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Acetyl alkannin from *Alkannatinctoria* works synergistically along with commercial antibiotics against common human pathogens

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ABSTRACT

Natural products from plants have been in therapeutic use since many years. Novel molecules from plant sources have been isolated, characterized and used in managing different diseases. Alkannatinctoria (Family: Boraginaceae) is an important herb stated in Siddha medicine, which is used to cure many ailments alone and in formulations. The present study comprises isolation of Acetyl Alkannin, from hexane extract of the root bark of A. tinctoria, through silica gel column chromatography. Structure of the compound was elucidated through MS and H^1, C^{13} NMR. The pure compound was analyzed for its antimicrobial action against common human pathogens, where it showed considerable Minimum Inhibitory Concentrations (MIC) against most of the pathogens. Significant activity was observed against Bacillus subtilis (MIC: 28 µg/ml), Enterococcus faecalis (MIC: 28 µg/ml), Escherichia coli (MIC: 12 µg/ml), Pseudomonas aeruginosa (MIC: 12 µg/ml). Studies on its combination with other commercial antibiotics depicted synergistic patterns against P. aeruginosa with most of the antibiotics. Also, it worked both synergistically and additively against microbes with other antibiotics. Thus, it could be used in combination with other antimicrobial agents in managing critical microbial infections.

Key words: Acetyl Alkannin, Antimicrobial Synergy, *Alkannatinctoria*, Napthoquinones.

INTRODUCTION

Alkannatinctoria (L.)Tausch is distributed widely in India, Europe and western Asia. Its root has been used as a botanical drug for ulcers, inflammation, and wounds [1]. It is called asSurulpattai in Tamil and is used in formulation of Ratan jot [2].Previous phytochemical studies on this plant have resulted in the isolation of a series of naphthoquinone pigments, includingalkannin and its derivatives [3–6]. Some of these compounds show biological properties such as cytotoxic, antimicrobial, antileishmanial, and anti-inflammatory activities [7, 8].Microbes in recent days are constantly becoming more resistant due to generous use of antibiotics [9]. Combinations of drug molecules are now suggested as potential antimicrobial agents [10], since they are highly effective in action against resistant pathogens [11]. Therefore, reassignment of already established antimicrobials along with novel ones would probably reduce the global problem of microbial resistance. Thus, in this present study we studied the antimicrobial patterns of a potent compound Acetyl Alkannin in combination with commercial pathogens against various human pathogens encountered commonly.

MATERIALS AND METHODS

Collection of plant material and extraction

Root bark of *Alkannatinctoria*was obtained from local market in Thanjavur, India. The plant was authenticated by Dr. Jayendran, Department of Botany, Government Arts College, Ootacamund, India. A voucher specimen (JDB1622) was deposited in Government Arts College, Ootacamund, India. The root barks were shade dried and ground to fine powder and used for extraction. Extracts were prepared by soaking 1 kg of plant material in Hexaneat room temperature for 24 h and repeated thrice with the residue. The extract was filtered through Whatman No.1 filter paper, and then all the filtrates were pooled up successively and concentrated under vaccum by Rotary evaporator (Buchi® Rotavap R-210).

Chemicals and Instrumentation

All reagents were purchased from Sigma-Aldrich. TLC was monitored with silica gel-precoated aluminum sheets (Type 60 F254, Merck, Darmstadt, Germany) and the spots were visualized in the ultraviolet light chamber, Iodine chamber, 5% MeOH-H_2SO_4 mixture. Elemental analyses were carried out on an automatic Flash EA 1112 Series, CHN Analyzer (Thermo). All melting points are measured using Buchi-545. ^{1}H NMR and ^{13}C NMR spectra were determined on a Bruker-400 NMR spectrometer and chemical shifts were expressed as part per million against TMS as internal reference. Mass spectra were recorded on Agilent 1200 (Liquid Chromatography), Agilent 6320 (Quadrupole Mass Analyzer) spectrophotometer.

Compound isolation

Hexane extract (35g) from root barks of *Alkannatinctoria* was taken for column chromatography with silica gel (60-120 mesh) (150 g) packed in a glass column of 4 x 45 cm with bed height of 30 cm. Elution was started with Hexane, followed by increasing ethyl acetate (EA) - hexane combinations (5, 10, 20, 40 and 80% EA in hexane) and finally with EA followed by MeOH. The column elution was monitored by TLC and fractions were pooled based on similar TLC profiles. In total, 7 fractions were collected and were concentrated under reduced pressure in a rotary evaporator. Fraction 3 yielded a red colored precipitate - Acetyl Alkannin (AA).

Antimicrobial studies

Pathogens and antibiotics used

Staphylococcus aureus (MTCC 96, 3160), *Bacillus subtilis*(MTCC 441), *Enterococcus faecalis* (MTCC 439), *Escherichia coli* (MTCC 723), *Vibrio cholera* (MTCC 3904), *Klebsiella pneumonia* (MTCC 432), *Proteus vulgaris* (MTCC 426), *Proteus mirabilis* (MTCC 425), *Shigella dysenteriae* (ATCC 23513), *Pseudomonas aeruginosa* (MTCC 741, 1688) were used for the antimicrobial studies. Commercial antibiotics were purchased from Sigma-aldrich, India which included Amoxicillin (AMX), Ampicillin (AMP), Methicillin (MET), Ciprofloxacin (CIP), Gentamicin (GEN), Chloramphenicol (CHL), Azithromycin (AZM), Erythromycin (ERY), Tetracycline (TET), Polymyxin B (PMB). All pathogens were maintained on nutrient agar slants at 4^{o}C.

Inoculum preparation

All procedures for determination of Antimicrobial activity were done and inoculum size was standardized according to the National Committee for Clinical Laboratory Standards guidelines [12]. Mueller Hinton Broth (MHB; HiMedia, Mumbai, India) was used to prepare inoculum and grown in incubator orbital shaker at 37^{o}C for 4-8 h until the cultures attained turbidity of 0.5 McFarland Unit. Inoculum size was adjusted and standardized to 5×10^5 CFU ml^{-1} throughout the experiments.

Determination of antibacterial efficacy

The minimum inhibitory concentrations (MICs) were determined for hexane extract of *Alkannatinctoria* and isolated bioactive compound by broth dilution method. However, tests for purified isolated compound were carried out in triplicate using ResazurinMicrotitre Assay (REMA) [13] with some modifications. Stock solutions of samples at 500µg/ml were prepared by dissolving the samples in 10% Dimethyl sulphoxide (DMSO). The test samples were diluted in MH broth. The concentration range was 3.9-500 µg/ml. In 96-well microtiter plates, 100 µl of each of the compound dilutions was added to a mixture of 90 µl of MHB and 10 µl of bacterial inoculum. The negative control consisted of 100µl of 10% DMSO, 90 µl of MHB and 10µl of cell suspension; the positive control had the addition of ciprofloxacin (3.9-500 µg/ml). Upon the incubation of the test plates at 30°C for 24 h, cell viability was determined by the addition of 15 µl of a 0.01% (wt/vol) Resazurin solution to each of the wells, following an extra incubation period of 2 h at 30°C. Viable microorganisms reduced the blue dye to a pink color, which was detected

by fluorescence scanning using a microfluorimeter (FLX-800 fluorimeter, BioTek, Winooski, VT) set to an excitation/emission profile of 530 nm/590 nm.

Combination studies

To study the interaction of isolated bioactive compound with other antimicrobial agents, combinations of the compound with commercial antibiotics were assessed by the checkerboard test [14]. Pure compound combined with antibiotics at concentrations ranging from 1/32 × MIC to 4 × MIC were prepared in MHB with standard inoculum size of 5×10^5 CFU ml^{-1}. Minimum bactericidal concentrations (MBCs) were also found for the combinations prepared. The fractional inhibitory concentration index (FICI) was found as the sum of the FICs of each of the drugs. FIC is defined as the MIC of each drug used in combination divided by the MIC of the drug when used alone. The interaction was defined as synergistic if the FIC index was less than or equal to 0.5; additive if the FIC index was greater than 0.5 and less than or equal 1.0; indifferent if the FIC index was greater than 1.0 and less than or equal to 2.0, and antagonistic if the FIC index was greater than 2.0. All experiments were done in triplicates and data represented in arithmetic average.

RESULTS AND DISCUSSION

Collection of plant material and extraction

The plant material was the root bark of *Alkannatinctoria*, which was dark red, fine textured powder after grinding. Total extract obtained was 35 g which was directly used for further compound isolation. Extraction of active molecules from plant extracts has been in vogue since decades. *Alkannatinctoria* is such a high potential medicinal plant which contains range of shikonins and napthoquinones [3]. It has found its use in ancient folk medicine and Siddha Medicine in the Tamil name of Surulpattai [2]. Thus, isolation of pure active molecules from it could provide better study of its medicinal properties.

STRUCTURAL ELUCIDATION

Acetyl alkannin (1): Molecular formula: $C_{18}H_{18}O_6$,Calculated *m/z* (M+ H^+) - Observed- 330.1108,Expected- **330.1103, ^{1}H-NMR (400MHz, $CDCl_3$):** δ 12.56 and 12.40 (each1H, s, 5- and 8-OH), 7.19 (2H, s, H-6 and 7), 6.98 (1H, s,H-3), 6.03 (1H,ddd, J ¼ 7.4, 4.6, 0.9 Hz, H-1`), 5.13 (1H, t, J =7.6 Hz, H-3`), 2.64 and 2.59 (2 × 1H, 2 × m, H-2'), 2.13 (3H, s,H-2``), 1.69 (3H, s,H-5`), 1.57 (3H, s, H-6`); **^{13}C-NMR (100MHz, $CDCl_3$):**δ 176.82 and 178.32 (C-1 and C-4), 169.87 (C-1''), 167.08 and 167.61 (C-5 and C-8), 148.35 (C-2), 136.23 (C-4'), 132.84 and 133.00 (C-6 and C-7), 131.84 (C-3), 117.82 (C-3'), 111.70 and 111.96 (C-9 and C-10), 69.65 (C-1'), 32.97 (C-2'), 25.88 (C-5'), 21.06 (C-2''), 18.06 (C-6')

Acetyl alkannin (AA) has been isolated from Alkannatinctoria for the first time although it has been reported to be isolated from *Rheum palmatum* [15]. It is a derivative of Alkannin, which has beenused for centuries as a natural red dye and is used in Chinese popular folk medicine for its anti-inflammatory and antitumor activities [16]. AA has also showed markedly potent activities against both HCT 116 and Hep G2 cells [15].

Determination of antibacterial efficacy

Antibacterial action was initially performed with the hexane extract where it showed considerable activity. Then, antibacterial experiments were done with pure Acetyl alkannin (AA) at different concentrations. Minimum inhibitory concentrations (MICs) of AA showed significant antibacterial efficacy against most of the pathogens (Table 1). Significant activity was observed against *Bacillus subtilis* (MIC: 28 µg/ml), *Enterococcus faecalis* (MIC: 28 µg/ml), *Escherichia coli* (MIC: 12 µg/ml), *Pseudomonas aeruginosa* (MIC: 12 µg/ml). The MIC values were in acceptable range [17].AA has never been studied for its antimicrobial efficiency in combination with other antibiotics against pathogens. However, extracts of *Alkannatinctoria* and its related molecule Alkannin is shown to exhibit antimicrobial properties [3].

Combination studies

The isolated compound, (AA) combined with other commercial antibiotics to form binary combinations, exhibited acceptable activity against most of the pathogens. The vital observation grasped was that AA worked synergistically in many cases, especially with Tetracycline against most of the pathogens (FICI<0.5) and additively against the rest of the pathogens (FICI: 0.5-1.0). In addition, Chloramphenicol and Erythromycin evidenced synergistic activity against *P.mirabilis* (FICI-0.72). Against *P. aeruginosa*, AA blended well with most of the antibiotics to yield maximum synergism. Additive combinations were also plenty (especially Amoxicillin, Ciprofloxacin against most of

pathogens), compared to non-interactive and antagonistic combinations (Table 2).Eventhough, AA has not been evaluated for its combined antimicrobial effect with other antibiotics; such studies were done previously [18]. Synergistic patterns reveal more effective routes in eliminating microbial resistance and thus, efficient therapeutic management of pathogenic diseases.

Table 1: Minimum inhibitory concentration of different components of *Alkannatinctoria*

Pathogen	Type	MIC (µg/ml)			
		HE	1	AA	3
Staphylococcus aureus (MTCC 96)	Gram Positive	300	72	42	80
Staphylococcus aureus (MTCC 3160)		240	56	32	42
Bacillus subtilis (MTCC 441)		>120	48	28	36
Enterococcus faecalis (MTCC 439)		240	48	28	32
Escherichia coli (MTCC 723)	Gram Negative	80	24	12	16
Vibrio cholera (MTCC 3904)		240	46	32	72
Klebsiella pneumonia (MTCC 432)		300	40	28	28
Proteus vulgaris (MTCC 426)		>300	72	42	48
Proteus mirabilis (MTCC 425)		>300	72	42	52
Shigella dysenteriae (ATCC 23513)		>240	38	20	32
Pseudomonas aeruginosa (MTCC 741)		>120	36	16	48
Pseudomonas aeruginosa (MTCC 1688)		200	32	24	42

HE- Hexane extract, AA- Acetyl alkannin.

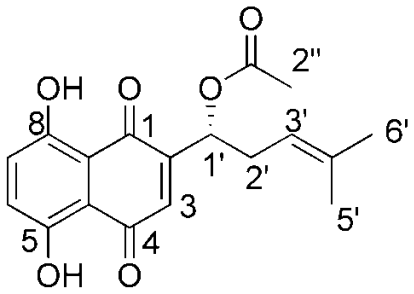
Table 2: Combination effects of Acetyl alkannin with commercial antibiotics

Pathogen		Antimicrobial Agents (MIC/MBC)(µg/ml)										
		AA[£]	AMX[#]	AMP	MET	CIP	GEN	CHL	AZM	ERY	TET	PMB
Staphylococcus aureus	**I**[*]	42	26	45	5	8	4	22	16	5	15	32
	C		22	20	4	6	10	18	22	8	5	18
	F		1.36	0.92	1.36	0.89	2.73	1.24	1.89	1.79	0.45	0.99
	R		**A**[$]	**A**	**N**	**A**	**T**	**N**	**N**	**N**	**S**	**A**
Staphylococcus aureus	**I**	32	60	60	8	12	14	30	22	8	22	50
	C		24	52	8	6	12	22	30	14	6	52
	F		1.15	2.49	1.25	0.68	1.23	1.42	2.3	2.18	0.46	2.6
	R		**A**	**N**	**N**	**A**	**N**	**N**	**T**	**T**	**S**	**T**
Bacillus subtilis	**I**	28	12	20	16	6	4	12	16	12	12	16
	C		12	10	16	6	6	14	5	6	8	4
	F		1.42	0.85	1.5	1.2	1.7	1.6	0.5	0.7	0.9	0.47
	R		**N**	**A**	**N**	**N**	**N**	**N**	**S**	**A**	**A**	**S**
Enterococcus faecalis	**I**	28	18	18	24	6	20	6	14	18	18	12
	C		12	14	8	4	14	6	8	12	8	5
	F		1.0	1.2	0.6	0.8	1.2	1.2	0.8	1.0	0.7	0.5
	R		**A**	**N**	**A**	**S**	**N**	**N**	**A**	**A**	**A**	**S**
Escherichia coli	**I**	12	12	16	12	6	6	6	12	12	12	10
	C		4	10	8	4	6	4	6	10	10	5
	F		0.6	1.4	1.3	1	1.5	1	1	1.6	1.6	0.9
	R		**A**	**N**	**N**	**A**	**A**	**A**	**A**	**N**	**N**	**A**
Vibrio cholera	**I**	32	15	8	24	4	3	4	20	40	30	8
	C		6	6	8	4	6	4	10	10	20	2
	F		0.5	0.9	0.5	1.1	2.18	1.1	0.8	0.5	1.2	0.3
	R		**S**	**A**	**S**	**A**	**T**	**N**	**A**	**S**	**N**	**S**
Klebsiella pneumonia	**I**	28	40	24	20	12	6	6	8	12	46	2
	C		14	22	10	6	6	3	4	14	24	1
	F		0.8	1.7	0.8	0.7	1.2	0.6	0.6	1.6	1.3	0.5
	R		**A**	**N**	**A**	**A**	**N**	**A**	**A**	**N**	**N**	**S**

Proteus vulgaris	**I**	42	25	40	30	16	12	12	12	40	46	18
	C		16	18	20	8	8	4	6	12	14	10
	F		1	0.8	1.1	0.6	0.8	0.4	0.6	0.5	0.6	0.7
	R		**A**	**A**	**N**	**A**	**A**	**S**	**A**	**S**	**A**	**N**
Proteus mirabilis	**I**	42	32	36	30	14	10	18	16	46	42	20
	C		18	22	26	8	8	12	20	28	32	22
	F		0.9	1.1	1.4	0.7	0.9	0.9	1.7	1.2	1.5	1.6
	R		**A**	**N**	**N**	**A**	**A**	**A**	**N**	**N**	**N**	**T**
Shigella dysenteriae	**I**	20	16	20	18	4	4	22	10	20	20	10
	C		12	12	12	6	4	12	3	5	5	3
	F		1.3	1.2	1.2	1.8	1.2	1.1	0.5	0.5	0.5	0.5
	R		**N**	**N**	**N**	**N**	**N**	**N**	**S**	**S**	**S**	**S**
Pseudomonas aeruginosa	**I**	16	30	18	12	6	4	6	12	20	16	6
	C		8	4	2	2	1	2	4	12	4	2
	F		0.7	0.4	0.2	0.4	0.3	0.5	0.5	1.3	0.5	0.4
	R		**A**	**S**	**S**	**S**	**S**	**S**	**S**	**N**	**S**	**S**
Pseudomonas aeruginosa	**I**	24	28	12	12	8	4	8	8	24	24	4
	C		16	8	4	3	6	3	8	14	12	1
	F		1.2	1	0.5	0.5	1.7	0.5	1.3	1.1	1	1
	R		**N**	**A**	**S**	**S**	**N**	**S**	**N**	**N**	**A**	**A**

[ł]*AA- Acetyl Alkannin*[#]***Commercial Antibiotics*** *–AMX- Amoxicillin, AMP- Ampicillin,MET- Methicillin,CIP- Ciprofloxacin, GEN-Gentamycin, CHL- Chloramphenicol, AZM- Azithromycin, ERY- Erythromycin, TET- Tetracycline, PMB-Polymyxin-B.*
[*]*I-Individual, C- Combination, F- Fractional inhibitory concentration index (FICI), R – Results.*
[$]***Observations****: **S**-Synergistic, **A**- Additive, **N** – No Interaction, **T**- Antagonistic.**FICI**- <0.5 (Synergy), 0.5 - 1.0 (Additive), 1.0 - 2.0 (No interaction), >2.0 (Antagonistic)*

Fig. 1: Structure of Acetyl Alkannin



CONCLUSION

The present study substantiates the isolation of a bioactive compound Acetyl Alkannin (AA), from hexane extract of *Alkannatinctoria* (root bark) through column chromatography. Characterization of the compound is done through H^1-NMR, C^{13}- NMR, MS and the molecule was further advanced to analysis of antimicrobial activity where it depicted significant Minimum inhibitory concentrations against common human pathogens. Combination of AA with commercial antibiotics revealed that AA worked synergistically and additively along with most of the antibiotics against both Gram positive and Gram negative bacteria. Thus, it is substantiated that acetyl alkannin isolated for the first time from root bark of *Alkannatinctoria* exhibits antimicrobial activity and significant combination effect against common human pathogens.

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