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RESEARCH ARTICLE

Identification of New Bioactive Compounds by Gc-Ms and Estimation of Physiological and Biological Activity of Kala Dhaman (*Cenchrus setigerus*)

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ABSTRACT

The present study was carried out to determine the possible bioactive components (sterols) in acetone extracts of *Cenchrus setigerus* using GC-MS analysis and *in-vivo* estimation of nitrogen assimilatory enzymes (NR, ALT, AST, GS, GDH and GOGAT) of seedlings and antibiotic activity of root extracts in various polar solvents of *Cenchrus setigerus* were done and antibiotic activity was tested against the G-ve bacteria including *Proteus merabilis, Klebsiella pnemoniae, Agerobacterium tumefaciens* and one fungi *Aspergillus niger* using disk diffusion method followed by determination of minimum inhibitory concentrations (MIC) by broth dilution method, against sensitive bacteria. Results reveal that various steroids from the above said plant were identified. The prevailing compounds (sterols) in the acetone extract of *C. setigerus* were 9,19-Cycloergost-24(28)-en-3-ol, 4,14-dimethyl-, acetate, (3β, 4α, 5α)- (14.39%), 2-Hexadecen-1-ol, 3,7, 11,15-tetramethyl-, [R-[R*,R*-(E)]]- (phytol) (1.27%), Cholest-4-en-3-one (2.36%) and β-Tocopherol (2.07%). All nitrogen assimilatory enzymes, GS showed highest activity followed by NR and highest antibiotic activity was exhibited by the isopropyl alcohol and chloroform extract against *P. merabilis* and *K. pnemoniae* after the GAA extract, The Glacial acetic acid extract was the most active, showing maximum Zone of inhibition (antibacterial effects) and *A. tumefaciens* (plant pathogen) was more sensitive to the extracts. Gentamycin and ketoconazole, the standard antibiotics were used to inhibiting these bacteria and fungi.

Key words: Antibiotic activity, Minimum Inhibitory Concentration (MIC), nitrogen assimilatory enzymes, *Cenchrus* grass, Zone of inhibition.

1. INTRODUCTION

The chemical analysis of isopropyl alcohol extract of *Cenchrus setigerus* showed a mixture of long-chain hydrocarbons, carboxyl esters, alcohols, acids, alkaloids, steroids, amino and nitro compound etc. Phytochemical screening using the pharmacognostic methods revealed the presence of flavonoids, steroids and alkaloids. Taking into consideration of the medicinal importance of this plant, the isopropyl alcohol extract of *C. setigerus* was analyzed for the first time using GC–MS. This work will help to identify the compounds (sterols) of therapeutic value. GC–MS is the best technique to identify the bioactive constituents of long chain hydrocarbons, alcohols, acids, ester, alkaloids, steroids, amino and nitro compound etc. Nitrogen assimilation is a vital process controlling plant growth and development. Inorganic nitrogen is assimilated into the amino acids glutamine, glutamate, asparagine, and aspartate, which serve as important nitrogen carriers in plants. The enzymes glutamine synthetase (GS), glutamate synthase (GOGAT), glutamate dehydrogenase (GDH) and aspartate aminotransferase (AST) are responsible for the biosynthesis of these nitrogen-carrying amino acids. The assimilation of inorganic nitrogen onto carbon skeletons has marked effects on plant productivity, biomass, and crop yield [1]. Nitrogen deficiency in plants has been shown to cause a decrease in the levels of photosynthetic structural components such as chlorophyll and ribulose bisphosphate carboxylase (rubisco), with resulting reductions in photosynthetic capacity and carboxylation efficiency [2] Because, enzymes involved in the assimilation of nitrogen into organic form in

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plants are crucial to plant growth, they are also effective targets for herbicide development [3].

The use of higher plants and their preparation to treat infectious and non-infectious disease is an age old practices and are the only method available in the past. Though the use of natural sources like plant material for curing diverse forms of ailments leads to human civilization, the scientific analysis of different natural sources for their possible medicinal potency is comparatively recent origin [4]. Various plant extracts can serve both as potential antimicrobial crude drugs as well as a source of new anti-infective agents [5].

Cenchrus setigerus (Kala Dhaman) are gaining attention in various field of research, as they are best suited to the present environmental conditions. This grass is more competitive under the conditions of high temperature, solar radiation and low moisture [6] and is more efficient at gathering CO_2 and utilizing nitrogen from the atmosphere and recycled N in the soil [7-8]. This grass has excellent soil binding capacity which helps to conserve soil in desert areas [9]. However, *C. setigerus* is most suitable and highly nutritive grass for desert environmental conditions, still no antimicrobial work yet have been done on this grass.

The objectives of this investigation was carried out to determine the possible bioactive compounds of *C. setigerus* using GC-MS analysis in acetone extract, *in vivo* estimation of nitrogen assimilatory enzymes of selected plant and antimicrobial activity of root extracts in various polar solvents were done.

2. MATERIALS AND METHODS

2.1. Identification of Components by GC-MS:

Interpretation on mass spectrum of GC-MS was done using the database of National Institute of standard and Technology (NIST) having more than 62,000 patterns. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library [10]. The name, molecular weight and structure of the components of the test materials were ascertained.

2.2. Nitrogen assimilatory enzymes:

2.2.1. Enzymatic activity: For *in vivo* studies seeds of *Cenchrus setigerus* were grown in 12" earthenware pots. Pots were filled with 8kg of a mixture of garden soil and goat manure in the ratio of 3:1. Pots were watered everyday. After two weeks of sowing thinning was done and 3-4 plants of uniform size were selected in each pot. Leaf samples were collected for biochemical analysis [11].

2.2.2. Preparation of enzyme extract: All operations for preparing the enzyme extracts were performed at 4°C. Plant material (*in vivo*-leaves) were homogenized using appropriate buffer in pre-chilled pestle mortar and centrifuged at 10,000 rpm for 20 min. The supernatant collected was used for all the enzyme assays and metabolites estimation.

2.2.3. Nitrate reductase (NR) assay (E.C.1.6.6.2): The estimation of this enzyme was done using Wray and Filner's method [12]. Nitrate is di-azotised by sulfanilamide and then reacted with N-1, n-ephthyl ethylene diamine dihydrochloride (NEDD) to produce azo dye (Pink colour) that is measured.

2.2.4. Alanine (ALT) and Aspartate (AST) aminotransferases assay (E.C.2.6.1.2 and E.C.2.6.1.1): ALT and AST were assayed using Bergmeyer's method [13]. Enzyme activity was calculated in terms of µmol of pyruvate released per mg of protein per minute.

2.2.5. Glutamine Synthetase (GS) (E.C.6.3.1.2): GS was assayed using a modification method of Elliot [14] as described by Boland *et al*., [15]. Activity was expressed in terms nM V-glutamate/nm NADH oxidized/mg of protein/min or nM/gfwt/hr.

2.2.6. Glutamate Synthase (GOGAT) (E.C.1.4.1.14): GOGAT activity was assayed by following the oxidation of the reduced coenzyme NADH [16]. This reduced coenzyme absorbs light at 340 nm. Enzyme activity was expressed as n-mol NADH oxidized/mg of protein/min.

2.2.6. **Glutamate dehydrogenase (GDH) (E.C.1.4.1.4):** GDH was assayed according to the method given by Boland *et al*., [15]. Enzyme activity was expressed as nM NADH oxidized/mg of protein/minute.

2.3. Antimicrobial Activity:

2.3.1. Plant material: Roots of *C. setigerus* (RUBL-5299) were collected in the month of August 2009 from the CAZRI, Jodhpur (Rajasthan). Plants samples were identified and deposited in the herbarium, department of botany, university of Rajasthan, Jaipur.

2.3.2. Preparation of extracts: Crude extracts of roots of *C. setigerus* were prepared with a series of non polar to polar solvents by hot extraction method. Different extracts were then screened for antimicrobial activity by disc diffusion Assay [17] against a few medically important bacteria and fungi.

2.3.3. Micro-organisms: The organisms used in this study were three G-ve bacteria and one fungus, viz., *Proteus merabilis* (MTCC-3310),

Klebsiella pnemoniae (MTCC-4030), *Agerobacterium tumefaciens* (MTCC-431) and *Aspergillus niger* (MTCC-282). Selected microorganisms were procured from IMTECH, Chandigarh, India.

2.3.4. Preparation of test pathogens and Disc diffusion assay: Bacterial strains were grown and maintained on NA medium, while fungi were maintained on SDA medium. DDA was performed for screening by standard method [18]. Activity index for each extract was calculated.

$$\text{Activity index (AI)} = \frac{\text{Inhibition Zone of the sample}}{\text{Inhibition Zone of the standard}}$$

2.3.5. Serial dilution method: MIC was determined as the least extract concentration which inhibited the growth of the test organisms. Bacterial and fungal suspensions were used as negative control, while broth containing standard drug was used as positive control.

2.3.6. Determination of Minimum bactericidal/ fungicidal concentration (MBC/MFC): Equal volume of the various concentration of each extract and nutrient broth mixed in micro-tubes to make up 0.5ml of solution. 0.5ml of McFarland standard of the organism suspension was added to each tube [19]. The tubes were incubated aerobically and MBC was determined by sub culturing and further incubated for 24 h. The highest dilution that yielded no single bacterial colony was taken as the MBC.

2.3.7. Total activity (TA) determination: Total activity is the volume at which the test extract can be diluted with the ability to kill microorganisms. It is calculated by dividing the amount of extract from 1 g plant material by the MIC of the same extract or compound isolated and is expressed in ml/g.

$$\text{Total Activity} = \frac{\text{Extract per gram dried plant part}}{\text{MIC of extract}}$$

3. RESULTS AND DISCUSSION

3.1. GC-MS analysis: GC-MS was done using the database of National Institute of standard and Technology (NIST). Results reveal that various steroids from the above said plant were identified. The prevailing compounds (sterols) in the acetone extract of *C. setigerus* were 9,19-Cycloergost-24(28)-en-3-ol, 4,14-dimethyl-, acetate, (3β, 4α, 5α)- (14.39% retention time-33.821 min. and area-3577706) (**Fig 1**), Phytol (1.27% retention time-18.315 min. and area-314880) (**Fig 2**), Cholest-4-en-3-one (2.36% retention time-36.596 min. and area-584906) (**Fig 3**) and β-Tocopherol (2.07% retention time-30.016 min. and area-513025) (**Fig 4**). However, till date there was no report on the presence of sterols from acetone extract of *C. setigerus.*

Figure 1: Mass Spectrum of 9,19-Cycloergost-24(28)-en-3-ol, 4,14-dimethyl-, acetate, (3β, 4α, 5α)- (RT- 33.821 min.)

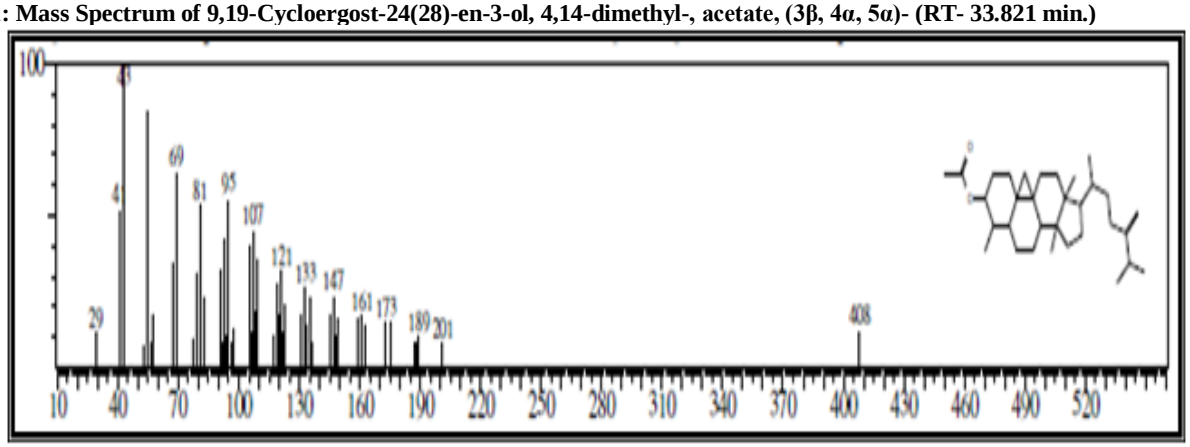


Figure 2: Mass Spectrum of Phytol- (RT- 18.315 min.)

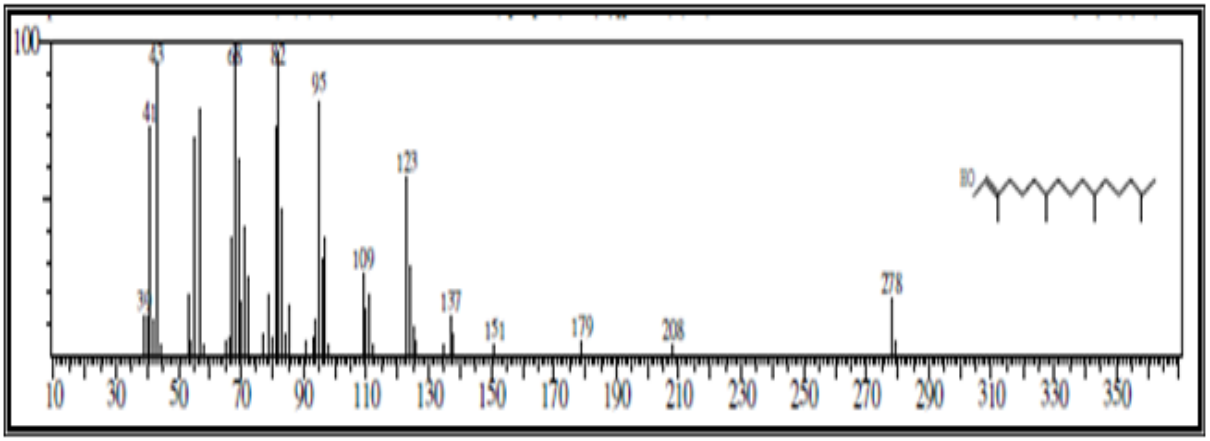


Figure 3: Mass Spectrum of Cholest-4-en-3-one (RT- 36.596 min.)

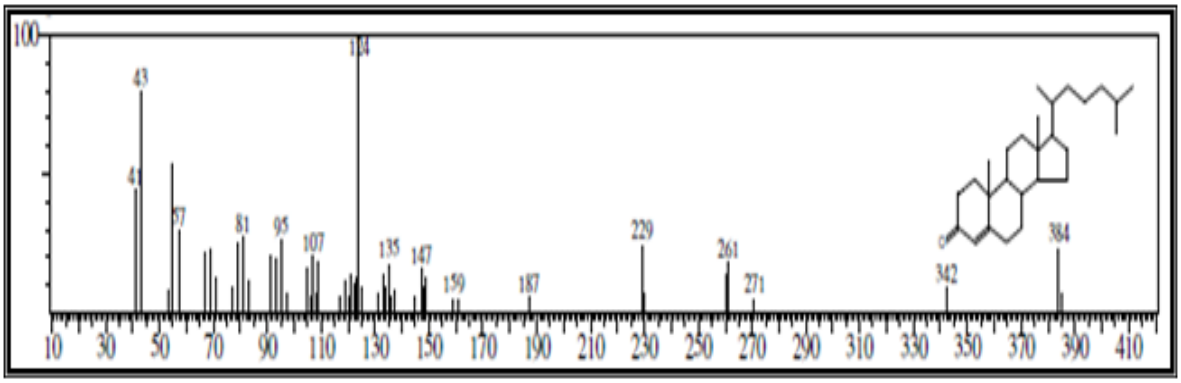
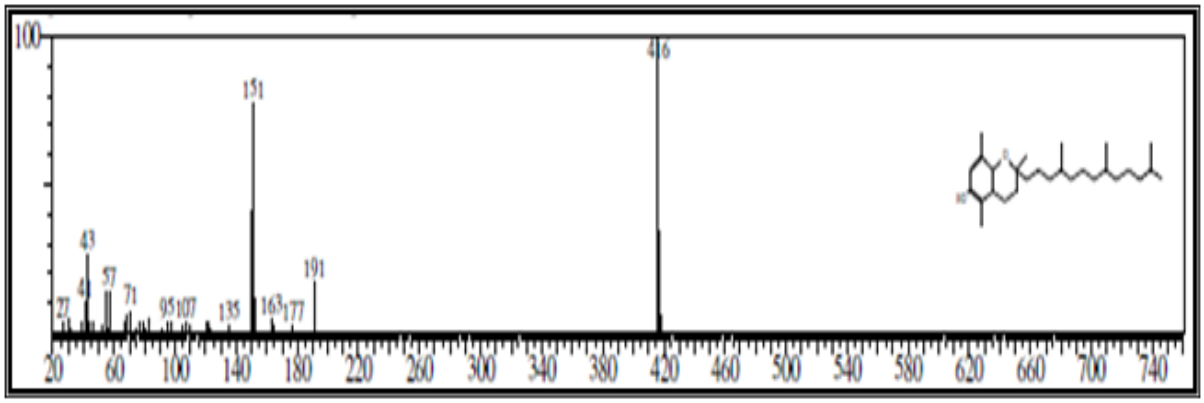
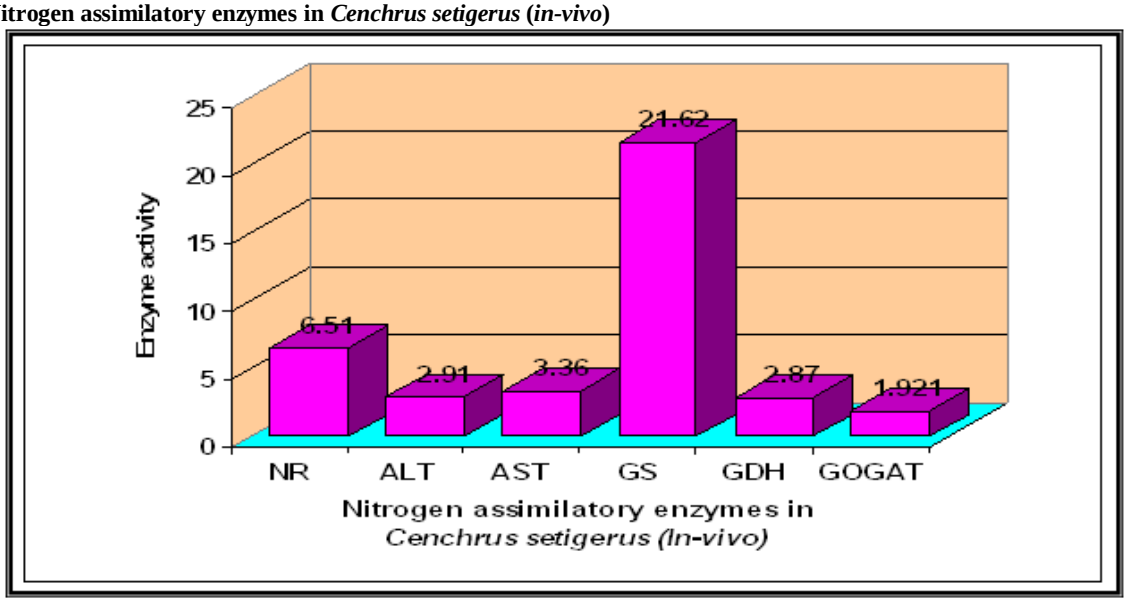


Figure 4: Mass Spectrum of β-Tocopherol (RT- 30.016 min.)



3.2. Nitrogen assimilatory enzymes: In the present investigation, above all nitrogen assimilatory enzymes, GS showed highest activity 21.62±0.154 (per g F. wt./min) followed by NR 6.51±0.145 (nm/mg of protein/min) (**Graph 1**). GS appears throughout the plant and animal kingdom. The enzyme has high affinity for ammonia and plays an important role in assimilating ammonia formed as a result of nitrogen fixation. NR the essential enzyme for the assimilation of nitrate is known to be influenced by wide range of environmental factors [20]. Stress conditions can also decrease NR activity as reported in water stress plants [21].

Graph 1: Nitrogen assimilatory enzymes in *Cenchrus setigerus* (*in-vivo*)



3.3. Antimicrobial Activity:

3.3.1. Zone of inhibition (ZOI) and Activity Index (AI): Antimicrobial activity (assessed in terms of ZOI and AI) of the root extracts in different polar solvents, tested against selected microorganisms were recorded. Most susceptible organism in the investigation was *A. tumefaciens* against which, most of the plant extracts showed inhibition zone supported by Singariya *et al.*, [22-23]. But, according the zone of inhibition *P. merabilis* was the most susceptible organism. The assay was repeated thrice and mean of the three experiments were recorded. The diameter of the ZOI was measured, commercial disc of antibiotics were used as positive control (standard) and experiment was done thrice (**Table 1**).

Table 1: Zone of Inhibition (mm)* and Activity index of root extracts of *Cenchrus setigerus*

Polar Solvents	Bio-activity of Root extracts of *Cenchrus setigerus* against pathogens							
	Proteus merabilis		*Klebsiella pnemoniae*		*Agerobacterium tumefaciens*		*Aspergillus niger*	
	ZOI	AI	ZOI	AI	ZOI	AI	ZOI	AI
Water	-	-	-	-	10.67±0.21	0.534	-	-
Acetic acid	27.67±0.22	2.306	25.83±0.25	1.292	22.83±0.22	1.038	-	-
Ethanol	-	-	9.33±0.24	0.467	-	-	-	-
Acetone	7.50±0.64	0.833	9.33±0.28	0.467	13.83±0.24	0.629	-	-
Ethyl acetate	9.67±0.29	0.691	9.33±0.26	0.467	10.67±0.28	0.667	-	-
Chloroform	10.33±0.21	1.148	10.5±0.64	0.525	10.83±0.26	0.542	-	-
Isopropyl alcohol	12.33±0.21	1.370	10.83±0.27	0.542	12.67±0.24	0.845	-	-
Benzene	-	-	-	-	10.17±0.24	0.848	-	-
Toluene	-	-	-	-	-	-	-	-
Petrolium ether	-	-	-	-	7.17±0.27	0.448	-	-
Hexane	-	-	-	-	8.67±0.22	0.434	-	-

All values are mean ± SD, n-3, ZOI-Zone of Inhibition (mm±S.D.), AI-Activity index

3.3.1.1. *Proteus merabilis*: Isopropyl alcohol extract show highest activity, ZOI-12.33±0.21 mm, AI-1.370 and followed by chloroform extract show ZOI-10.33±0.21 mm, AI-1.148, against *P. merabilis* after GAA extracts.

3.3.1.2. *Klebsiella pnemoniae*: Isopropyl alcohol and chloroform extract show highest activity after GAA extract, ZOI-10.83±0.27 mm, AI-0.542 and ZOI-10.50±0.64 mm, AI-0.525 respectively against *K. pnemoniae*.

3.3.1.3. *Agerobacterium tumefaciens*: Acetone and isopropyl alcohol extract show highest activity after GAA extracts, ZOI-13.50±0.24 mm, AI-0.629 and ZOI-12.67±0.24 mm, AI-0.845 respectively against *A. tumefaciens*.

3.3.1.4. ***Aspergillus niger*:** There was absence of antifungal activity.

3.3.2. MIC and MBC/MFC: MIC and MBC/MFC values (**Table 2**) were evaluated for those plant extracts, which were showing activity in disc diffusion assay. In the present investigation lowest MIC value 0.234 mg/ml was recorded for GAA extracts against *K. pnemoniae*, *P. merabilis* and *A. tumefaciens* indicating significant antimicrobial potential of test extracts. MIC and MBC/MFC values were found equal for GAA extracts showing bactericidal properties of test extracts.

Table 2: MIC and MBC/MFC of root extracts of *Cenchrus setigerus*

Polar Solvents	Bio-activity of root extracts of *C. setigerus* against pathogens							
	Proteus merabilis		*Klebsiella pnemoniae*		*Agerobacterium tumefaciens*		*Aspergillus niger*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC
Water	-	-	-	-	3.75	7.5	-	-
Acetic acid	0.234	0.469	0.234	0.468	0.234	0.468	-	-
Ethanol	-	-	7.5	15	-	-	-	-
Acetone	7.5	15	3.75	7.5	1.875	3.75	-	-
Ethyl acetate	7.5	7.5	3.75	3.75	1.875	3.75	-	-
Chloroform	3.75	7.5	1.875	3.75	3.75	3.75	-	-
Isopropyl alcohol	3.75	3.75	3.75	7.5	1.875	3.75	-	-
Benzene	-	-	-	-	0.938	0.938	-	-
Toluene	-	-	-	-	-	-	-	-
Petrolium ether	-	-	-	-	15	15	-	-
Hexane	-	-	-	-	7.5	15	-	-

MIC-Minimum inhibitory concentration (mg/ml); MBC-Minimum bactericidal concentration (mg/ml); MFC-Minimum fungicidal concentration (mg/ml)

3.3.3. Total activity: Total activity indicates the volume at which extract can be diluted with still having ability to kill microorganism. Most of the extracts showed high values of TA against *P. merabilis*, *K. pnemoniae* and *A. tumefaciens* were 205.13 ml, which prove the potential to inhibit the growth of the test microorganisms, even at low concentration (**Table 3**).

Table 3: Total activity of root extracts of *C setigerus* in different polar solvents against tested pathogens.

Polar Solvents	Total activity of root extracts of *C. setigerus* against pathogens			
	Proteus merabilis	*Klebsiella pnemoniae*	*Agerobacterium tumefaciens*	*Aspergillus niger*
Water	-	-	5.73	-
Acetic acid	205.13	205.13	205.13	-
Ethanol	-	5.13	-	-
Acetone	4.43	8.85	17.71	-
Ethyl acetate	2.03	4.05	8.11	-
Chloroform	8.59	17.17	8.59	-
Isopropyl alcohol	10	10	20	-
Benzene	-	-	9.28	-
Toluene	-	-	-	-
Petrolium ether	-	-	1.91	-
Hexane	-	-	1.28	-

3.3.4. Phyto-chemical estimation: The phyto–chemical estimation for the roots of *C. setigerus* were carried out according to Farnsworth [24] wherein the consistency was found to be sticky in the high polar solvent extracts whereas the low polar solvent extracts were found to be non-sticky (**Table 4**) which supported by Singariya *et. al*, [25] But, water, ethanolic and ethyl acetate extracts were found to be non-sticky. The yield (%) of the extracts was also analyzed where in the highest yields were recorded for GAA and ethanolic extracts, 4.80 and 3.85 respectively.

Table 4: Phyto-chemical estimation of root extract of *C. setigerus* in different polar solvents

Polar Solvents	Primary Phyto-chemical estimation of Root extracts of *C. setigerus*		
	Total Yield (mg/10gm±S.D.)	Color	Consistency
Water	215±12.94	Dark brown	Nonsticky
Acetic acid	480±15.55	Dark green	Sticky
Ethanol	385±11.21	Dark brown	Non sticky
Acetone	332±08.11	Dark brown	Sticky
Ethyl acetate	152±12.58	Yellow	Nonsticky
Chloroform	322±13.42	Brown	Sticky
Isopropyl alcohol	375±08.42	Dark brown	Sticky
Benzene	087±08.86	Yellow	Nonsticky
Toluene	207±11.56	Brown	Nonsticky
Petrolium ether	287±15.89	Very light Yellow	Nonsticky
Hexane	096±12.24	Colorless	Nonsticky

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