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Antimicrobial and Cytotoxic activity of rhizome extract of *Acorus calamus* (Bojho) in combination with different antimicrobial agents: Synergistic Effects

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Abstract

Acorus calamus (sweet flag) is a monocot plant found in wetland, have the scented leaves and rhizomes. Various active bio-ingredients of *A. calamus* had been studied and characterized and some of them are known for antimicrobial and antitumor activities. The dry rhizomes were powdered ethanolic extraction was performed in a Soxhlet apparatus. The extract was dried and re-suspended to sterile distilled water and sterilized by membrane filtration. The synergist affects against bacteria, fungi, helminths were evaluated and cytotoxic assay was performed. The minimum inhibitory concentrations of plant extract was found to be 50µg/ml for the vancomycin resistance *Staphylococcus aureus*, for extended spectrum beta lactamase *Escherichia coli*, Methicillin resistant *S. aureus*, were 100 µg/ml. Synergistically cefixime and cefpodoxime both antibiotics are found to be effective against all strains of *S. aureus* and *E. coli* except VRSA. The antifungal characteristics were found to be effective when agar cup diffusion were performed in combination with fluconazole antifungal drug. The combination of plant extract was more effective anthelminthic drug than the anthelminthic drug alone. Similarly, the plant extract has lethal concentration 50 (LC_{50}) was found to be 173.3 µg / ml and LC_{90} was 555. 4 µg / ml on brine shrimp. The *A. calamus* has potential characteristics to be as antimicrobial and antitumor medicine when used synergistically with antimicrobial agents.

Keywords: *A. calamus*, antibacterial, antifungal, antihelminthic, cytotoxic, brine shrimp

Introduction

Acorus calamus Linn. (Bojho in Nepali) of a family: Araceae is a semi-evergreen perennial herbal plant with scented rhizomes, arching tapered reed-like leaves and yellow-green flowers and indigenous to Nepal and India. The rhizome of *A. calamus* plant has been used since ancient times for its beneficial role as the brain tonic. It is highly valued for its rhizome and fragrant oils to be used as natural medicine. It is reported that *A. calamus* possesses many medical benefits including antimicrobial, anthelmintic, antidiarrheal, antioxidant, antiulcer, analgesic and more activities (Rajput *et al*., 2014). The powdered rhizome of *A. calamus* is said to perform diaphoretic, expectorant and also said to cure tuberculosis as well as heart and lungs cancer (Small and Catling, 2000).

Tremendous numbers of chemical constituents are found in various parts of *A. calamus* and the major composition is β-asarone (Patra and Mitra, 1981; Tamas *et al*., 1996; Rana *et al*., 2013). The rhizomes of are an important commercial commodity and of considerable medicinal and spicy value

and is being sold in Asian and European countries from street shop to commodities shopping centers (Foster, 1999). The crude and purified extracts of rhizome and other different parts were studied to have a good effect against gram positive and negative bacteria in vitro. Similarly, in vitro antibacterial actions against many bacterial, fungal pathogens and superbugs, as well as anticancer and antihelminthic properties, are reported (Devi and Ganjewala, 2009).

The irrational use of commercial antimicrobial agents for the therapeutic system had developed trouble due to the development of antimicrobial resistance by microorganisms. Hence during these two decades, many of scientists are searching both new types of synthetic chemical antimicrobial agents as well as a plant-based natural product. The plant compositions have been found to be effective against different microbes like bacteria, fungi, protozoans, helminths as well as anti-tumor activity too (Cowan, 1999). The herbal product may be highly affected if used synergistically with other chemotherapeutic agents in varying concentrations (Upadhyay *et al*., 2014).

Combinations of antibiotic are common in an allopathic therapeutic system and even available in the commercial market too but the synergistic effect of the plant extract and allopathic drug against different microbes are still in practice very less commonly (Gurib-Fakim, 2006). Several types of research showing the activity of hexane and methanol extract of the rhizome of *Acorus* species significantly inhibited various drug-resistant strains of Staphylococcus aureus while tested with Ampicillin, Chloramphenicol and benzyl benzoate in vitro (Kim *et al*., 1998). For a certain strain of Extended-Spectrum Beta-Lactamase producing E. coli, in vitro synergism was observed between the respective crude extracts of *A. calamus* with either tetracycline or ciprofloxacin (Ahmad and Aqil, 2007).

However irrational use of antimicrobial agents is generating great threats of the emergence of new microbial strains of bacteria, fungi and other microbial agents. Resistance development is an even bigger problem since the bacterial resistance is often not restricted to the specific antibiotic prescribed, but generally extends to other compounds of the same class. Bacterial resistance and its rapid increase is a major concern of global public health and are emerging as one of the most significant challenges to human health (WHO, 2002). Treating microbial infections by chemical antimicrobial agents (viz: antibiotics, antifungal, antiviral, antiparasitic and antihelminthic) are useful but their haphazard use has led to a frightening resistance among microorganisms as well as led to re-emergence of old infectious diseases (Cohen, 1992). Hence, the main aim of the research was to study the antibacterial, antifungal, antihelminthic activity of crude extract of only *A. calamus* and its mixture of different concentrations with antibiotics, antifungal drug and antihelminthic drug as well as to evaluate the cytotoxic activity on hatched brine shrimp.

Materials and Methods

Sample Collections

The rhizomes of *A. calamus* were collected from the periphery of Dharan-14, Hattisar Sunsari, Nepal from the altitude of 1148 ft from the sea level. The earthy matter of rhizome was removed by washing and taken to the Microbiology laboratory of Central Campus of Technology, Dharan, Nepal. The plant species was verified from the herbarium collection of the Department of Botany, Post Graduate Campus, Biratnagar, Nepal.

Extraction

The rhizome was dried in a hot air oven at 60^0C of constant temperature for three days and the dryness was confirmed by obtaining the constant weight while weighing three times at different intervals. The dried rhizome was chopped into small pieces and fine powder was made with a mechanical grinder. The powder (20 gram) was filled in a thimble (HiMedia India) and 100 ml ethanol was used for the extraction in a Soxthlet Apparatus. The extraction of ethanol soluble bioactive compounds of *A. calamus* was extracted by succilation using a soxhlet apparatus till 10 cycles of siphoning.

Solvent Evaporation

The ethanol was evaporated by keeping the extract-solvent solution in a petridish in a hot air oven maintained at 60^0C and the dried. The dried extract was dissolved in a sterile distilled water to make the concentration of 1600µg per ml of stock solutions.

Phytochemical Screening

The major biomolecules such as alkaloids, Carbohydrates, Amino Acid, Reducing Sugar, Tannins, Phenolic Compound, Saponins, Flavonoids and Terpenoids were screened according to the common phytochemical methods described by (Kokate, 2003).

Biological Evaluation

The synergistic effect against control bacteria using ATCC *Staphylococcus aureus* and ATCC *Escherichia coli* as well as clinically isolated drug resistant bacteria used for the study were Methicillin resistant *S. aureus* (MRSA), Vancomycin resistant *S. aureus* (VRSA), Extended spectrum beta lactamase (ESBL) *E. coli*. Cefixime and Cefpodoxime antibiotics were used for the synergistic effect with plant extract. The antifungal assay of plant extract was performed on *Candida albicans* and *Cryptococcus neoformans* and fluconazole was used for the synergistic effect with plant extract. The antibacterial and antifungal assays were performed by agar cup diffusion methods. All bacterial and fungal members were identified by conventional microbiological methods. Antihelminthic assay performed by keeping the live adult earthworm in

different concentration of extracts and synergistically with albendazole. The cytotoxicity of extract was performed against live hatched napuli of *Artemia salina* (Brine Shrimp) by keeping it on different concentrations of extract for 24 hour and the percentage of live napuli and the lethal concentration (LC_{50} and LC_{90}) was determined.

Results

Physical and Phytochemical properties: The percentage lost of moisture was 71.943 and the percentage of extract yield was 4.03. The phytochemicals present were alkaloids, carbohydrate, glycosides, saponins, phytosterols, phenols, flavonoids, amino acids and diterpines but the protein was absent. The color of the extracts was dark brown when in a dried form. The pH was 6.9 when dissolved in sterile distilled water. The extract was hygroscopic when opened, moisture is absorbed.

Antibacterial Test

The antibacterial tests were performed by agar cup method (6 mm) and 100µl of sample was kept on the cup of agar. The antibacterial assay was determined by measuring the diameter of zone of inhibition of bacteria on the MHA plates and the result shows that the plant extract is more effective against ATCC *S. aureus*, MRSA and ATCC *E. coli*. All the bacterial growth was inhibited by the plant extract higher than 200 µg/mL and concentration below 50µg/mL did not show the growth (Table 1).

Synergistic (Extract and Cefodoxime Antibiotic) Effect Against Selected Bacteria

Synergistic effect of the plant extract and Cefpodoxime (30 µg / mL) antibiotics are found to be inhibitory to most of the bacterial strains and highest zone of inhibition was shown by ATCC *S. aureus* followed by non ESBL *E. coli*. The zone of inhibition of VRSA was not observed but MRSA are inhibited to 26 mm diameter by combination of 800 µg/mL of concentration. Muller Hinton Agar for *Staphylococcus aureus*, MRSA and VRSA were prepared by making the NaCl concentration 4% (Table 2).

Table 1: Minimum inhibitory concentration of selected bacteria by *A. calamus* crude extract

***A. calamus* extract concentration**	**Mean Zone of Inhibitions in millimeter (mm)**					
	ATCC* *E. coli*	**ESBL† *E. coli***	**Non ESBL *E. coli***	**VRSA‡**	**MRSA±**	**ATCC *S. aureus***
800 µg / mL	22	18	20	16	23	24
400 µg / mL	22	14	19	17	19	17
200 µg / mL	20	10	20	15	15	13
100 µg / mL	13	R	13	14	12	12
50 µg / mL	R	R	R	14	R	10
25 µg / mL	R	R	R	R	R	R

*American Type Culture Collections
† Extended Spectrum Beta Lactamases
±Methicillin Resistant *Staphylococcus aureus*
‡Vancomycin Resistant *Staphylococcus aureus*
R: Resistance

Table 2: MIC determination by plant extract and cefpodoxime antibiotics

S.N.	**Combination of Cefpodoxime (30 µg /mL) and extract of *A. calamus***	**Zone of Inhibitions in millimeter (mm)**					
		ATCC *E. coli*	**ESBL *E. coli***	**Non ESBL *E. coli***	**VRSA**	**MRSA**	**ATCC *S. aureus***
1	800 µg / mL	24	16	25	R	26	34
2	400 µg / mL	24	13	21	R	16	30
3	200 µg / mL	22	13	18	R	13	25
4	100 µg / mL	21	11	15	R	11	23
5	50 µg / mL	21	11	11	R	11	23
6	25 µg / mL	18	11	12	R	R	22
7	×	18	13	15	R	R	22
8	Sterile Distilled Water	-	-	-	-	-	-

Synergistic Antibacterial Study Using Cefixime Antibiotic

The data shown in Table 3 shows the synergistic effect of extract with cefixime is found to be effective against all strains of bacteria taken in the study. Most of the zones of inhibition were found to be more than 24 mm in combination of plant extract with cefixime antibiotics.

Antifungal Tests

Candida albicans and *Cryptococcus neoformans* fungal strains were used for the study. The zone of inhibition obtained between the *C. albicans* and *C. neoformans* are almost similar in diameter (Table 4).

Anthelmintic Tests

The antihelminthic activity of the albendazole as a antihelminthic drug and the plant extract at different concentrations are performed in twice replication and mean paralysis time (assumed when tail parts stoped motility) and mean death time were measured when the motility was completely lost. When using the drugs synergistically against the earthworms in a petridish containing 40 mL of drug and plant extract, the paralytic time of two concentrations containing 1600 and 800 µg / mL were 18 minute and 21 minute respectively. the lowest concentration containing combination of 50 µg / mL of extract with 100 µg / mL of albendazole took almost 2 hours to be paralyzed and 2 and half hour to die (Table 5).

Table 3: MIC determination by plant extract and cefixime antibiotics

S.N.	Combination of Cefixime (30 µg /mL) with *A. calamus* extract (µg / mL)	Zone of Inhibitions in millimeter (mm)					
		ATCC *E. coli*	ESBL *E. coli*	Non ESBL *E. coli*	VRSA	MRSA	ATCC *S. aureus*
1	800	31	30	31	24	32	29
2	400	27	27	31	19	32	24
3	200	27	27	31	15	30	21
4	100	23	24	28	12	21	16
5	50	21	21	27	11	21	16
6	25	19	20	26	R	20	13
7	×	20	20	19	13	20	15
8	Sterile Distilled Water	-	-	-	-	-	-

Table 4: MIC determination by plant extract and fluconazole against fungi

S.N.	Combination of antifungal agent and Plant extract (µg / mL)		Zone of Inhibition	
	A. calamus extract	Fluconazole	*Candida albicans*	*Cryptococcus neoformans*
1	800	No	13	14
2	400	No	13	14
3	200	No	13	13
4	100	No	10	11
5	50	No	-	9
6	25	No	-	-
7	800	30	23	20
8	400	30	21	21
9	200	30	17	17
10	100	30	17	17
11	50	30	16	15
12	25	30	17	14
13	×	30 (Control)	16	15
14	Sterile Distilled Water		-	-

Table 5: Antihelminthic activity on 100 µg of albendazole and different concentration of plant extract.

S.N.	Concentrations (µg / mL)		Mean Paralysis Time in minute	Mean Death Time in minute
	Albendazole	Plant Extract		
1	100	1600	18	85
2	100	800	21	108
3	100	400	84	133
4	100	200	74	118
5	100	100	124	150
6	100	50	-	-
7	Normal Saline		-	-

Similarly, while taking 1600 µg /mL of plant extract with 200 µg / mL of albendazole, the earthworm is paralyzed more rapidly than the concentration taking 100 µg / mL of albendazole with similar concentration of plant extract (Table 6).

Cytotoxic Tests

Brine Shrimp lethality assay: On brine shrimp lethality assay, concentration above 400 µg / mL had shown the survival rate of 0% followed by the concentration of 200 µg / mL of plant extract to 70%. On graphical representation of the death rate of naupli the lethal concentration 50 (LC_{50}) was found to be 173.3 µg / mL and LC_{90} was 555. 4 µg / mL (Table 7).

On graphical representation of the death rate of naupli the lethal concentration 50 (LC_{50}) was found to be 173.3 µg / mL and LC_{90} was 555. 4 µg / mL (Fig. 1).

Table 6: Antihelminthic activity on 200 µg of albendazole and different concentration of plant extract.

S.N.	Concentrations (µg / mL)		Mean Paralysis Time (Minute)	Mean Death Time (Minute)
	Albendazole	Plant Extract		
1	200	1600	18	94
2	200	800	34	89
3	200	400	66	109
4	200	200	62	105
5	200	100	80	116
6	200	50	94	151
7	Normal Saline		-	-

Table 7: The percentage survival of Naupli with plant extract

S.N.	*A. calamus* extract concentration (µg / mL)	Peccentage (%) Death of Naupli after 24 hours
1	800	100
2	400	100
3	200	70
4	100	40
5	50	30
6	25	10
7	0	0

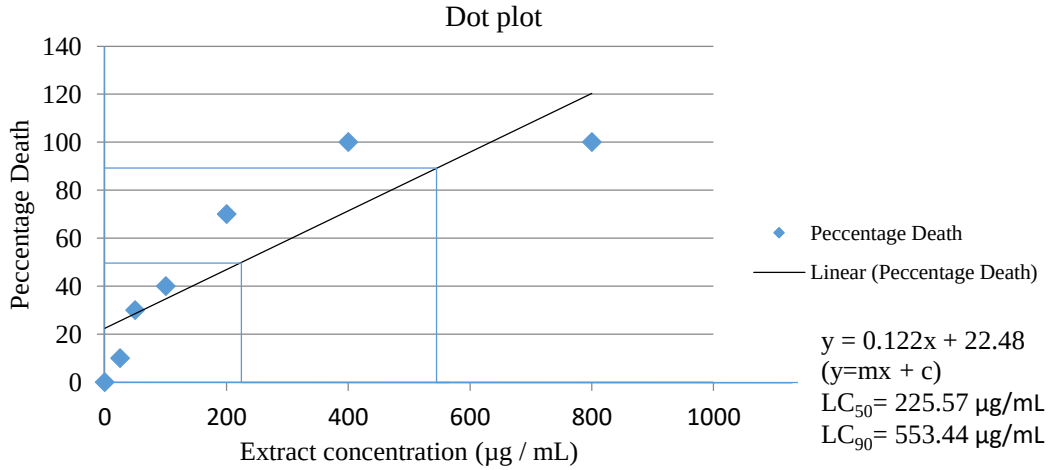

Fig. 1: Determination of LC50 and LC 90 by dot plot

LC: Lethal Concentration
LD: Lethal Dose

Discussions and Conclusions

The antibacterial effect of ethanol crude extract of *A. calamus* is studied against different variety of bacteria as well as multi drug resistance bacteria like MRSA, VRSA and ESBL *E. coli.* The synergistic effect by using the plant extract and antibiotics were one of new concepts in the therapeutic system. The mixture of different concentration

of plant extract with antibiotics like cefixime and cefpodoxime have good effect on MDR bacteria like methicillin resistance *Staphylococcus aureus* (MRSA), vancomycin resistance *S. aureus* (VRSA) and extended spectrum beta lactamases (ESBL) *E. coli* as well as inhibition of clinically important fungal specimens were with fluconazole as antifungal agents in combination.

Few researchers studied in vitro antibacterial action against *S. aureus, E. coli, Shigella dysenteriae, S. sonnei* and few other drug resistance bacteria with an alcoholic extract of the rhizome of *A. calamus*. A significant synergistic effect of the crude extract of *A. calamus* with antibiotics like cefuroxime, chloramphenicol, or tetracycline has been observed in previous researches and similar findings were observed. In vitro synergistic antimicrobial effect was observed on *E. coli* that produced β-lactamase between the crude extracts of *A. calamus* with tetracycline or ciprofloxacin (Phongpaichit *et al*., 2005; Ahmad and Aqil, 2007; Devi and Ganjewala, 2009; Rajput *et al*., 2014).

Antibiotics and other antimicrobial agents are chemical agents used against different microbial infections and these agents payback due to huge researches and discovery of new drugs. However irrational use of antimicrobial agents is generating great threats of emergence of new microbial strains of bacteria, fungi and other microbial agents. Resistance development is an even bigger problem since the bacterial resistance is often not restricted to the specific antibiotic prescribed, but generally extends to other compounds of the same class. Bacterial resistance and its rapid increase is a major concern of global public health and are emerging as one of the most significant challenges to human health (World Health, 2002). Treating microbial infections by chemical antimicrobial agents (viz: antibiotics, antifungal, antiviral, antiparasitic and antihlminthic) are useful but their haphazard use has led to an frightening resistance among microorganisms as well as led to re-emergence of old infectious diseases (Isturiz and Carbon, 2000).

Antifungal activity test is performed by agar cup diffusion method and the diameter of zone of inhibition is measured to evaluate the activity of extract (Pattnaik *et al*., 1997). Antifungal activity of rhizome and leaves extract of *A. calamus* showed marked effect against *Aspergillus niger, A. flavus, Microsporum canis* with significant zone of inhibition with MIC value 2-4 mg/mL. *A. flavus* is more sensitive but *Penicillium chrysogenum* is less sensitive with ethyl acetate extract. A good activity against *Cryptococcus gastricus* and *Candida albicans* with MIC value ranged 4-5 mg/mL for rhizome extract and 6-8 mg/ mL for leaves extract (Lee *et al*., 2004; Phongpaichit *et al*., 2005).

Albendazole and rhizome extract was found to have good combination at 100µg/mL of albendazole and 1600 µg/mL of plant extract against Indian earthworm (*Pheretima posthuma*). Since it has the similar anatomical features with the human intestinal helminthes and the anthelminthic assay can be done by keeping the earthworm in a petridishes containing various dilutions of plant extract, antihelminthic drug or their combinations (for synergistic effect) in vitro (McGaw *et al*., 2000; Ghosh, 2006). A synergistic anthelmintic activity ethanolic extract of rhizomes of *A. calamus* had been studied with root part of *Vitex negundo*. But the activity of *A. calamus* extract with albendazole was noticed better than with extract of *V. negundo* (Deb *et al*., 2013).

The cytotoxic assay on brine shrimp was LC_{50} and LC_{90} were 225.54 µg / mL and LC_{90} was 553.44 µg / mL respectively. Brine shrimp is a model organism for use in cytoxicity assays, despite the recognition that it is too robust an organism to be a sensitive indicator species of pollution and anticancer activity. The brine shrimp lethality assay was first studied by Michael *et al*. (Sorgeloos *et al*., 1978; Krishnaraju *et al*., 2005). *Artemia* are hatched using brine shrimp eggs in a sterile artificial seawater (prepared using sea salt 38 g/L and adjusted to pH 8.5 using 1N NaOH) under constant aeration for 48h. Active nauplii after hatched are used for the cytotoxic assay. Fixed number of nepuli can be used to test with fixed concentration of the plant extract and the motility is observed using a hand lens (Meyer *et al*., 1982a). Though the β-asarone, has recognized carcinogenic effects, anticarcinogenic activation of α-asarone has been reported on human carcinoma cells (Hu and Ji, 1986).

Antimicrobial synergistic effect is a common in treatment system in allopathic therapeutic system. Some antibiotics in combination are usually available in commercial market too but the synergistic effect of the plant extract and allopathic drug against different microbes are still not in practice (Gurib-Fakim, 2006). Several researches showing the activity of hexane and methanol extract of rhizome of *Acorus* species significantly inhibited various drug resistant strains of *Staphylococcus aureus* while tested with ampicillin, chloramphenicol and benzyl benzoate in vitro. For a certain strain of ESBL producing *E. coli*, in vitro synergism was observed between the respective crude extracts of *A. calamus* with either tetracycline or ciprofloxacin (Pattnaik, *et al*., 1997; Ahmad and Aqil, 2007).

Use of natural products from different herbal plants and their extracts may be a novel approach for treatment as antimicrobial agents. Their extracts in combination with different antibiotics as synergistic therapy against resistant microbes may generate an important ideas for future prospective for treatment. Combination therapy may be supportive and helpful for patients with serious infections caused by drug resistant pathogens. Since natural products are not harmful, this study is highly essential for the future prospective of treatments by using herbal medicine to

decrease the use of chemical antimicrobial agents (Newall *et al*., 1996).

Conflict of Interest

The authors declare that there is no conflict of interest with present publication.

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