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Phytochemical Screening and Antibacterial Activity of *Cryptocoryne spiralis* var. *spiralis* and *Cryptocoryne retrospiralis* (Roxb) Kunth

Wadkar SS[1], Shete CC[1], Inamdar FR[1], Gurav RV[2], Patil KS[1] and Ghosh JS[1*]

[1]*Department of BioTechno, Smt. K. W. College, Sangli, Maharashtra, India*

[2]*Department of Botany, Shivaji University, Kolhapur, Maharashtra, India*

[*]**Corresponding author:** Ghosh JS, Department of BioTechno, Smt KW College, Sangli, Maharashtra, India, Tel: 9850515620; E-mail: ghoshjai@gmail.com

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Abstract

Phytochemical screening and their antibacterial activity of plant are important parameters which lead to the isolation of new and novel compounds. *Cryptocoryne spiralis* (Retz) Fischer ex Wydler *var. spiralis* (CSS) and *Cryptocoryne retrospiralis* (Roxb) Kunth (CR), leaf and rhizome have been selected for phytochemical screening to identify the different classes of secondary metabolites. Antibacterial activity of different solvent extracts of leaf and rhizome were carried out by using agar well diffusion method. Minimum inhibitory concentration (MIC) was determined by serial dilution technique. The inhibitory effect was studied using the growth pattern of these test organisms. GC-MS analysis was also done to determine the secondary metabolite profile. Phytochemical screening showed the presence of active compounds such as alkaloids, coumarins, flavonoids, saponins, tannins and glycosides. The ethanolic and methanolic extracts of both rhizome and leaf of both the species of *Cryptocoryne* showed good antimicrobial activity against Gram positive bacteria. Ethanolic extract of rhizome of CSS was found with highest inhibition efficacy in terms of its MIC (200 µg/ml) against *Micrococcus aureus* (NCIM 2802) and *Bacillus subtilis* (NCIM 2045). This is primarily due to the presence of Neomenthol, Menthol, Santalol, Cis- alpha Santalol, and Bicyclo (2, 2, 1) heptane, 2-methyl-3-methylene-2-(4-methyl-3-pentenyl)-, (1S-exo) in the ethanolic extracts of rhizome of CSS and CR respectively. These extracts revealed the presence of bio-active constituents which are known to exhibit Med properties so it may act as effective sources of natural antimicrobials.

Keywords: MIC; GC-MS; Glycosides; *C. spiralis var. spiralis*; *C. retrospiralis*

Introduction

Plant kingdom harbors an inexhaustible source of active ingredients invaluable in the management of many diseases. These active components of herbal remedies have an advantage of being in combination with many other substances that appear to be inactive [1]. In recent years, multiple drug resiatance has developed due to indiscriminate use of existing antimicrobial drugs in the treatment of infectious diseases [2]. This has led to search for new antimicrobial substances from various sources like the Med plants. The screening of plant extracts and plant products for antimicrobial activity has shown that they represent a potential source of novel antibiotic prototypes [3]. A number of evidences have been accumulated to demonstrate the promising potentials of Med plants used in various traditional, complementary and alternative systems [4]. The need of the hour is to screen a number of Med plants for promising antimicrobial activity.

Cryptocoryne spiralis (Retz) Fischer ex Wydler var. *spiralis* and *Cryptocoryne retrospiralis* (Roxb) Kunth (Araceae) are commonly found at margins of ditches or sandy soil of the river, gravelly river beds also growing on open situations on plateaus of higher altitude. They are widely distributed in India from Maharashtra to North Karnataka, Kerala, Coromandel Coast, Pondicherry, Bengal and Assam.

The rhizome of these plants are used by the traditional healers for the treatment of diarrhoea, fever and jaundice, burns and boils [5,6]. Prasad et al. showed that, *Cryptocoryne spiralis var. spiralis* was widely used as a substitute of highly expensive *Aconitum heterophyllum* Wall. for the treatment of diarrhea as well as cough, abdominal complaints, fever and in case of remedy for infantile vomiting [7].

In the present investigation, two species of *Cryptocoryne* genus i.e., *Cryptocoryne spiralis var. spiralis* and *Cryptocoryne retrospiralis* were selected to screen bioactive phytochemical and their antibacterial property.

Materials and Methodology

Extraction of plant material

The rhizome and leaves of *C. spiralis* var. *spiralis* (CSS) and *C. retrospiralis* (CR) were cleaned, chopped and dried and ground separately to fine powder. Solvent and aqueous extraction of these powders was done by using solvents like acetone, ethanol and methanol in 1:10 (w/v) ratio for 24 h with shaking on rotary shaker at 150 rpm speed. Extracted material was filtered through Whattman filter paper No. 1. After filtration, extracts were again dried and concentrated by evaporating the solvent completely in water bath at the range of boiling points of solvents. The dried extracts were resuspended in 10% DMSO and stored at 4˚C [8].

Test organisms

Total six bacterial strains were used for this study out of which two were Gram positive, *Bacillus subtilis* (NCIM 2045), *Micrococcus aureus* (NCIM 2802) while, four were Gram negative, *Pseudomonas aeruginosa* (NCIM 2036), *Escherichia coli* (NCIM 2832), *Salmonella*

typhi (NCIM 2501) and *Klebsiella pneumoniae* (NCIM 2883). Bacterial strains were obtained from NCIM, Pune, Maharashtra (India). The test organisms were sub cultured on nutrient agar medium at 37˚C for 24 h and preserved at 4˚C as a stock culture

Screening of antibacterial activity

Sensitivity of different bacterial strains to extracts from rhizome and leaf of different *Cryptocoryne* species was measured in terms of zone of inhibition using agar diffusion assay [9]. The plates containing Mueller-Hinton agar media were spreaded with 0.2 ml of the respective inoculums equivalent to McFarland 0.5 (15×10^7cfu/ml) turbidity standard. Wells were bored out from agar plates using a sterilized stainless steel borer and filled with 0.1 ml (500 µg) of the extract. The plates were incubated at 37˚C up to 48 h and diameter of resultant zone of inhibition was measured. The bacteria with a clear zone of inhibition were considered to be sensitive. The experiments were performed in triplicate.

Determination of minimum inhibitory concentration (MIC)

The MIC of *Cryptocoryne* rhizome and leaf extracts was determined by agar diffusion assay [9]. The plates containing Nutrient agar media were seeded with 0.2 ml of the respective inoculums (15×10^7organisms/ml). Wells were prepared in agar plates using a sterilized stainless steel borer and extracts of different concentrations prepared by serial dilution technique in different solvents were loaded. Each plate contained three wells including control as 10% DMSO.

Growth pattern of test organisms

The effect of rhizome and leaf extracts of *C. spiralis* var. *spiralis* (CSS) and *C. retrospiralis* (CR) on growth pattern of test organisms was studied [10]. One set of experiment where nutrient broth contains the respective solvents added with test organism i.e., solvent extract, while in another set of experiment nutrient broth contains rhizome or leaf extract only. The flasks were incubated for 24 h at 37˚C in incubator shaker but growth was monitored by measuring absorbance at 540 nm at every 30 min interval.

Phytochemicals analysis

The qualitative tests for phytoconstituents tannins, alkaloids, coumarins, saponins, steroids, flavonoid and glycosides in the acetone, ethanol, methanol and aqueous extracts were performed according to Firdouse, Alam and De et al. with some modifications [11,12].

Gas Chromatography-Mass Spectroscopy (GC-MS) analysis

GC-MS analysis was carried out by Shimadzu 2010 MS Engine (Shimadzu Corporation, Japan). The temperature was enhanced to 200˚C with a rate 10˚C min^{-1} and then linearly raised to 280˚C at a rate of 20˚C min^{-1}. Helium was used as sample carrier gas with flow rate of 1 ml min^{-1}. The initial temperature of column was stabilized at 80˚C for 2 min by oven. The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS library. Measurement of peak areas and data processing were carried out by Turbo-Mass.

Results and Discussion

Screening of antibacterial activity

It was found that both species have significant antibacterial activity with rhizome and leaf extracts, although the inhibitory activity was found to be strain specific (Table 1). Ethanol and methanol extracts showed relatively significant antibacterial activities against both Gram-positive and Gram-negative bacterial strains tested. Ethanol extracts from rhizome of CSS (22 mm), ethanol and methanol extract from rhizome of CR (20 mm) showed highest activity against *Micrococcus aureus* among all the bacteria. Ethanolic extracts from leaf of CSS (20 mm) and both ethanolic and methanolic extract from leaf of CR (18 mm) showed highest activity against *Micrococcus aureus* among all the bacterial strains studied. Aqueous extracts from rhizome and leaf of both species showed very poor inhibitory activity against all bacterial cultures except *M. aureus.* Present study showed that ethanol was the better solvent for extraction as compared to methanol, acetone and water.

Organism Name	Zone of inhibition (mm)											
	Leaf						Rhizome					
	CSS			CR			CSS			CR		
	A	E	M	A	E	M	A	E	M	A	E	M
B. subtilis	16	18	17	15	18	16	16	21	18	14	16	15
M. aureus	18	20	18	17	18	18	18	22	20	18	20	20
P. aeruginosa	12	15	14	12	15	14	15	17	16	13	15	14
E. coli	13	18	15	14	17	15	16	18	18	15	18	16
S.typhi	16	18	17	13	15	14	17	20	18	14	16	15
K. pneumoniae	15	17	16	14	15	15	16	19	18	14	18	16

Table 1: Antibacterial activity of organic solvent extracts of CSS and CR rhizome and leaf. E: Ethanol, A: Acetone, M: Methanol.

Determination of minimum inhibitory concentration (MIC)

The lowest concentration of the extract which showed zone of inhibition against respective organisms was taken as MIC. Better efficacy of both ethanol and methanol extracts of CSS, and CR rhizome and leaf were further supported by MIC results. The MIC values of ethanol extracts of rhizome were ranging from 200-400 µg/ml for CSS and 250-450 µg/ml for CR. Methanol extracts showed MIC values ranging from 250-400 µg/ml for CSS while 250-450 µg/ml for CR (Table 2). The highest MIC values were observed for acetone extracts from rhizome (550 µg/ml) of CR against P. aeruginosa. The highest MIC value (450 µg/ml) was found with ehanol extracts from rhizome of CR against P. aeruginosa and acetone extract from rhizome of CR against *B. subtilis*. The lowest MIC value for ethanolic extract (200 µg/ml) was observed for CSS against *M. aureus* and *B. subtilis* while, (250 µg/ml) acetonic, methanolic extract of CSS and ethanolic, methanolic extract of CR against *M. aureus.*

Organisms	MIC (µg/ml)											
	Leaf						Rhizome					
	CSS			CR			CSS			CR		
	A	E	M	A	E	M	A	E	M	A	E	M
B. subtilis	400	300	350	400	350	300	300	200	350	450	300	350
M. aureus	300	250	250	350	250	300	250	200	250	300	250	250
P. aeruginosa	500	400	400	500	400	450	500	400	400	550	450	450
E. coli	400	350	350	450	300	350	300	300	300	350	300	350
S.typhi	350	300	350	450	400	400	350	300	350	400	300	300
K. pneumoniae	350	300	400	400	350	400	400	350	350	350	300	300
CSS: *C. spiralis var. spiralis, C: C. retrospiralis*. A: Acetone, E: Ethanol, M: Methanol												

Table 2: Minimum inhibitory concentration (MIC) (µg/ml) of organic solvent extracts of CSS and CR rhizome and leaf.

The MIC values of ethanol extracts of leaf were ranged from (250-400 µg/ml) for both CSS and CR. Methanol extracts showed MIC values ranged from (250-400 µg/ml) for CSS and (300-450 µg/ml) for CR. (Table 2). The highest MIC values were observed for acetone extracts of leaf (500 µg/ml) of both CSS and CR against *P. aeruginosa*. The lowest MIC value for ethanol extracts of leaf (250 µg/ml) for CSS and CR while, for methanolic extract of leaf of CSS (250 µg/ml) was observed against *M. aureus*. This highlights the equal effectiveness of ethanol and methanol extracts in comparison to agar diffusion assay.

Growth pattern of test organisms

The objective of this study was to find out exactly what was the response of the test organism test organisms growing in presence of acetone, ethanolic and methanolic extracts. The results are as shown in Figures 1 and 2.

It can be noted that all the organisms had retarded growth rate in presence of ethanol and methanol extracts from rhizome and leaf of *C. spiralis var. spiralis* and *C. retrospiralis*. However, the retardation of growth was higher in ethanolic extracts as compared to methanolic extracts. Further *M. aureus* is least inhibited by the acetone extracts from the rhizome of all species and in presence of the acetone extracts from the leaf of all species the growth of *M. aureus*, *E. coli* and *S. typhi* is not affected significantly except that it retards to some extent. Therefore, best antimicrobial activity is seen with the ethanolic extract, followed by methanolic extract and the least is by the acetone extracts from the respective rhizome and leaf of all species (Figures 1 and 2).

Phytochemicals analysis

The qualitative phytochemical analysis of acetone, ethanol, methanol and aqueous rhizome and leaf extracts of CSS and CR were presented in Table 3. All phytochemical compounds viz. glycosides, flavonoids, tannins, alkaloids, steroids and saponins were found present in all the solvent extracts of CSS and CR.

Gas Chromatography-Mass Spectroscopy (GC-MS) analysis

GC-MS analysis of ethanol extracts of *Cryptocoryne* species is represented in Table 4 [13-21]. The important bioactive compounds detected are cyclo hydrocarbon Bicyclo (2.2.1) heptanes, 2-methyl-3-methylene-2-(4-methyl-3-pentenyl)-(1S-exo), 3-Butoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris (trimethylsiloxy) tetrasiloxane, cyclohexasiloxane, dodecamethyl, 2-Ethoxy-3 chlorobutane, santalol, cis-alfa santalol, trans beta santalol, neomenthol, menthol. These phytocompounds were reported responsible for various pharmacological actions. The presence of various bio-active compounds detected in GC-MS analysis using the ethanolic extracts justifies the use of plant for pharmacological action. The antibacterial activity and phytochemical evaluation in alcoholic, aqueous and petroleum ether extract of *Cryptocoryne spiralis* reported by Das et al. [22]. Madhavan et al. and Prasad et al. also support the present investigation [7,23].

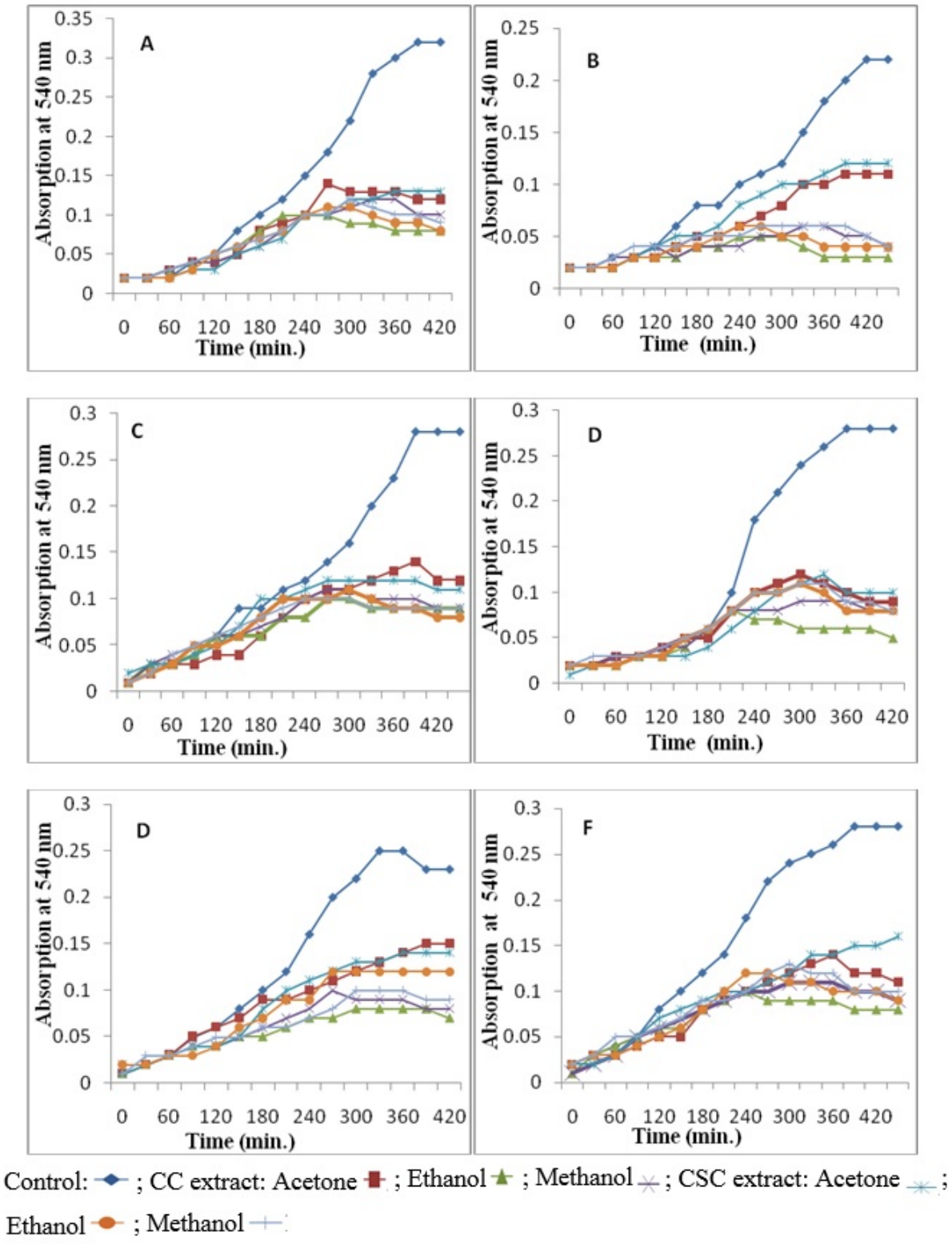

Figure 1: Growth pattern of microorganisms A) *B. subtilis* B) *M. aureus* C) *P. aeruginosa* D) *E. coli* E) *S. typhi* F) *K. pneumoniae* in presence of acetonic, ethanolic and methanolic extracts from rhizome of *C. spiralis var. spiralis* (CSS) and *C. retrospiralis*, (CR).

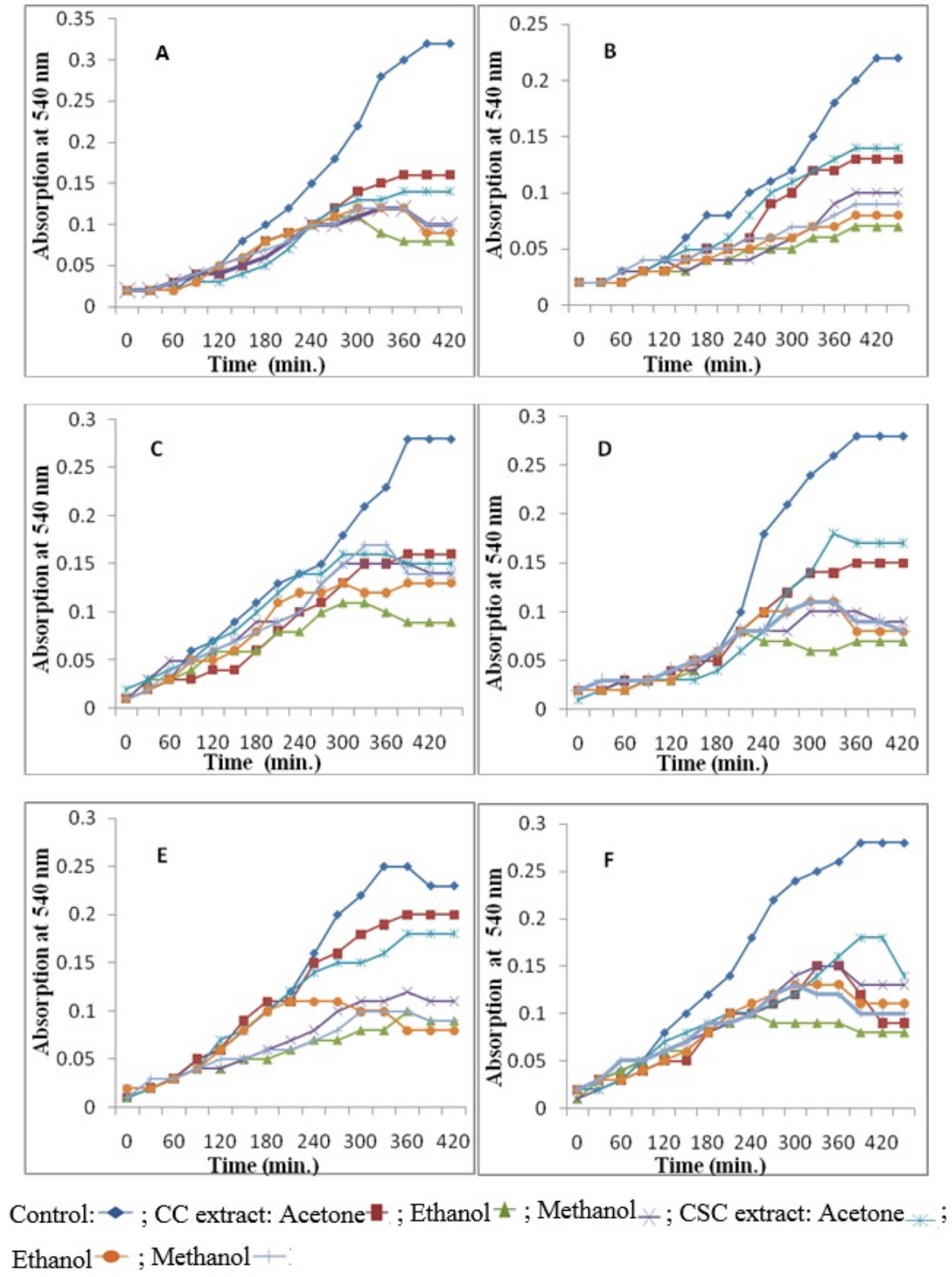

Figure 2: Growth pattern of microorganisms A) *B. subtilis* B) *M. aureus* C) *P. aeruginosa* D) *E. coli* E) *S. typhi* F) *K. pneumoniae* in presence of acetonic, ethanolic and methanolic extracts from leaf of *C. Spiralis var. spiralis* (CSS) and *C. retrospiralis*, (CR).

Bioactive Compound Names	Leaf								Rhizome							
	CSS				CR				CSS				CR			
	A	E	M	W	A	E	M	W	A	E	M	W	A	E	M	W
Tannins	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-
Alkaloids	+	+	+	-	+	+	+	-	+	+	+	-	+	+	+	-
Coumarins	+	+	+	-	+	+	+	-	+	+	+	-	+	+	+	-
Saponins	+	+	-	-	+	+	+	-	+	+	-	-	+	+	+	-
Steroides	-	+	-	-	-	+	+	-	-	+	-	-	-	-	+	-
Flavonoides	+	+	-	-	+	+	+	-	+	+	-	-	+	+	+	-
Glycosides	+	+	+	-	+	+	+	-	+	+	+	-	+	+	+	-
A: Acetone, E: Ethanol, M: Methanol, W: Aqueous, + Present, - Absent																

Table 3: Phytochemical analysis of aqueous organic solvent extracts of CSS and CR leaf and rhizome.

Peak	R. Time (min.)	Name of compound	Activity reported against
C. spiralis var. spiralis			
1	16.853	Neomenthol	*B. subtilis, E. coli, S. mutans*
2	16.955	Menthol	*S. typhimurium, P. aeruginosa*
3	24.867	Cis, alpha Santalol,	*E. coli, P. aeruginosa, K. pneumoniae, S. aureus*
4	25.531	Santalol	
5	33.986	Alpha Santalol	
6	34.666	Bicyclo [2.2.1] heptane, 2-methyl-3-methylene-2-(4-methyl-3-pentenyl)-, (1S-exo)	*E.Coli, P. aeruginosa, B. subtilis, S.aureus*
C. retrospiralis			
1	18.692	Cyclohexasiloxane, dodecamethyl	*P. vulgaris, K. pneumoniae, S. flexneri, P. aeruginosa*
2	20.284	3-Butoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane	*B. subtilis, E. coli, S. typhimurium, S. fexneri.*
3	25.542	Santalol	*E. coli, P. aeruginosa, K. pneumoniae, S. aureus*
4	24.934	Cis, alfa Santalol	

Table 4: Phytocomponents identified in ethanolic rhizome extract of *Cryptocoryne* species using GC-MS analysis.

Conclusion

The demonstrable antimicrobial activity of the rhizome extracts of the *C. spiralis var. spiralis* and *C. retrospiralis* implies that, these plants have a potential for preparation of alternative medicines for infections caused by microorganisms which have become resistant to the current therapeutic measures, which is an essential requirement of the present medical practice. This is more emphasized by its antibacterial properties against *Micrococcus aureus* which is a very common wound infecting organism and equally resistant to many of the common medicines used to treat its infections both in humans and in animals. *Pseudomonas aeruginosa* is the next wound infecting organism but notorious for its resistance to the medicines used, is also found susceptible to these extracts and this is very significant application. The results of the present study reveal the fact that the organic solvent extracts exhibited greater antimicrobial activity because the antimicrobial principles were extracted only through the organic solvent medium [24]. The present study supports the use of *Cryptocoryne* rhizome in the traditional system of medicine.

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Disclosure Statement

No potential conflict of interest was reported by the authors.

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