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Original Article

ANTIBACTERIAL CAPACITY AND IDENTIFICATION OF BIOACTIVE COMPOUNDS BY GCMS OF ALLIUM CEPA

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ABSTRACT

Objective: Plants offer a novel source for the isolation of a wide variety of medicinal agents. *Allium cepa* commonly known as onion is very well known medicinal plants and we investigated the antibacterial activity of different extracts and their phytochemical analysis by gas chromatography mass spectrometry (GCMS).

Methods: The extracts of *A. cepa* prepared in six different solvents was analyzed for antibacterial activity against nine American type cell culture (ATCC) reference bacterial strains i.e. *Shigella flexneri, Enterococcus faecalis, Staphylococus aureus, Proteus mirabilis, Salmonella typhi, Serratia marcescens, Klebsiella pneumonia, Escherichia coli, Pseudomonas aeruginosa* by using the agar well diffusion method. GCMS analysis also has been carried out for their phytochemical analysis.

Results: The results obtained from agar well diffusion assay showed the zone of inhibition range from 10 ± 0.76 to 26 ± 0.76 mm for different extracts. The methanol extract was found most potent against *K* pneumonia and *S*. marcescens with the zone of inhibition of 26 ± 0.76 mm for both strains. Minimum inhibitory concentration (MIC) values were in the range of 1.87 to 7.5 mg/ml and the MIC values for *K*. pneumonia and *S*. marcescens was 1.87 mg/ml. A total of 43 compounds were identified by GCMS analysis. Out of them dodecanoic acid was found common in all extracts.

Conclusion: It is concluded that Allium cepa have good antibacterial activity so it can be used for the treatment of various infectious diseases.

Keywords: Antibacterial activity, GCMS, Allium cepa, Bioactive compounds

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INTRODUCTION

Medicinal plants are being used for the treatment of different kind of diseases since ancient times. Plants synthesize a number of chemical compounds which are not directly involved in plant growth, but responsible for the different biological activities and provide protection from predators such as insects, fungi and herbivorous [1]. These chemicals are known as secondary metabolites. The secondary metabolites isolated from plants act in the human body in the similar way of the chemical compounds of allopathic drugs in their mechanism of action [2] Moreover, day by day the pathogens are being resistant against the synthetic drug, due to which herbal medicines can be an effective source for treatment of diseases with lesser side effects [3-4].

A. cepa has been used as spices, vegetables, ornamentals and as medicines for curing and treatment of various diseases. The Allium genus comprises of more than 700 species, widely distributed all over the world [5] and known for their flavor, easy growth and long storage time. It is one of the civilization's oldest medicines and described as the dynamite of natural foods. Allium species are characterized by their rich content of sulfur compounds that are responsible for their organoleptic characteristics [6-7] and contributes to the antioxidant and antimicrobial activities [8]. Many studies have been done for its uses in the treatment of different diseases. The bulb is the main and most commonly used part of the onion. The bulb of A. cepa has been reported to possess various activities like antihelmintic, antibacterial, anti-inflammatory, antiseptic, antispasmodic, carminative, diuretic, expectorant, febrifuge, hypoglycemic, hypotensive, lithintropic, stomachic and tonic [9-11]. Quercetin is one of the major flavanol beside other phytochemicals present in the A. cepa which has antioxidant, antibacterial, urease inhibition and anti melanogenesis activities [12-13]. Diallyl disulphide, polyphenols and anthocyanins are the other major components present in A. cepa [14].

Keeping in view of the importance of this plant, the present study has been carried out to check the antibacterial activity of different extracts of *A. cepa* against different bacteria. GCMS analysis also has been carried out for their phytochemical analysis.

MATERIALS AND METHODS

Chemicals and reagents

Nutrient agar, peptone water and streptomycin discs were purchased from HiMedia, India. Resazurin was purchased from Sigma-Aldrich Chemicals Private Limited, India. The solvents used for the preparation of plant extracts were purchased from sisco research laboratory (SRL), India. All solvents and chemicals, purchased were of analytical grade.

Preparation of plant extracts

Onions (red type) were purchased from the local market of Rohtak (28.8909 °N and 76.5796 °E), Haryana, India. These were peeled off, followed by washing and subjected to shade dry. The dried material was ground in an electrical grinder to obtain powder form. The powdered material (50 gms) was extracted with six organic solvents i.e. acetone, benzene, chloroform, ethyl acetate, methanol, and petroleum ether (1:10) using cold percolation for 48-72 h. The obtained extracts were filtered using Whatman No. 1 filter paper and then concentrated using rotary evaporator at 40 °C.

Antibacterial activity

The antibacterial activity of the extracts was analyzed against nine different american type cell culture (ATCC) reference bacterial strains i.e. *Shigella flexneri* ATCC 12022, *Enterococcus faecalis* ATCC 29212, *Staphylococus aureus* ATCC 259323, *Proteus mirabilis* ATCC 43071, *Salmonella typhi* ATCC 13311, *Serratia marcescens* ATCC 27137, *Klebsiella pneumonia* ATCC 700603, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 by using agar well diffusion method of Perez et al. (1990) with slight modifications [15]. The bacterial strains were obtained from Department of microbiology of Pt. B. D. Sharma University of Health Sciences, Rohtak, Haryana, India.

Minimum inhibitory concentration (MIC)

MIC was determined using micro broth dilution method using 96 multi-well microtitre plates following the method of Sarker *et al.*

(2007) with slight modifications [16]. Resazurin was used as indicator dye (270 mg/40 ml dH₂O), purple colour of indicator dye reduced in the presence of living bacteria into pink/colourless. In the absence of living bacteria the colour of the indicator remains purple. The lowest concentration at which colour change occurred was taken as MIC.

GCMS analysis

Phytochemical analysis of all six extracts of *A. cepa* was carried out using GCMS analyzer (BRUKER SCION 436-GC SQ). Extracts were dissolved in methanol (HPLC grade) and filtered through WhatmanTM FILTER DEVICE (0.2 μ m). Helium (99.99%) was used as carrier gas, at flow rate of 1 ml per minute in split mode. RESTEK Rtx®-5 (Crossbond® 5% diphenyl/95% dimethyl polysiloxane) with 30 m length, 0.25 μ m df and 0.25 mm ID column was used for separation of phytochemicals. 2 μ l of the sample was injected to the column. The injector temperature was 280 °C. The temperature of oven was started at 70 °C and hold for 2 min and then raised at a rate of 7 °C per minute up to 320 °C; hold for 1 min. Temperature of ion sources was maintained at 250 °C. The mass spectrum obtained by electron ionization at 70eV and detector operates in scan mode 30 to 500 Da atomic units. Total running time was 38.71 min including 3 min solvent delay.

RESULTS AND DISCUSSION

Yield of extracts

The measured yield of the extracts and % age of extracted values have been given in table 1.

Table 1: The yield of A	cepa extracts and %age of extracted value
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S. No.	Extracts	Yield of extracts (g)	%age of extracted value (Quantity of extract obtained × 100/Weight of dried powder)
1	Methanol	5.68	11.36
2	Acetone	4.69	9.38
3	Ethyl acetate	3.45	6.9
4	Chloroform	0.870	1.5
5	Benzene	0.746	1.4
6	Petroleum ether	0.480	0.96

Antibacterial activity

In the present study antimicrobial activities of extracts of A. cepa was investigated against reference bacterial strains at different concentrations by agar well diffusion assay. All the extracts showed good antibacterial activity with the zone of inhibition diameter ranging from 10 to 26 mm for different bacteria (table 2). The methanol extract showed highest antibacterial activity at all concentration as compared to other different extracts. Methanol extract of A. cepa showed concentration dependent activity. The concentration of 40 mg/ml was found to be more effective. Highest zone of inhibition was reported against K. pneumonia ATCC 700603 and S. marcescens ATCC 27137. Similarly, acetone extract showed 17 mm and 15 mm zone of inhibition against S. flexneri ATCC 12022 and E. coli ATCC 25922 respectively. The outcomes of our study indicate that A. cepa have significant antimicrobial potential which supports the study of Palaksha et al., [17]. It has been reported that the antimicrobial activity of A. cepa due to the presence of organosulfur and phenolic compounds [18].

Antibacterial activity of extracts of *A. cepa* has been analyzed against *Bacillus subtillis* and *S. aureus* by Sable *et al.* and the zone of inhibition measured were 8 mm and 9 mm respectively. *A. cepa*

extract in combination with Zingiber officinale extract showed the zone of inhibition of 13 mm and 11 mm against B. subtillis and S. aureus respectively [19]. Thus, the synergistic effects may increase the potential of the A. cepa. Antagonistic effect of the onion extracts has been checked against bacterial isolates i.e. E. coli, S. aureus, Streptococcus pneumonia and Streptococcus pyogenes with the inhibition zone of 17, 19, 17 and 20 mm respectively [20]. Kim et al. have studied the effect of A. cepa extracts on the oral pathogenic bacteria i.e. Streptococcus mutans, Streptococcus sobrinus, Porphyromonas gingivalis and Prevotella intermedia and found that extracts were active against all of these bacteria [21]. Shakurfow et al. have analyzed the antibacterial activity of cold water and organic solvent extracts against Listeria monocytogenes and found that the cold water extract was more effective than the organic solvent extract [22]. Santas et al. have checked the antibacterial activity of ethyl acetate extract of A. cepa against four Gram+ve (Bacillus cereus, S. aureus, Microcroccus luteus and Listeria monocytogenes) and two Gram-ve bacteria (E. coli and P. aeruginosa).

They observed that the extract inhibit the Gram+ve bacteria effectively while Gram-ve were found resistant [23]. On the contrary, in the present study the methanol extract of *A. cepa* was more effective against the Gram-ve bacteria.

Bacterial strain	Methanol	Acetone	Ethyl acetate	Chloroform	Petroleum ether	Benzene	Streptomycin
S. flexneri	20±0.76*	17±1.00	10±0.57	-	14±0.57	-	21±0.57
E. feacalis	14±0.57	13±1.00	10±0.76	11±1.00	12±1.00	11±0.57	23±0.57
S. aureus	16±0.76	-	12±0.76	-	12±1.00	-	26±0.76
P. mirabilis	20±0.57	-	-	11±0.57	-	11±0.76	23±1.00
S. typhi	15±1.00	12±0.57	-	-	-	-	19±1.00
S. marcescens	26±0.76	11±0.76	10±1.00	12±0.57	10±0.76	12±0.76	20±0.57
K. pneumonia	26±0.76	10±0.57	17±1.00	-	10±0.57	-	19±0.57
E. coli	23±0.76	10±0.57	12±0.57	10±0.76	-	-	18±0.57
P. aeruginosa	20±0.57	15±1.00	10±0.57	12±0.57	-	-	23±0.76

Table 2: The zone of inhibition in mm against differ	ent bacterial strains
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*The zone of inhibition showed as mean±Standard deviation (n=3)

MIC values of different extracts against tested microbes have been shown in table 3. The results showed that MIC values of *A. cepa* extracts varied from 1.87 mg/ml to 7.50 mg/ml. Lowest MIC value was

observed for methanol extract (1.87 mg/ml) against *K. pneumoniae* and *S. marcescens*. It means that *A. cepa* methanol extract possess the highest antimicrobial activity as compared to other extracts.

Bacterial strain	Methanol	Acetone	Ethyl acetate	Chloroform	Petroleum ether	Benzene
S. flexneri	5.0	3.75	7.5	-	3.75	-
E. feacalis	3.75	5.0	7.5	7.5	5.0	5.0
S. aureus	7.5	-	5.0	-	5.0	-
P. mirabilis	3.75	-	-	7.5	-	5.0
S. typhi	-	7.5	-	-	-	-
S. marcescens	1.87	7.5	7.5	5.0	7.5	5.0
K. pneumonia	1.87	7.5	3.5	-	7.5	-
E. coli	7.5	7.5	5.0	7.5	-	-
P. aeruginosa	5.0	5.0	3.75	5.0	-	-

Table 3: MIC values (in mg) of different extracts against different bacteria

The MIC values are showed as mean (n=3)

GCMS analysis

Organic compounds of different extracts were identified by GCMS analysis and spectra result was matched with the National Institute of Standards and Technology (NIST) MS library. The lists of identified phytochemicals from different extracts (methanol, acetone, ethyl acetate, chloroform, benzene, petroleum ether) have been given in table 4. The main phytochemicals identified by matching the spectra with NIST library were dodecanoic acid, methyl tetradecanoate, tetradecanoic acid, pentadecanoic acid, 14methyl-, methyl ester, methyl stearate, eicosanoic acid, methyl ester, di(2-ethylhexyl)adipate, 9-octadecenamide, (Z), 9-octadecenamide, (Z), tetracontane, 3,5,24-trimethyl, beta.-sitosterol. The spectra of the GCMS are given in fig. 1-6. The GCMS analysis revealed the presence of fatty acids and esters. Many fatty acids isolated from plants have been reported for their antimicrobial activity. Dodecanoic acid, also known as lauric acid has been isolated from coconut oil possesses good antibacterial activity against *S. aureus*, Bacillus cereus, Salmonella thypimurium and E. coli [24]. Pthalic acid, also known as benzoic acid has been tested against bacterial strain and zone of inhibition were ranged from 15 to 18 mm [25]. Odiba et al. had extracted the beta sitostirol from honey bee Propolis and studied its antibacterial activity against P. aeruginosa, E. coli, K. pneumonia, S. aureus, Streptococcus pyrogenes, Corynebacterium ulcerans, Bacillus subtillis, Shigella dysentariae, P. mirabilis, Candida albicans, Candida krusei and Candida tropicalis. They concluded that beta sitosterol showed the good antibacterial activity [26]. We have also reported the presence of beta sitosterol in A. cepa extracts. The GCMS analysis of extracts showed the existence of volatile compounds which are comparable to the study done by Lekshmi et al. [27]. The similar compounds reported in our study are octadecanoic acid, undecane, sitosterol, tetradecanal, dibutyl phthalate etc. Farag et al. identified the 39 volatile compounds from A. cepa and A. sativum using solid-phase micro-extraction coupled to GCMS and 38 non-volatile compounds by using UPLC/PDA/orbitrap-MS in methanol extracts [28]. In our study, most of the compounds identified in all six extracts were almost similar. However their quantities may differ as revealed from peak area and % of total. Maximum numbers of compounds (25) were identified in chloroform extract using NIST library.

Table 4: Phytoconstituents of six extracts screened by GCMS analysis with area and % of total

Name of compounds	Area			% of total						Formula			
	ACM	ACA	ACEA	ACC	ACPE	ACB	ACM	ACA	ACEA	ACC	ACPE	ACB	-
Dodecanoic acid, methyl ester	6.501e+7	6.490e+7	5.116 e+7	6.910e+7	5.934e+7	7.648e+7	10.016	12.909	7.422	6.798	8.650	6.284	$C_{13}H_{26}O_2$
Dodecanoic acid	2.912e+8	4.592e+7	9.358 e+7	1.230 e+8	7.029 e+7	1.293e+8	44.860	9.133	13.576	12.097	10.247	10.626	$C_{12}H_{24}O_2$
Methyl tetradecanoate	3.041e+7	3.224e+7	-	3.763 e+7	-	-	4.685	6.414	-	3.702	-	-	$C_{15}H_{30}O_2$
Tetradecanoic acid	5.040e+7	1.441e+7	-	1.505 e+7	1.088 e+7	-	7.766	2.867	-	1.481	1.587	-	$C_{14}H_{28}O_2$
Phthalic acid, hex-3-yl isobutyl ester	7.923e+6	-	-	-	-	-	1.221	-	-	-	-	-	$C_{18}H_{26}O_4$
Pentadecanoic acid, 14-methyl-, methyl	6.338e+7	7.851e+7	-	-	4.510 e+6	-	9.766	15.617	-	-	0.657	-	$C_{17}H_{34}O_2$
ester 9,12-Octadecadienoic acid, methyl ester, (E,E)	5.744e+7	-	1.964 e+8	-	-	-	8.850	-	28.492	-	-	-	$C_{19}H_{34}O_2$
Methyl stearate	1.053e+7	-	1.966 e+7	2.231 e+7	20.22e+7	2.555e+7	1.622	-	2.853	2.195	2.948	2.099	$C_{19}H_{38}O_2$
Tetracontane, 3,5,24- trimethyl	8.674e+6	-	9.829 e+6	-	-	-	1.336	-	1.426	-	-	-	C43H88
1-Decanol, 2-hexyl	1.006e+7	-	-	-	-	3.214e+7	1.550	-	-	-	-	2.641	$C_{16}H_{34}0$
Eicosanoic acid, methyl ester	5.628e+6	-	1.531 e+7	1.244 e+7	-	2.582e+7	0.867	-	2.221	1.224	-	2.122	$C_{21}H_{42}O_2$
Di(2- ethylhexyl)adipate	7.241e+6	5.209e+6	7.188 e+6	7.489 e+6	8.299 e+6	-	1.116	1.036	1.043	0.737	1.210	-	$C_{22}H_{42}O_4$
Bis(2-ethylhexyl) phthalate	2.558e+7	2.605e+7	2.952 e+7	3.084 e+7	2.778 e+7	2.042e+7	3.941	5.182	4.283	3.034	4.050	1.678	$C_{24}H_{38}O_4$
9-Octadecenamide, (Z)	1.561e+7	2.899e+7	1.143 e+7	1.402 e+7	9.950 e+6	-	2.405	5.766	1.658	1.379	1.451	-	C ₁₈ H ₃₅ NO
Phenol, 4-propyl	-	1.179e+7	2.214e+7	-	-	-	-	2.346	3.212	-	-	-	C9H120
13-Octadecenoic acid, methyl ester	-	1.179e+8	-	-	-	-	-	23.454	-	-	-	-	$C_{19}H_{36}O_2$
Hexadecanoic acid, 15-methyl-, methyl ester	-	1.417e+7		2.206 e+7	-	-	-	2.818	-	2.170	-	-	$C_{18}H_{36}O_2$
Hentriacontane	-	9.817e+6	-	1.016 e+7	1.505 e+7	-	-	1.953	-	0.999	2.194	-	$C_{31}H_{64}$
Tritetracontane	-	1.080e+7	2.040 e+7	2.354 e+7	-	-	-	2.148	2.959	2.316	-	-	C43H88
Heneicosanoic acid, methyl ester	-	8.505e+6	-	-	-	-	-	1.692	-	-	-	-	$C_{22}H_{44}O_2$
Oleanitrile	-	3.350e+7	-	-	-	-	-	6.664	-	-	-	-	$C_{18}H_{33}N$
Phenol, 2-propyl	-	-	2.214e+7	-	-	-	-	-	3.212	-	-	-	$C_9H_{12}O$

Tridecanoic acid, 12-	-	-	3.185 e+7	-	-	4.079e+7	-	-	4.621	-	-	3.351	$C_{15}H_{30}O_2$
methyl-, methyl ester													
1,2-	-	-	8.522 e+6	-	8.880 e+6	-	-	-	1.236	-	1.294	-	$C_{16}H_{22}O_4$
Benzenedicarboxylic													
acid, bis(2-													
methylpropyl) ester													
Hexadecanoic acid.	-	-	1.020 e+8	-	-	1.993e+8	-	-	14.804	-	-	16.374	$C_{17}H_{34}O_2$
methyl ester													
Hexadecanoic acid,	-	-	-	-	7.521 e+6	-	-	-	-	-	1.096	-	C18H36O2
ethyl ester													01030 0 2
(R)-(-)-14-Methyl-8-	-		1.086 e+7						1.576			-	C17H320
hexadecyn-1-ol			1.000 017						1.570				01/11320
Docosanoic acid,			6.518 e+6						0.946			-	C23H46O2
	-	-	0.310 6+0	-	-	-		-	0.940	-	-	-	C231146O2
methyl ester			1.021 e+7	1 1 1 (7	7.021	2.122e+7			1.482	1.098	1 0 2 2	1 000	C U O
9,19-Cyclolanostan-3-	-	-	1.021 e+7	1.116 e+7	7.021 e+6	2.122e+7	-	-	1.482	1.098	1.023	1.990	$C_{32}H_{54}O_2$
ol,acetate, (3. beta.)					= (10)								
betaSitosterol	-	-	7.344 e+6	1.264 e+7	5.612 e+6	1.742e+7	-	-	1.064	1.243	0.818	4.431	C29H50O
9,19-Cyclolanost-24-	-	-	1.562 e+7	2.231 e+7	1.651 e+7	4.632e+7	-	-	2.267	2.195	2.407	3.806	C32H52O2
en-3-ol, acetate													
2-Methoxy-4-	-	-	-	2.066e+7	-	-	-	-	-	2.032	-	-	$C_9H_{10}O_2$
vinylphenol													
3(2H)-Furanone, 2-	-	-	-	2.043 e+7	-	-	-	-	-	2.010	-	-	$C_{11}H_{18}O_2$
hexyl-5-methyl													
11,14-	-	-	-	3.144 e+8	-	-	-	-	-	30.929	-	-	C19H34O2
Octadecadienoic acid.													01)10102
methyl ester													
(Z)6,(Z)9-	-			1.565 e+7		1.600e+7				1.539		2.958	C15H28O
Pentadecadien-1-ol				1.505 0.7		1.0000017				1.557		2.750	01311280
Pentadecanoic acid,					5.645 e+6						0.823		C16H32O2
		-	-	-	5.045 640	-		-	-	-	0.023	-	C161132O2
methyl ester			3.185 e+7	-	3.240 e+7				4.621		4.723	-	C15H30O2
Tridecanoic acid, 12-	-	-	3.103 0+7	-	3.240 847	-		-	4.021	-	4.723	-	C151130O2
methyl-, methyl ester					(01)						0.004		C U O
Methyl 11-		-	-	-	6.816 e+6	-	-	-	-	-	0.994	-	$C_{17}H_{32}O_2$
hexadecenoate													
Methyl 12,13-	-	-	-	-	2.272 e+8	-	-	-	-	-	33.121	-	$C_{15}H_{26}O_2$
tetradecadienoate													
Oleic Acid	-	-	-	-	7.245 e+6	-	-	-	-	-	1.056	-	$C_{18}H_{34}O_2$
Docosanoic acid,	-	-	-	-	1.494 e+7	-	-	-	-	-	2.178	-	$C_{24}H_{48}O_2$
ethvl ester													
9 12-	-	-	-	-	-	5.011e+8	-	-	-	-	-	41.171	C17H30O2
Hexadecadienoic acid,													
methyl ester													
Hexadecanoic acid, 2-	-	-	-	-	-	4.221e+7	-	-				3.469	C19H38O4
hydroxy-1-													31330- F
(hydroxymethyl)ethyl													
ester													
63161													

ACM-Allium cepa methanol extract, ACA-Allium cepa acetone extract, ACEA-Allium cepa ethyl acetate extract, ACC-Allium cepa chloroform extract, ACPE-Allium cepa petroleum ether extract, ACB-Allium cepa benzene extract

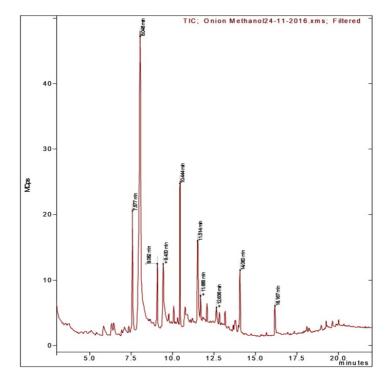


Fig. 1: GCMS spectra of methanol extract

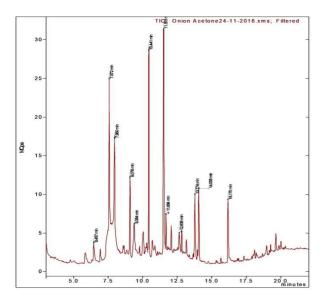


Fig. 2: GCMS spectra of Acetone extract

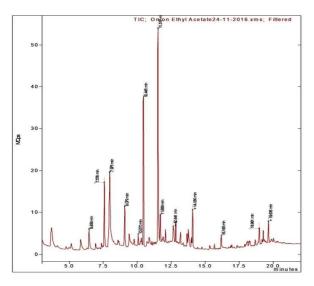


Fig. 3: GCMS spectra of ethyl acetate extract

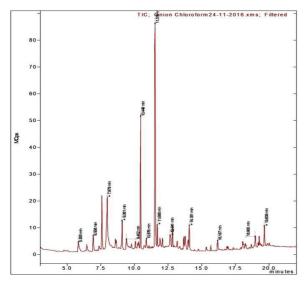


Fig. 4: GCMS spectra of chloroform extract

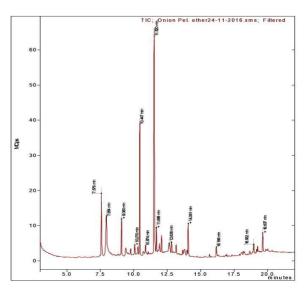


Fig. 5: GCMS spectra of petroleum ether extract

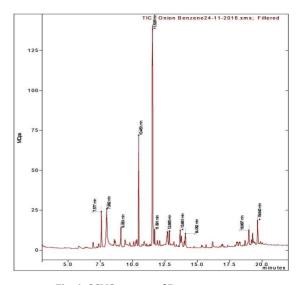


Fig. 6: GCMS spectra of Benzene extract

In this study, the common compounds identified were dodecanoic acid (methyl ester), dodecanoic acid and bis (2-ethylhexyl) phthalate in all the extracts. The maximum amount of dodecanoic acid was 44.86 % in methanol extract. The % of total dodecanoic acid (methyl ester) and bis (2-ethylhexyl) phthalate was comparable in all extracts which lies in the range of 6.284% to 12.91%. The highest antibacterial activity was found in methanol extract of *A. cepa* which may be due to the presence of higher amount of dodecanoic acid in comparison to other extracts. The highest antibacterial activity of methanol extract may also be due to the presence of volatile and non-volatile secondary metabolites. Further, research is required to study the effectiveness of purified active components from methanol extract of *A. cepa*.

CONCLUSION

It is concluded that *A. cepa* methanol extract has shown good antibacterial activity against different bacterial strains. GCMS analysis of different extracts revealed the presence of 43 phytochemicals. The findings of this work support the vision that this plant could provide biologically active natural drugs which may be useful for the treatment of bacterial infectious diseases.

ACKNOWLEDGEMENT

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ABBREVIATIONS

ATCC: American Type Cell Culture, GCMS: Gas Chromatography Mass Spectrometry, MIC: Minimum Inhibitory Concentration.

AUTHORS CONTIBUTIONS

Dushyant Sharma has prformed experimentation work, data collection, and drafted the manuscript. Reena Rani has made significant involvement in the interpretation of data and revising the manuscript. Monika Chaturvedi participated in the design of the study and performed the statistical analysis. Jaya Parkash Yadav helped in designed the study and manuscript.

CONFLICT OF INTERESTS

There is no conflict of interest between authors.

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