Minimum Inhibitory Concentration (MIC). Related to this, a higher antimicrobial concentration will kill the organisms. This is called the Minimum Bactericidal Concentration (MBC). MBC of the tested compounds were determined by serial dilution methods[3] like MIC determinations. MIC was determined after 24 h of incubation but MBC was determined after 96 h of incubations. After 96 h the test tubes which remained clear (no bacterial growth) containing medium with bacterial inoculums and the specific amount of test compounds were examined for bacterial mortality that is bactericidal property. The test for bacterial mortality was done by culture the test tube contents on sterile nutrient agar medium and no growth was the conformity of bactericidal property. After 96 h the test tubes remained clear as no viable cells were present in them.

Preparation of bacterial suspension for testing: All the bacteria were grown overnight in flasks containing 80 mL Tryptone Soya Broth, TSB (Oxoid), with shaking, at 30°C. The culture was centrifuged at 4000 rev min^{-1} for 10 min. The resulting cell pellets were pooled and resuspended in 0.1% peptone water. The density of the suspension was adjusted to approximately 10^7 to 10^8 CFU mL^{-1} by comparing to a McFarland 0.5 $BaSO_4$ standard.

RESULTS AND DISCUSSION

Antibacterial potency: In the present study of disc diffusion assay method the activity was evaluated by the diameter of zone of inhibitions produced by the discs containing test samples after 24 h of incubations of cultural Petri dishes at 37°C. At the concentration of 30 μg $disc^{-1}$ the test samples did not exhibit remarkable antibacterial activity in comparison with the standard kanamycin. At this concentration IAA, NAA, 2,4-D and GA gave zone of inhibitions between 09 to 13 mm whereas, standard kanamycin gave zone of inhibitions between 25 to 30 mm (Table 1). Especially, NAA as well as IAA exhibited remarkable potent antibacterial activity against the tested bacterial species at the high concentration of 150 μg $disc^{-1}$ and they gave zone of inhibitions at 26-35 mm (Table 1). Though IAA and NAA gave nearly similar antibacterial activity against *Staphylococcus aureus*, *Psedomonas aeroginosa*, *Shigella dysenteriae*, *Salmonella typhi* and *Klebsiella pneumoniae* by comparison of zone of inhibitions but NAA gave slightly more activity than the IAA which against the other tested species *Streptococcus β-haemolyticus*, *E. coli* and *Shigella flexneri* (Table 1). 2,4-D and GA gave promising antibacterial activity with the zone of inhibitions of 15-27 mm against the tested bacteria and they also gave nearly similar activity to each other at higher concentration of 150 μg $disc^{-1}$ (Table 1).

Minimum inhibitory concentration: Indoleacetic acid (IAA) gave the same MIC value at 64 μg mL^{-1} against the tested pathogens *Streptococcus β-haemolyticus*, *Staphylococcus aureus*, *Pseudomonas aeroginosa*, *Shigella flexneri* and *Klebsiella pneumoniae* (Table 2). Against *Shigella dysenteriae* indole acetic acid exhibited minimum bacteriostatic concentration at 32 μg mL^{-1} whereas, it displayed maximum bacteriostatic concentration at 128 μg mL^{-1} against the two tested species *Salmonella typhi* and *E. coli* (Table 2). Naphthaleneacetic acid (NAA) showed its minimum inhibitory concentration at 32 μg mL^{-1} against the four tested pathogens and it also gave maximum bacteriostatic concentration at 64 μg mL^{-1} against other four pathogens (Table 2).

Table 1: Comparative antibacterial effects of plant hormones (IAA, NAA, 2,4-D and GA) and standard kanamycin (K)

	Diameter of zone of inhibition (mm)								
	IAA		NAA		2,4-D		GA		K
Test organisms	30 (μg $disc^{-1}$)	150 (μg $disc^{-1}$)	30 (μg $disc^{-1}$)	150 (μg $disc^{-1}$)	30 (μg $disc^{-1}$)	150 (μg $disc^{-1}$)	30 (μg $disc^{-1}$)	150 (μg $disc^{-1}$)	30 (μg $disc^{-1}$)
S. β-haemolyticus	10	30	13	35	10	19	10	17	29
Staphylococcus aureus	12	28	12	29	12	22	11	20	25
Psedomonas aeroginosa	11	27	13	30	12	24	11	22	28
Shigella dysenteriae	12	29	11	31	11	27	12	23	27
Shigella flexneri	11	26	12	34	10	24	11	21	27
Salmonella typhi	09	28	11	29	11	18	10	16	28
E. coli	10	30	13	35	10	17	10	15	30
Klebsiella pneumoniae	11	31	13	33	11	23	10	19	29

IAA: Indoleacetic acid, NAA: Naphthaleneacetic acid, 2,4-D: 2,4- Dichlorophenoxyacetic acid, GA: Gibberellic acid

Table 2: Comparative MIC and MBC values of the tested hormones (IAA, NAA, 2,4-D and GA) and standard kanamycin (K)

	IAA		NAA		2,4-D		GA	
Test organisms	MIC (μg mL^{-1})	MBC (μg mL^{-1})	MIC (μg mL^{-1})	MBC (μg mL^{-1})	MIC (μg mL^{-1})	MBC (μg mL^{-1})	MIC (μg mL^{-1})	MBC (μg mL^{-1})
Streptococcus β-haemolyticus	64	256	32	256	64	512	64	512
Staphylococcus aureus	64	512	32	512	128	512	128	512
Psedomonas aeroginosa	64	512	64	512	64	512	128	512
Shigella dysenteriae	32	256	32	256	64	256	64	512
Shigella flexneri	64	512	32	256	64	512	64	512
Salmonella typhi	128	512	64	512	128	512	128	512
E. coli	128	512	64	256	128	512	128	512
Klebsiella pneumoniae	64	256	64	256	64	512	128	512

MIC: Minimum Inhibitory Concentration, MBC: Minimum Bactericidal Concentration, IAA: Indoleacetic acid, NAA: Naphthaleneacetic acid, 2,4-D: 2,4-Dichlorophenoxyacetic acid, GA: Gibberellic acid

MIC values of Dichlorophenoxyacetic acid (2,4-D) were 64, 128, 64, 64, 64, 128, 128 and 64 μg mL^{-1}, respectively against the tested species (Table 2). Gibberellic acid (GA) gave similar bacteriostatic kinetics like 2,4-D against the tested pathogens except for *Pseudomonas aeroginosa* and *Klebsiella pneumoniae* where GA gave comparatively more values 128 and 128 μg mL^{-1} instead of 64 and 64 μg mL^{-1} for 2,4-D.

Minimum bactericidal concentration: Indoleacetic acid (IAA) gave eight MBC values (same or different) against the eight tested bacterial strains (Table 2). Naphthaleneacetic acid also gave eight MBC values against the tested pathogens, *Streptococcus β-haemolyticus*, *Staphylococcus aureus*, *Pseudomonas aeroginosa*, *Shigella dysenteriae, Shigella flexneri*, *Salmonella typhi*, *E. coli* and *Klebsiella pneumoniae* (Table 2). Except *Shigella dysenteriae* 2,4-Dichlorophenoxyacetic acid gave the same MBC value of 512 μg mL^{-1} against other seven tested organisms whereas, it gave the MBC value of 256 μg mL^{-1} against *Shigella dysenteriae* (Table 2). Against all the tested bacterial strains Gibberellic acid (GA) showed same bacterial killing kinetics as it gave the same MBC value of 512 μg mL^{-1} (Table 2).

The bactericidal and bacteriostatic terminology originates from whether the antimicrobial's mechanism is based on inhibiting cell wall formation (bactericidal) or inhibiting bacterial metabolism or ribosomal protein synthesis (bacteriostatic). Pharmacologists have taught us that some antimicrobials are bactericidal and some are bacteriostatic. These terms are slight misnomers since all antimicrobials are potentially bactericidal and bacteriostatic at different concentrations[16]. The idea is that if cell wall formation is blocked, the organisms will lyse and perish, but if metabolism or protein synthesis is blocked, the organisms merely slow down. While this is true to some degree, bactericidal or bacteriostatic outcomes are dependent on the concentration of the antimicrobial agent as well. A low dose of a bactericidal compound may only inhibit bacterial growth, while a high dose of a bacteriostatic compound will be bactericidal.

In the present study we evaluated the bacteriostatic and bactericidal characteristics of four plant hormones IAA, NAA, 2,4-D and GA against both gram positive and gram negative pathogens. The results revealed interesting findings. All the plant hormones tested in this study were significant antibacterialy active at high concentration of 150 μg $disc^{-1}$. Bacteriostatic concentrations MIC of the tested compounds vary significantly with their own bactericidal concentrations MBC against the tested pathogens which are an interesting findings in the present study. In the previous IAA and its derivates believed to have some roles as anticancer agent[8,9,15]. IAA also reported to have antifungal property[8,17]. 2,4-D was claimed as potent herbicide[2] and it also reported to have some antifungal property[18]. No reports of antibacterial activity of these four compounds had cited in previous literature so it is the first report of bacteriostatic and bacteriocidal characteristics of these four plant hormones. These results may be helpful to interpret the mechanism of antibacterial actions against the tested pathogenic bacteria which may explore new type of bacteria killing kinetics.

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