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Research Article

# Isolation of some new steroids and evaluation of bio-activity of *Cenchrus ciliaris*

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**ABSTRACT**

Cenchrus ciliaris L. (Poaceae) is most suitable and highly nutritive grass for desert environmental conditions; still no bioactive compound identified and no antimicrobial work yet have been done on this grass. To determine the possible bioactive components (steroids) of C. ciliaris and in vivo estimation of metabolites, photosynthetic pigments of seedlings and antimicrobial activity of extract in various polar solvents from the seeds of C. ciliaris. New compounds were isolated by GC-MS analysis. Antimicrobial activity was evaluated against Proteus merabilis, Klebsiella pnemoniae, Agerobacterium tumefaciens and Aspergillus niger; using disk diffusion method followed by MIC by broth dilution method. Estimation of metabolites (total soluble sugar, soluble protein, proline and total phenolics), photosynthetic pigments (chlorophyll-a, chlorophyll-b and carotenoids) of seedlings were done with well established methods. Four new steroids from C. ciliaris were first time identified in the isopropyl alcohol extract such as 4,22-Stigmastadiene-3-one (4.76%), fagarsterol (lupeol) (1.94%), δ4-sitosterol-3-one (stigmast-4-en-3-one) (1.93%) and ethyl iso-allocholate (0.49%). The highest antimicrobial activities (in terms of zone of inhibition in mm) were exhibited by the ethyl acetate and water extract against P. merabilis (18.50 ± 0.64 and 15.5 ± 0.64 respectively) after the glacial acetic acid extract. Total soluble sugar and chlorophyll-a showed highest content.

**Keywords:** Antibacterial activity; photosynthetic pigments; total soluble sugar; *Cenchrus* grass; Lupeol; Ethyl iso-allocholate; proline

## INTRODUCTION

The chemical analysis of isopropyl alcohol extract of *Cenchrus ciliaris* L. (Poaceae) 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. ciliaris* was analyzed for the first time using GC-MS. This work will help to identify the compounds (steroids) 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. 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 (Sikinner, 1955). Antimicrobial resistance to anti microbial agents has lead to treatment failure and the shift of medical care from orthodox to herbal medicine. Most of the herbal medicines in use await validation of their claimed effects and possibly the development of novel antimicrobial drugs from them (Otimentin et al*.,* 2008). Natural plants derived compounds contribute a lot in fight against pathogens (Vyvyan, 2002). Various plant extracts can serve both as potential antimicrobial crude drugs as well as a source of new anti-infective agents (Rios, 2005).

*Cenchrus ciliaris* ($C_4$ grass) is gaining attention in various field of research, as this is best suited to the present environmental conditions. $C_4$ grasses are more competitive under the conditions of high temperature, solar radiation and low moisture (Agrawal, 2007). This grass is more efficient at gathering $CO_2$ and utilizing nitrogen from the atmosphere and recycled N in the soil (Bessman, 1956; Singariya, 2009). This grass has excellent soil binding capacity which helps to conserve soil in desert areas (Sinha et al., 1996). However, *C. ciliaris* is most suitable and highly nutritive grass for desert environmental conditions.

Proline may protect protein structure and membrane from damage and to reduce enzyme denaturation (Saradhi et al., 1995). Alternatively proline accumulation

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has also been proposed to nitrogen storage (Larcher, 1995). Carotenoids act as accessory light-harvesting pigments as well as for the protection of choroplasts from light mediated stress (Siefermann-Harms, 1987). Carotenoids protect photosynthetic apparatus through two important ways: (a) β-carotenoids directly quenches both triple Chl ($^1Chl^*$) and singlet oxygen ($^1O_2^*$). (b) The xanthophylls cycle involving a reversible conversion from violaxanthin and antheraxanthin to zeaxanthin and quenches $^1Chl^*$ (Gilmore & Govindjee, 1999).

## MATERIAL AND METHODS

### 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. The name, molecular weight and structure of the components of the test materials were ascertained (Muthulakshmi et al., 2012).

**Photosynthetic pigments and metabolite: (a) *In-vivo* studies:** For *in vivo* studies seeds of *C. ciliaris* were grown in 12" earthenware pots. Pots were filled with 8 kg 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 (Singariya et al., 2009).

**(b) Preparation of extract:** All operations for preparing the 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 metabolites estimation.

**(i) Estimation of soluble protein:** Soluble proteins were estimated by the method of Bradford (1976). This involves the binding of Coomassie Brilliant Blue G-250 (CBB-G-250) to proteins.

**(ii) Estimation of proline:** Bates et al., (1973) method was used for estimated of proline content. The extracted proline was made to react with ninhydrin in acidic conditions to form the chromophore (red colour), which was read at 520nm.

**(iii) Estimation of total soluble sugars:** Total soluble sugars were estimated in accordance with Mc-Ready et al. (1950) method.

**(iv) Total Phenolics:** The phenol in extracts determined according to Folin-Ciocalteu procedure (Singleton & Rossi, 1995).

**(v) Photosynthetic pigments:** 500 mg of leaves were homogenized in 5 ml of 80 % acetone centrifuged for 20 min at 10,000 g and the supernatant was used for estimation of photosynthetic pigments. Absorbance was recorded at 663, 645 and 470 nm against 80 % acetone as blank. Arnon's (1949) method was used for calculation of chlorophyll content.

**[3] Antimicrobial Activity: (a) Plant material:** Seeds of *Cenchrus ciliaris* (variety: CAZRI-358) were collected in the month of August 2009 from the CAZRI, Jodhpur, Rajasthan. The collected plant materials were transferred immediately to the laboratory and powdered with the help of grinder (Hussain et al., 2010).

**(b) Preparation of extracts:** Crude extracts of seeds of *C. ciliaris* were prepared with a series of non polar to polar solvents by hot extraction method (Harborne, 1984) in Soxhlet assembly. Different extracts were then screened for antimicrobial activity by disc diffusion Assay (Cruickshank, 1968) against a few medically important bacteria and fungi. The fraction showing best activity was then used for determining of MIC by tube dilution method (Deborah et al., 2006) and minimum bactericidal/fungicidal concentration (MBC/MFC).

**(c) Micro-organisms:** The organisms used in this study were three Gram-negative 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.

**(d) 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 (Singariya et al., 2012a). Activity index for each extract was calculated (Table 3).

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

**(E) Serial dilution method:** MIC was determined as the least extract concentration which inhibited the growth of the test organisms (Singariya et al., 2011b). Bacterial and fungal suspensions were used as negative control, while broth containing standard drug was used as positive control.

**(F) 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 (Bhattacharya et al., 2009). 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 (Akinyemi et al., 2005).

**(G) 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 (Singariya et al., 2011c).

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

## RESULTS AND DISCUSSION

***[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 (steroids) in the isopropyl alcohol extract of *C. ciliaris* were 4,22-Stigmastadiene-3-one (4.76% retention time-37.297 min. and area-1499155) (fig.-1), fagarsterol (lupeol) (1.94% retention time-36.935 min. and area-610475) (fig.-2) it is used as anti cancers drug, δ4-sitosterol-3-one (stigmast-4-en-3-one) (1.93% retention time-38.783 min. and area-608167) (fig.-3) and ethyl iso-allocholate (0.49% retention time-29.081 min. and area-233057) (fig.-4) it has antimicrobial, Diuretic and anti-inflammatory activity. However, till date there was no report on the presence of steroids from *C. ciliaris.*

**[2] Photosynthetic pigments and metabolite: (a) Metabolites:** *In vivo* study of *C. ciliaris* reveals that total soluble sugar shows highest content 16.82±0.032

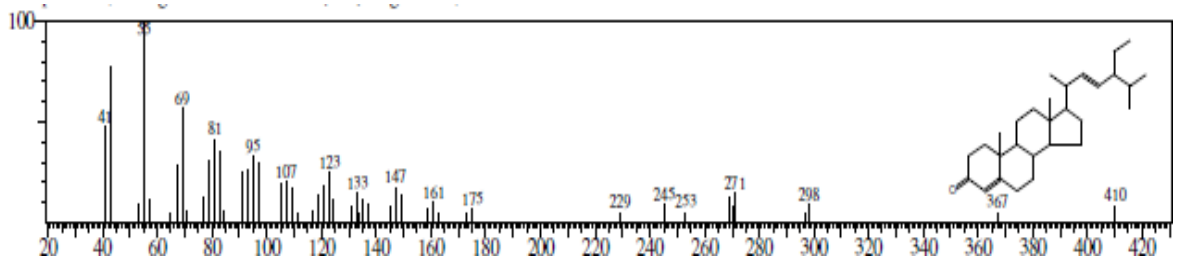


**Figure 1: Mass Spectrum of 4,22-Stigmastadiene-3-one (RT- 37.297 min.)**

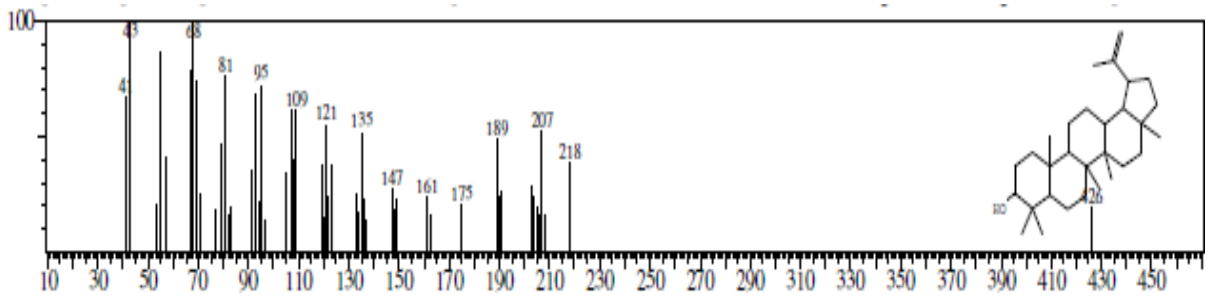


**Figure 2: Mass Spectrum of Fagarsterol (Lupeol) (RT- 36.935 min.)**

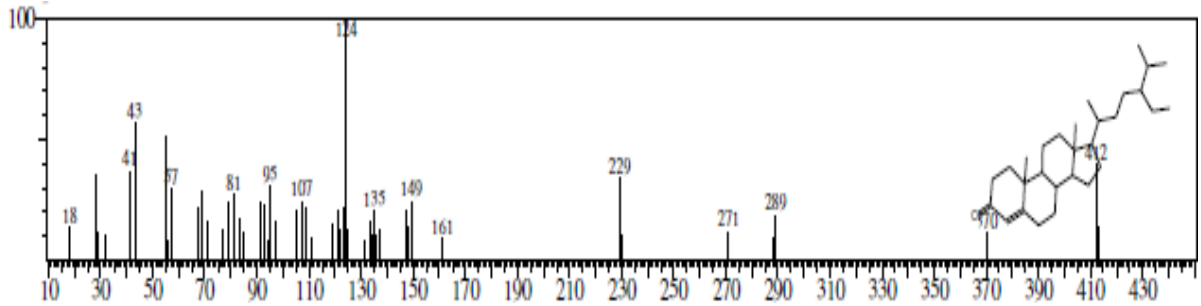


**Figure 3: Mass Spectrum of Delta.4-Sitosterol-3-one (RT- 38.783 min.)**

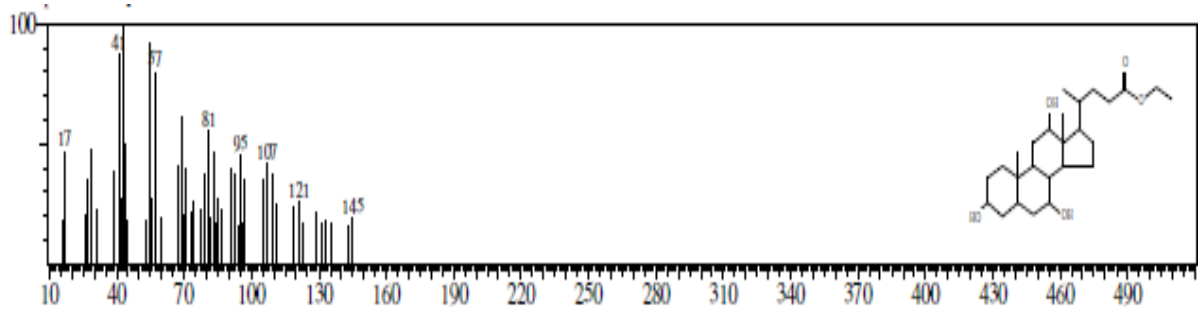


**Figure 4: Mass Spectrum of Ethyl iso-allocholate (RT- 29.081 min.)**

**Table 1: Different metabolites of Cenchrus ciliaris**

| S.No. | Metabolites | Parameter | *Cenchrus ciliaris* (*In-vivo*) |
|---|---|---|---|
| 1. | Soluble Protein | (mg/g F. wt.) | 10.51±0.034 |
| 2. | Total Soluble Sugar | (mg/g F. wt.) | 16.82±0.032 |
| 3. | Proline | (m mol/g F. wt.) | 0.066±0.016* |
| 4. | Total Phenolics | (mg/g F. wt.) | 12.225±0.104 |

* SD value X10$^{-3}$

**Table 2: Photosynthetic Pigments of Cenchrus ciliaris**

| S.No. | Pigments | Parameter | *Cenchrus ciliaris* (*In-vivo*) |
|---|---|---|---|
| 1. | Chlorophyll a | (mg/g F. wt.) | 0.947±0.016 |
| 2. | Chlorophyll b | (mg/g F. wt.) | 0.495±0.031 |
| 3. | Carotenoids | (mg/g F. wt.) | 0.197±0.008 |

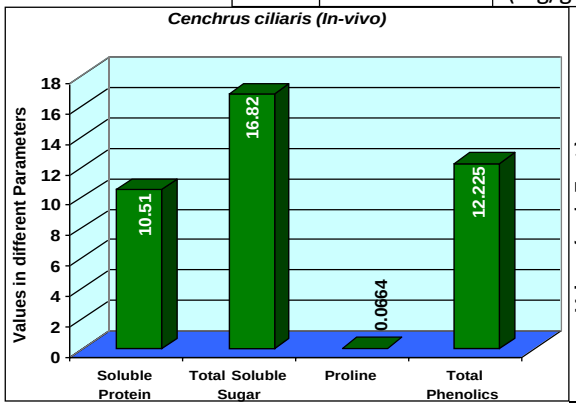


**Figure 5: Different metabolites of Cenchrus ciliaris (*In vivo*)**

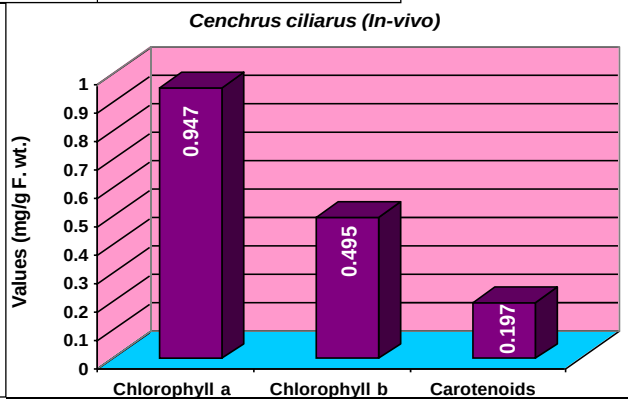


**Figure 6: Photosynthetic Pigments of Cenchrus ciliaris (*In vivo*)**

**Table 3: Zone of Inhibition (mm)* and Activity index of seed extracts of Cenchrus ciliaris**

| Polar Solvents | Bio-activity of seed extracts of *Cenchrus ciliaris* against pathogens | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *Proteus merabilis* | | *Klebsiella pnemoniae* | | *Agerobacterium tumefaciens* | | *Aspergillus niger* | |
| | ZOI | AI | ZOI | AI | ZOI | AI | ZOI | AI |
| Water | 15.5±0.64 | 1.292 | 9.33±0.25 | 0.467 | 8.67±0.24 | 0.542 | - | - |
| Acetic acid | 31.67±0.25 | 2.639 | 40.83±0.29 | 2.042 | 32.5±0.64 | 2.031 | - | - |
| Ethanol | - | - | 7.17±0.23 | 0.359 | 11.67±0.24 | 0.834 | - | - |
| Acetone | 8.17±0.27 | 0.681 | 9.67±0.24 | 0.484 | 8.67±0.27 | 0.619 | - | - |
| Ethyl acetate | 18.50±0.64 | 2.313 | 9.17±0.23 | 0.459 | 9.67±0.24 | 0.806 | - | - |
| Chloroform | - | - | 9.5±0.64 | 0.475 | 10.67±0.21 | 0.593 | - | - |
| Isopropyl alcohol | 7.17±0.24 | 0.598 | 9.17±0.62 | 0.459 | 12.67±0.22 | 0.905 | - | - |
| Benzene | 8.17±0.26 | 1.021 | 7.17±0.26 | 0.359 | 9.33±0.26 | 0.666 | - | - |
| Toluene | 7.33±0.29 | 0.611 | 9.67±0.24 | 0.484 | 10.33±0.26 | 0.430 | - | - |
| Petrolium ether | - | - | - | - | 8.33±0.24 | 0.694 | - | - |
| Hexane | - | - | - | - | - | - | - | - |

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

(mg/g F. wt.) (table 1) followed by total phenolics 12.225±0.104 (mg/g F. wt.) among all the metabolites and Proline was recorded as 0.0664±0.016 (m mol/g F. wt.) (graph 1).

**(b) Photosynthetic Pigments:** In case of Photosynthetic Pigments Chlorophyll a, Chlorophyll b and Carotenoids were estimated (mg/g F. wt.) (table 2). Result of this study reveals that highest activity was reported by chlorophyll a (0.947±0.016) followed by chlorophyll b (0.495±0.031) (graph 2).

***[3]* Antimicrobial Activity: (A) 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 (table 3). In the present study total 11 extracts of seeds of selected grass were tested for their bioactivity, among which 10 extracts showed significant antimicrobial potential against test microbes most susceptible organism in the investigation was *A. tumefaciens* against which, most of the plant extracts showed inhibition zone. But, according the zone of inhibition *P.*

**Table 4: MIC and MBC/MFC of seed extracts of C. ciliaris in different polar solvents**

| Polar Solvents | Bio-activity of seed extracts of *C. ciliaris* against pathogens | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *P. merabilis* | | *K. pnemoniae* | | *A. tumefaciens* | | *A. niger* | |
| | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MFC |
| Water | 1.875 | 1.875 | 3.75 | 7.5 | 7.5 | 15 | - | - |
| Acetic acid | 0.234 | 0.234 | 0.117 | 0.117 | 0.234 | 0.234 | - | - |
| Ethanol | - | - | 15 | 15 | 1.875 | 1.875 | - | - |
| Acetone | 7.5 | 15 | 7.5 | 15 | 7.5 | 15 | - | - |
| Ethyl acetate | 1.875 | 3.75 | 3.75 | 3.75 | 3.75 | 7.5 | - | - |
| Chloroform | - | - | 3.75 | 7.5 | 1.875 | 1.875 | - | - |
| Iso propyl alcohol | 7.5 | 15 | 3.75 | 7.5 | 0.938 | 1.875 | - | - |
| Benzene | 7.5 | 15 | 7.5 | 15 | 1.875 | 3.75 | - | - |
| Toluene | 7.5 | 15 | 3.75 | 7.5 | 3.75 | 7.5 | - | - |
| Petrolium ether | - | - | - | - | 7.5 | 7.5 | - | - |
| Hexane | - | - | - | - | - | - | - | - |

**Table 5: Total activity of seed extracts of Cenchrus ciliaris in different polar solvents**

| Polar Solvents | Total activity of seed extracts of *Cenchrus ciliaris* against pathogens | | | |
|---|---|---|---|---|
| | *P. merabilis* | *K. pnemoniae* | *A. tumefaciens* | *A. niger* |
| Water | 26.93 | 13.47 | 6.73 | - |
| Acetic acid | 183.33 | 366.67 | 183.33 | - |
| Ethanol | - | 1.21 | 9.71 | - |
| Acetone | 9.13 | 9.13 | 9.13 | - |
| Ethyl acetate | 23.73 | 11.87 | 11.87 | - |
| Chloroform | - | 14.29 | 28.59 | - |
| Iso propyl alcohol | 1.36 | 2.72 | 10.88 | - |
| Benzene | 2.49 | 2.49 | 9.97 | - |
| Toluene | 3.24 | 6.48 | 6.48 | - |
| Petrolium ether | - | - | 4.24 | - |
| Hexane | - | - | - | - |

**Table 6: Phyto-chemical estimation of seed extracts of C. ciliaris in different polar solvents**

| Polar Solvents | Primary Phyto-chemical estimation of seed extracts of *C. ciliaris* | | |
|---|---|---|---|
| | Total Yield (%) | Color | Consistency |
| Water | 505±18.37 | Coffee | Sticky |
| Acetic acid | 479±13.57 | Brick red | Sticky |
| Ethanol | 182±17.82 | Yellow | Nonsticky |
| Acetone | 685±11.78 | Light yellow | Nonsticky |
| Ethyl acetate | 445±14.89 | Dark green | Nonsticky |
| Chloroform | 536±18.87 | Colorless | Nonsticky |
| Iso propyl alcohol | 102±9.86 | Colorless | Nonsticky |
| Benzene | 187±13.49 | Yellow | Nonsticky |
| Toluene | 243±11.56 | Brown | Sticky |
| Petrolium ether | 318±16.83 | Light cream | Nonsticky |
| Hexane | 290±15.68 | Brick red | Sticky |

*merabilis* was the most susceptible organism. Maximum antimicrobial activities were observed by glacial acetic acid (GAA) and ethyl acetate extracts in *C. ciliaris.*

**(i) *Proteus merabilis*:** Ethyl acetate extract show highest activity after GAA extracts, ZOI- 18.50 ± 0.64 mm, AI-2.313 and followed by water extract show ZOI- 15.50 ± 0.64 mm, AI- 1.292, against *P. merabilis*.

**(ii) *Klebsiella pnemoniae*:** Acetone and toluene extract show highest activity after GAA extracts, ZOI- 9.67 ± 0.24 mm, AI- 0.484 and followed by chloroform extract show ZOI- 9.50 ± 0.64 mm, AI- 0.475, against *K. pnemoniae*.

**(iii) *Agerobacterium tumefaciens*:** Isopropyl alcohol extract show highest activity after GAA extracts, ZOI- 12.67 ± 0.22 mm, AI- 0.905 and followed by ethanolic extract show ZOI-11.67 ± 0.24 mm, AI- 0.834, against *A. tumefaciens*.

**(iv) *Aspergillus niger*:** There was absence of antifungal activity.

**(B) MIC and MBC/MFC:** MIC and MBC/MFC values (Table 4) were evaluated for those plant extracts, which were showing activity in disc diffusion assay. The range of MIC and MBC/MFC of extracts recorded was 0.117-15 mg/ml. In the present investigation lowest MIC value 0.117 mg/ml was recorded for GAA extracts against *K. pnemoniae* followed by 0.234 mg/ml against *P. merabilis* and *A. tumefaciens* by the same indicating significant antimicrobial potential of test extracts. MIC and MBC/MFC values were found equal for glacial acetic acid extracts showing bactericidal properties of test extracts.

**(C) Total activity (TA):** 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* which prove the potential to inhibit the growth of the test microorganisms, even at low concentration. Maximum TA values calculated were 366.67 ml against *K. pnemoniae* followed by 183.33 ml against *P. merabilis* as well as *A. tumefaciens* (table 5).

**(D) Quantitative phyto-chemical estimation:** The quantitative phyto–chemical estimation for the seeds of *C. ciliaris* were carried out according to Farnsworth (1966) 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 which supported by Singariya et. al, (2011a), but, hexane and toluene extracts were found to be sticky. The yield (mg/10 gm ± S.D.) of the extracts was also analyzed where in the highest yields were recorded for *C. ciliaris* (685 ± 11.78) in acetone extracts (Table 6).

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