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Synergistic Interaction Between Synthetic and Natural Products: A Promising Tool for the Development of Environmentally Safe Potent Antimicrobial Agents

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Abstract: In order to combat resurgent and drug resistant microbial strains present binary approach is broad spectrum screening of already existing chemical entities or their analogs, which can be synthesized by "environment benign" methodology and exploitation of synergistic interaction between synthetic and natural products. Thus, a series of compounds, 1,5-Diphenyl pent–1,4–diene–3–one(A1); 1,9–Diphenylnon–1,3,6,8–tetraene–5–one(A2);1,5–bis(2–hydroxyphenyl)pent–1,4–diene–3–one(A3); 1,5–difuran pent–1, 4–diene –3–one(A4); 1,5–bis(4-dimethylaminophenyl)pent–1,4–diene–3–one(A5), consisting of α-β unsaturated carbonyl group,has been selected. The activity of compounds in isolation and in conjunction with natural products, *Nicotiana tobaccum* and Neem oil (*Azadirachta Indica*), against highly pathogenic and of extensive host range plant fungi, *Sclerotium rolfsii, Rhizoctonia bataticola* and *Fusarium udum* has been determined in terms of percentage of inhibition and as antibacterial agents against MTCCB 96 *Staphylococcus aureus* and clinically isolated resistant human pathogenic bacteria *Enterococcus faceium.* The present study summarizes that the combinations of natural and synthetic compound are more effective compared to just synthetic chemicals and/or less available natural products. It can be postulated that enhanced activity of combinations is due to synergistic interaction between synthetic and natural products that can be used as a promising tool for the designing of safer antimicrobial drugs.

Key words: Neem · *Azadirachta Indica* · Nicotiana tobaccum · Synergism · Antifungal · Antibacterial

INTRODUCTION

During the past five decades, microbial infection on human being as well as plants has become a serious global problem due to the emergence of resistant microbial strains [1-4]. In order to combat them enormous efforts are being made to synthesize more and more new compounds which can be used as potential antimicrobial agents and structural modifications of chemotherapeutics to which resistance has developed. However, the hazardous synthetic pathway and their exposure inevitably leads to environment contamination, bioaccumulation, biomagnifications and adverse effect on non target organisms. A call has therefore been made by environmentalists for their limited use diverting the attention of scientists worldwide towards products of natural origin [5]. Though natural products are rich source of less toxic antinfectives, their frequent use has been restricted due to structural complexity, less availability, instability and less potency as compared to synthetic chemicals.

It is thus desperate need of time to explore alternative strategies and environmentally benign technologies to develop potent and safer antimicrobial agents. In this regard, a binary approach consisting of broad spectrum screening of existing chemical entities and their derivatives, that can be synthesized by safer methodologies with no use of hazardous chemicals as solvent or catalysts and exploitation of synergistic interactions between synthetic and natural product to yield new agents to inhibit the growth of microbes has been adopted.

Synergism has been defined as a phenomenon in which two different compounds are combined to enhance their individual activity. In contrary to this if the combination results to a worsening effect it is called as

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antagonistic and the effect which is less than synergistic but not antagonistic as well is termed as additive or indifferent [6]. The scientific application of synergism can be traced back to 1940's [7]. Since then potentiated effect of combinations of different antibiotics or natural substances is exhaustively studied and well documented [8-13]. However, there is scant attention vis-à-vis synergism between synthetic and natural products. The present endeavour is to overshadow the availability problem of natural products by using them in conjunction with less environment compatible synthetic chemicals. This would enhance and improve the efficacy of natural products as well as reduce the concentration of synthetic chemicals.

For this intent, a selected series of compounds were screened in isolation and in conjunction of natural products, *Nictotiana tobaccum* and Neem (*Azadirachta Indica*) oil, against clinically isolated multi drug resistant pathogenic bacteria *Enterococcus faecium* and MTCCB 96 *Staphylococcus aureus* by disc diffusion method and against highly pathogenic and of extensive host range fungi *Sclerotium rolfsii, Rhizoctonia bataticola, Fusarium udum* by food poisoning method.

MATERIALS AND METHODS

Synthesis of Compounds: All the reagents used for synthesis were of high purity. The literature method [14] has been followed to synthesize all compounds at ambient temperature and pressure with no use of hazardous chemicals as catalyst. Detailed synthetic route, as summarized in scheme 1, is presented below.

0.01 Mole of requisite aldehyde was dissolved in 10 ml of ethanol and 10 ml 3M NaOH. To this reaction mixture, 0.02 moles of acetone was added slowly in an incubator-shaker at room temperature. After half an hour of continuous shaking the reaction mixture resulted to a clear pale yellow solution. Further shaking for about 10 minutes, cloudiness appeared which settled down slowly. The precipitate was then filtered out and re-crystallized by appropriate solvent.

$$R-CHO + CH_3COCH_3 \xrightarrow{NaOH} \underset{(A1-A5)}{R-CH=CH-CO-CH=CH-R}$$

(Scheme 1) (Table 1)

Physical and Spectral Data of Synthetic Compounds: The melting points were determined in open capillary tube and are uncorrected. The Infra red (I. R.) spectra of all the compounds were recorded on Perkin Elmer BX-II Spectrophotometer (Perkin Elmer, Boston MA) using Potassium bromide (KBr) pellets. The ultra violet (U.V.) Spectra were recorded on a UNICAM UV-4 Spectrophotometer using ethanol as solvent. CHN analysis is done with Element analysensysteme GmbH Vario EL (Table 2).

Table 1: The list of Compounds

R-CH=CH-CO-CH=CH-R		
R	Compound	IUPAC Nomenclature
C_6H_5-	(A1)	1,5-Diphenyl pent-1,4-diene-3-one
C_6H_5CH=CH-	(A2)	1,9-Diphenylnon-1,3,6,8-tetraene-5-one
o-OH-C_6H_4-	(A3)	1,5–bis(2–hydroxyphenyl) pent–1,4–diene–3–one
C_4H_3O-	(A4)	1,5-difuran pent-1,4-diene-3-one
p-$N(CH_3)_2$-C_6H_4-	(A5)	1,5-Bis[4-(N,Ndimethyl)phenyl] pent-1,4-diene-3-one

Microbial assay

Antifungal Screening: Determination of Percentage of Inhibition

Culture Media: Potato dextrose agar of HI MEDIA, MUO96 was used as culture medium.

Preparation of Test Compound: The compounds were dissolved in acetone. Combinations of these compounds with natural products, Neem oil (*Azadirachta Indica*) [N1] and *Nicotiana tobaccum* [N2], were prepared in 1:1 ratio.

Bioassay [15]: Single spore cultures of fungal species, grown and maintained on potato dextrose agar media at 28±1°C were used for the experiments. The compounds were mixed aseptically with PDA media and were sterilized. The medium with acetone was served as control. The Petri plates having 9 cm inner diameter were taken and 25 ml of the sterilized PDA containing test samples were plated in the centre. After solidification, 0.5 cm diameter agar discs taken from a 48 h old sporulated fungi culture was placed with the fungal side downward in the centre of each plate with the help of sterile cork borer and glass rod. The plates were incubated in dark at 28±1°C. Radial growths were determined by measuring the colony size along two diameters at right angles after 24 h interval. Three replicates for each treatment were prepared. The experiments were repeated three times. The percentage of inhibition was calculated using the following formula [16]: I = C – T / C x 100, where, I = percentage of inhibition; C = radial growth in control – disc diameter (cm); T = radial growth in treated – disc diameter (cm). The results of control assays together with the combination of synthetic compounds and natural compounds are shown in Table 3.

Table 2: Physical Data and Characterization of Compounds

Compound	Molecular Formula	CHN Analysis %obs. (% calc.)	Melting Point (^{0}C)	Yield (%)	IR Spectra cm^{-1}	UV Spectra
(A1)	$C_{17}H_{14}O$	86.6, 5.2 (87.2, 5.9)	110	88	1658.21 1601.46 3025.65	λ_{max}=300.5 nm, ε_{max} = 2.2
(A2)	$C_{21}H_{18}O$	89.0, 5.2 (88.1,5.5)	96	78	1653.76 1585.02 3025.15	λ_{max}=338.0 nm, ε_{max} = 0.6 λ_{max}=255.0 nm, ε_{max} = 0.8
(A3)	$C_{17}H_{14}O_3$	75.4, 5.1 (76.7, 5.0)	112	67	1671.78 1616.30 3355.62 3064.90	λ_{max}=327.0 nm, ε_{max} = 1.3 λ_{max}=282.5 nm, ε_{max} = 1.8
(A4)	$C_{13}H_{10}O_3$	72.0, 4.3 (72.9, 4.6)	118	75	1682.94 1606.94 3119.52	λ_{max}=312.0 nm, ε_{max} = 2.2
(A5)	$C_{21}H_{24}ON_2$	77.9,7.8,8.9 (78.2, 8.0, 8.6)	130	90	1674.37 1578.82 1331.93 2906.07	λ_{max}=300.5 nm, ε_{max} = 2.3

Table 3: Antifungal Screening

	Percentage of Inhibition		
Compounds	*F. udum*	*R. bataticola*	*S. rolfsii*
N1 (*Azadirachta Indica*)	11.11	44.44	13.33
N2 (*Nicotiana tobaccum*)	-	22.22	-
A1	-	44.44	10
A1 + N1	-	6.67	16.67
A1 + N2	41.11	-	24.44
A2	16.67	16.67	11.11
A2 + N1	-	6.67	16.67
A2 + N2	22.22	45.56	-
A3	-	61.11	25.55
A3 + N1	-	53.33	16.67
A3 + N2	-	-	-
A4	-	8.00	8.88
A4 + N1	-	6.67	24.44
A4+N2	22.22	53.33	52.22
A5	44.44	61.11	50
A5 + N1	22.22	13.33	27.77
A5 + N2	85.55	66.67	83.33

Table 4: Antibacterial Screening

	Inhibition zones	
Compounds	*E. faecium*	*S. auerus*
N1 (*Azadirachta Indica*)	-	5mm
N2 (*Nicotiana tobaccum*)	-	-
A1	-	-
A1 + N1	6mm	6mm
A1 + N2	6mm	6mm
A2	-	-
A2 + N1	-	6mm
A2 + N2	6mm	-
A3	12mm	13.5mm
A3 + N1	9.5mm	9mm
A3+N2	12mm	9mm
A4	-	-
A4 + N1	-	-
A4+N2	-	-
A5	5mm	-
A5 + N1	-	-
A5 + N2	-	-

Antibacterial Screening

Disc Diffusion Method

Culture Media: The Nutrient broth of HI MEDIA M 002 and Agar of HI MEDIA, RM 026 was used.

Microorganism: *E. faecium*, procured from all India Institute of Medical Sciences (AIIMS), New Delhi, India and MTCCB 96 *S. aureus* were maintained in laboratory by successive subcultures in fresh Nutrient agar media at 4°C and by liofilazation.

Preparation of Test Compound: The compounds were dissolved in DMSO.Combinations of these compounds with natural products, Neem oil (*Azadirachta Indica*) [N1] and *Nicotiana tobaccum* [N2], were prepared in 1:1 ratio

Bioassay: In this method [17] loop full of the given test strain was inoculated in 25ml of NB and was incubated for 18h in an incubator shaker (INNOVA 4300) at 37°C in order to obtain an active colony of bacterial strain. The plates were prepared by dissolving 13 g of NB and 20 g of Agar in 1000ml of distilled water. In order to proceed 28-30 ml of autoclaved NB Agar media was added into (100mm) diameter Petri plates, inoculation of test strain was done by spreading(100µL per plate).Then Whatman filter paper (No.1) sterile discs previously soaked in a known concentration of test compounds (A1-A5) were placed in nutrient agar media. The combinations of natural compounds and synthetic compounds were kept in 1:1 ratio using the same solvent. The antibacterial activities were determined by the inhibition zone formed by these compounds (Table 4).

RESULTS AND DISCUSSION

Spectral Analysis: The structure of the compounds were established on the basis of their spectroscopic data. IR spectra of compounds gave characteristic bands at 2900-3100cm^{-1} & 1610-1570 cm^{-1} due to aromatic ring skeleton vibration, absorbance band for carbonyl group is found in the range of 1680-1650cm^{-1}, the wave number shifts from normal range of >C=O band of 1700cm^{-1} indicating the presence of conjugated carbonyl group, band near 2900-3100 cm^{-1} range indicates -CH=CH- skeleton. For A3 a band near 3355cm^{-1} signifies the presence of –OH group and in A5 the band at 1331cm^{-1} shows the presence of Tertiary aromatic amine.

The UV spectra shows n>π* transitions (λmax in the range of 300-340 nm) with an increment of wavelength indicating the presence of conjugated carbonyl group. The CHN results were also found in accordance with the calculated values.

Biological Assay: Present investigation has revealed magnificent results. The induction of significant antimicrobial activity in combinations consisting of biologically inactive components has been observed. The combination of even slightly bioactive compounds exhibited promising antimicrobial activity.

Antifungal Activity: Earlier preliminary evaluation of test compounds against *Sclerotium rolfsii, Rhizoctonia bataticola* and *Fusarium udum* using disc diffusion method has revealed a significant biological activity of compounds in isolation and in conjunction with natural products [18]. Encouraged by these results fungal growth inhibition was measured quantitatively in terms of percentage of inhibition by food poisoning method. The results revealed that compound A1 is inactive against *F.udum* and almost inactive against *S. rolfsii* when it was screened alone but its combination with N2 has shown synergistic influence on its activity with percentage of inhibition 41.11 and 24.44 respectively against the fungi. Similarly growth inhibitory activity of mildly active N2 has been enhanced from 22.22 to 45.56% when screened in combination with almost inactive A2 (percentage of inhibition of 16.67) against *R. bataticola.*

Compound A4, when tested alone, exhibited negligible activity against *R. bataticola* and *S. rolfsii* but its 1:1 combination with N2 shows pronounced synergistic interaction against all the test fungi. Strong synergistic interaction was also observed when compound A5, moderately active against *F.udum* and *S. rolfsii*, screened in conjunction with inactive N2, here the activity of combination is almost doubled. While against *R. bataticola* the activity of combination is found to be as active as the activity of A5, when tested alone. This clearly reveals broad spectrum effect of synergism.

Antibacterial Activity: Previous studies revealed bioactivity of test compounds, in isolation, against *E. faecium* [18]. The present investigation has been extended to study the antibacterial activity in conjunction with natural products against *S. arueus* and *E. faecium.* The results highlighted the occurrence of a pronounced synergism by the fact that although compounds A1 and A2 do not show antibacterial activity when tested alone, but the combination of either of them with N2 resulted in induction of activity against *E. faecium.* Also combination of N2 and A1 was effective against *S. aureus* as well. The potentiated effect is again visible in combination of mildly active N1 with A1 against both the bacteria and with A2 against *S. arueus.* The results also demonstrate additive or indifferent effect in combination of A3 with N2 where activity of A3 remains same.

CONCLUSION

The significance of our studies lies in the fact that synergism between natural products and synthetic compounds can improve the efficacy of natural products and minimize the use of synthetic chemicals to avoid the environmental impact. It can led us towards a greener and effective control measure for various microbial diseases causing huge damage to crops and life threatening effect on human beings. Synergistic interaction between natural

products and synthetic compounds can be used as a promising tool to yield new products with broad spectrum biological activity. Though exhaustive studies are still required to understand the mechanism of action and interaction in order to obtain products with improved efficacy, it is more effective and eco friendly approach to develop antimicrobial agent in future. There is also need for the optimization of this technique, which may allow the development of a pharmacologically and agriculturally acceptable antimicrobial agent or group of agents.

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