ORIGINAL ARTICLE

Identification of stigmasterol by preparative thin-layer chromatography, infrared, gas chromatography–mass spectrometry analysis, and antioxidant properties of *Cenchrus ciliaris* L.

Premlata Singariya[1], Mourya Krishan Kumar[2], Kumar Padma[1]

[1]Department of Botany, University of Rajasthan, Jaipur – 302 004, Rajasthan, India, [2]Rural Veterinary Polyclinic, Hingonia, Jaipur – 303 012, Rajasthan, India

Abstract

Aim: The present investigation was carried out to determine the possible bioactive components of *Cenchrus ciliaris*, *in vitro* antibacterial activity of methanolic extracts, and comparative estimation of antioxidant properties (*in vitro* and *in vivo*) of *C. ciliaris* (Poaceae). **Materials and Methods**: Antibacterial activity was evaluated against seven Gram-negative bacteria, two Gram-positive bacteria, and three fungi, using disk diffusion method followed by determination of minimum inhibitory concentrations by broth dilution method, against sensitive bacteria and fungi. Bioactive components determined using thin-layer chromatography, infrared spectra, and gas chromatography–mass spectrometry analysis, and antioxidant properties estimated in terms of peroxidase, catalases, polyphenol oxidase, superoxide dismutase, lipid peroxidation activity and carotenoids, and total phenolics contents. **Results and Discussion**: The most bioactive compound in *C. ciliaris* was stigmasterol (3.02%), Rf value (0.83). Most of the extracts at higher concentrations showed varying degrees of inhibitory activity against all bacteria. The highest activity was exhibited by the leaf extract against *Staphylococcus aureus*. *Escherichia coli*, *Agrobacterium tumefaciens,* and *Candida albicans* were the most sensitive pathogens which show maximum antimicrobial effects. Gentamycin and Ketoconazole, the standard antibiotics used were effective against the bacteria and fungi, respectively. Highest antioxidant properties of *C. ciliaris* were found to greater in *in vivo* than *in vitro*. **Conclusion**: *C. ciliaris* contains various bioactive compounds. Hence, it is recommended as a plant of phytopharmaceutical importance.

Key words: Antibacterial activity, *Cenchrus* grass, minimum inhibitory concentration, zone of inhibition

INTRODUCTION

The chemical analysis of methanolic extract of *Cenchrus ciliaris* showed a mixture of long-chain hydrocarbons, carboxyl esters, alcohols, acids, alkaloids, steroids, amino and nitro compound, etc. Stigmasta-5, 22-dien-3-ol (stigmasterol) was isolated and identified from the extract. 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 methanolic extract of *C. ciliaris* was analyzed for the 1st time using gas chromatography–mass spectrometry (GC–MS). This work will help to identify the compounds 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.[1]

Oxidative damages are caused by reactive oxygen species (ROS), and excess amounts of ROS are harmful to many cellular components including membrane lipids. ROS cause peroxidation of polyunsaturated fatty acids in the membranes.[2] However, ROS are inevitable byproducts from the essential aerobic metabolisms, and they need to be maintained under

Address for correspondence:
Dr. Premlata Singariya, P.No.-55, Shri Kalyan Nagar, Kartarpura, Jaipur – 302015, Rajasthan, India.
E-mail: premlatasingariya@gmail.com

Received: 15-12-2016
Revised: 20-12-2017
Accepted: 12-03-2018

sublethal levels for normal plant growth. Hence, plants are equipped with an array of enzymatic and non-enzymatic antioxidant molecules to alleviate cellular damage caused by ROS.[3] Multiple antioxidant enzyme systems are involved in the enzymatic scavenging of ROS. Superoxide dismutases (SODs) react with the superoxide radical to produce H_2O_2. Hydrogen peroxide is scavenged by catalases (CATs) and peroxidases (POXs).

C. ciliaris (C_4 grass) is gaining attention in various field of research, as they are best suited to the present environmental conditions. C_4 grasses are more competitive under the conditions of high temperature, solar radiation, and low moisture.[4] This grass has excellent soil-binding capacity which helps to conserve soil in desert areas[5] and is more efficient at gathering carbon dioxide and utilizing nitrogen from the atmosphere and recycled N in the soil.[6,7] However, *Cenchrus* is most suitable and highly nutritive grasses for desert environmental conditions.

EXPERIMENTAL DESIGN

Identification of Bioactive Components

Identification of components by GC–MS analysis

Interpretation on mass spectrum of GC–MS was done using the database of the 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.[8]

GC–MS analysis

GC–MS analysis of these extracts was carried out by following the method of Hema *et al*.[9] The GC–MS analysis of the extracts was performed using a GC–MS (Model; QP 2010 series, Shimadzu, Tokyo, Japan) equipped with a VF-5 ms fused silica capillary column of 60 m length, 0.25 mm dia., and 0.25 mm film thickness. Injection Mode: Split, flow control mode: Linear velocity, pressure: 173.3 kPa, linear velocity: 28.9 cm/s, purge flow: 3.0 mL/min, and split ratio: 10.0. For GC–MS detection [GC-2010], an electron ionization system with ionization energy of 70 eV was used. Helium gas (99.99%) was used as a carrier gas at a constant flow rate - total flow: 16.3 mL/min and column flow: 1.21 mL/min. Injector and mass transfer line temperature were set at 200 and 240°C, respectively. The oven temperature was programmed (column oven temp.: 100°C and injection temp.: 270°C) from 70°C to 220°C at 10°C/min, held isothermal for 1 min and finally raised to 300°C AT 10°C/min. 2 ml of respective diluted samples was manually injected in the splitless mode, with split ratio of 1:40 and with mass scan of 50–600 amu. Total running time of GC–MS is 48 min.[10] The relative % amount of each component was calculated by comparing its average peak area to the total areas; software adopted to handle mass spectra and chromatograms was a Turbo Mass. The relative percentage of the each extract constituents was expressed as percentage with peak area normalization.[11]

Separation of active compound from C. ciliaris extracts suspension by preparative thin-layer chromatography (PTLC)

The glass plates (20 cm × 20 cm) coated with silica gel "G" (0.4–0.5 mm thick and 45 g/80 ml distilled water) were dried at room temperature. The dried plates were activated at 100°C for 30 min in an oven and cooled at room temperature. Methanolic extracts were separately applied 1 cm above the edge of the plates along with standard reference compound (stigmasterol).[12]

Development of chromatogram

The development tank was saturated with suitable solvent system hexane, acetone (8:2), benzene, ethyl acetate (85:15 or 3:1), chloroform, methanol, and water (10:10:3) for the analysis of lipid present in plant extract, but hexane, acetone (8:2) gave better separation.[13]

Spot visualization and identification by TLC and infrared (IR) spectra

Few pieces of iodine crystals were kept in the tank and covered with glass plate to saturate the tank with iodine vapor. The plate was then kept in iodine vapor saturated tank and left for few hours.[14] Melting point and IR spectra of each of the isolated compound were taken and a comparison of TLC color reaction was made, which was found to be in accordance with those reported for authentic compounds.

For Antioxidant Activity

Field studies

For field 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 every day. After 2 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.

Laboratory studies

For laboratory studies, seeds of *C. ciliaris* were first sterilized by treating them with 0.01% mercuric chloride ($HgCl_2$) solution for 5 min, these sterilized seeds were then germinated in 9 cm Petri plates lined with double layer of filter paper in distilled water. These Petri plates were kept in bio-oxygen demand incubator at 28°C ± 2°C. A cool fluorescent light of 34.1 µmol/m^2/s PAR was given to the seeds. A 5-day-old seedlings were analyzed for different parameter.[7]

Preparation of enzyme extract

All operations for preparing the enzyme extracts were performed at 4°C. Plant materials (laboratory seedling and field condition 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.[7]

Estimation of antioxidant

The level of lipid peroxidation (LPO) was measured in terms of malondialdehyde content a product of LPO described by Heath and Packer.[15] The activity of CAT (CAT, EC.1.11.1.6), POX (POX, EC.1.11.1.7), and polyphenol oxidase (PPO) (PPO, EC.1.10.3.1) was assayed after the method of Chance and Maehly[16] with the some modification. SOD (SOD, EC.1.15.1.1) activity was estimated by the method of Beauchamp and Fridovich.[17] The total phenolics in extracts determined according to Folin–Ciocalteu procedure[18] Arnon's method[19] was used for calculation of carotenoids content.

RESULTS AND DISCUSSION

Identification of Bioactive Components

TLC plate of *C. ciliaris* extracts was visualized under ultraviolet lamp one of the spots gave characteristic fluorescence and their Rf values were comparable to their respective standard compound (stigmasterol – grayish-violet, Rf value - 0.83) [Figure 1]. The characteristic color was also developed when TLC plates were sprayed with anisaldehyde reagent (stigmasterol – grayish-violet) corresponding to their authentic standard compound. Melting point (stigmasterol 131–132°C) was also measured and compared with authentic standard compound.[12]

GC–MS Analysis

The GC–MS studied showed that the retention time and peaks of the isolated stigmasterol [Figure 2] were comparable with that of standard. The active principles with their retention time (34.342) and concentration (4% area) in the standard and sample are presented. In the present study, stigmasterol has been confirmed in *C. setigerus* of family Poaceae.[1,12,20,21]

Antioxidant Activity

In the present study, comparative estimation of antioxidant properties (laboratory and field conditions) of *C. ciliaris* in terms of POX, CAT, PPO, SOD, LPO activity and carotenoids, and total phenolics contents was done. POX, CAT, SOD activity and carotenoids, and total phenolics contents were found to greater in field, then laboratory conditions estimation, but LPO and PPO were show less activity in field estimation [Table 1 and Graph 1].

DISCUSSION

Stigmasterol is used as the precursor of Vitamin D_3[22] and in the manufacture of synthetic progesterone, a valuable

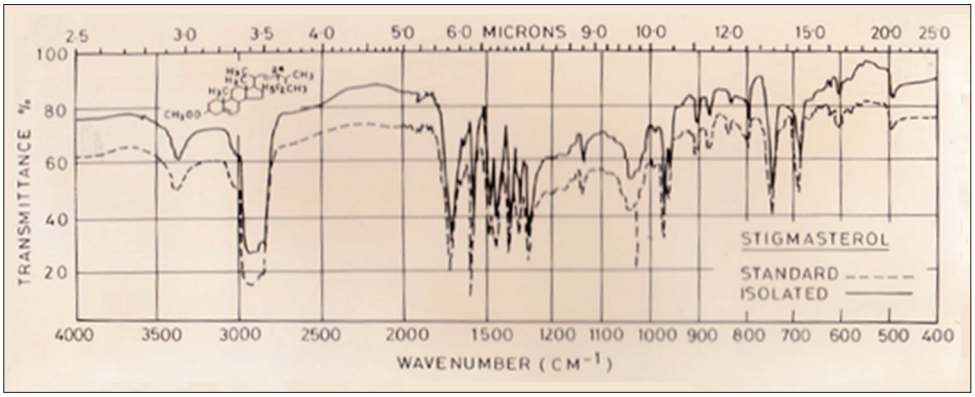


Figure 1: Superimposed infrared spectra of stigmasterol (standard and isolated) compound in *Cenchrus ciliaris*

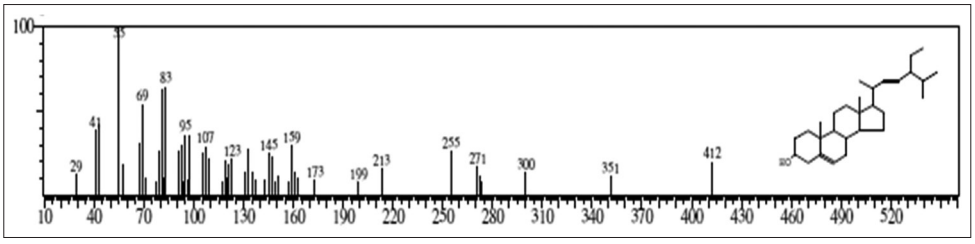
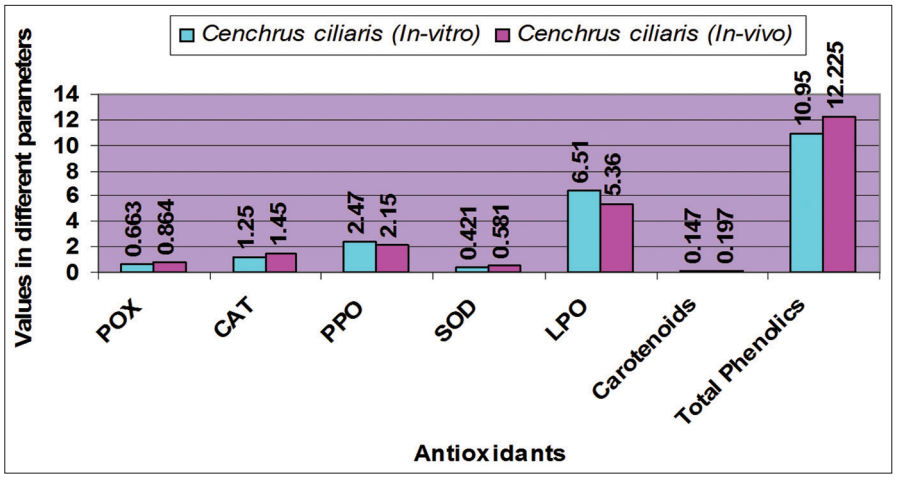


Figure 2: Mass spectrum of stigmasterol (RT: 34.342)



Graph 1: Comparative study of antioxidants of *Cenchrus ciliaris (in vitro* [laboratory conditions] and [field conditions] *in vivo*).

Table 1: Antioxidants of *C. ciliaris (in vitro*, laboratory conditions and field conditions, *in vivo*)

Antioxidants	Parameter	*C. ciliaris* (laboratory conditions)	*C. ciliaris* (field conditions)
POX	µmol H_2O_2/min/g F. wt.	0.663±0.043	0.864±0.047
CAT	Units/min/g F. wt.	1.25±0.047	1.45±0.041
PPO	min/g/F. wt.	2.47±0.035	2.15±0.035
SOD	µmol nitro blue toluene/s/g D. wt.	0.421±0.027	0.581±0.042
LPO	µmol MDA/g F. wt.	6.51±0.044	5.36±0.023
Carotenoids	mg/g F. wt.	0.147±0.042	0.197±0.008
Total phenolics	mg/g F. wt.	10.95±0.045	12.225±0.104

C. ciliaris: Cenchrus ciliaris, POX: Peroxidase, CAT: Catalase, PPO: Polyphenol oxidase, SOD: Superoxide dismutase, LPO: Lipid peroxidation, MDA: Malondialdehyde

human hormone that plays an important physiological role in the regulatory and tissue building mechanisms related to estrogen effects, as well as acting as an intermediate in the biosynthesis of androgens, estrogens, and corticoids.[23] Research has indicated that stigmasterol may be useful in prevention of certain cancers including ovarian, prostate, breast, and colon cancers. Studies with laboratory animals fed with stigmasterol suggest a decrease of 23% in the cholesterol absorption over a 6-week period.[20] It inhibits several proinflammatory and matrix degradation mediators typically involved in osteoarthritis-induced cartilage degradation.[24] It also exhibits potent antioxidant, hypoglycemic, and thyroid-inhibiting properties.[25]

As a part of the enzymatic component of antioxidant system of plant response, antioxidant enzymes contribute significantly to ROS detoxification. The coordinate function of antioxidant enzymes such as SOD, POX, CAT, and GR helps in processing of ROS and regeneration of redox ascorbate and glutathione metabolites.[26] Abiotic stress (water, salinity, heat, and heavy metal) induces generation of active oxygen species[27] causing LPO and consequently membrane injury, protein degradation, enzymes inactivation, pigment bleaching, and disruption of DNA strands.[28,29]

REFERENCES

1. Singariya P, Kumar P, Mourya KK. Isolation of new steroids and evaluation of bio-activity of kala dhaman grass (*Cenchrus setigerus*). Braz Arc of Bio Tech 2014;57:62-9.
2. Smirnoff N. Antioxidant systems and plant response to the environment. In Environment and Plant Metabolism: Flexibility and Acclimation. Oxford: Bios Scientific; 1995. p. 217-43.
3. Foyer CH, Noctor G. Oxygen processing in photosynthesis: Regulation and signalling. New Phytol 2000;146:359-88.
4. Agrawal P. Eco-physiological and Biochemical Studies Related to Drought Adaptation in Grasses of Indian Desert. Ph.D. Thesis. Jodhpur (Rajasthan), INDIA: ;2007.
5. Sinha RK, Bhatia S, Vishnoi R. Desertification Control and Rangeland Management in the Thar Desert of India. In: Rala Report No. 200; 1996. p. 115-23.
6. Bessman SP. In: Mc Elry D, Glass, B, editors. Ammonia Metabolism in Animals: Symposium on Inorganic Nitrogen Metabolism. Baltimore, Marylan: The Johns Hopkins Press; 1956.
7. Singariya P. Effect of sub-Optimal Environment and PGR's

on Metabolic Pattern of Certain Species of *Cenchrus*. Ph.D Thesis. Jodhpur (Rajasthan) INDIA: ;2009.
8. Singariya P, Mourya KK, Kumar P. Identification of some bio-active compounds of ethyl acetate extract of *Cenchrus ciliaris* by gas chromatography-mass spectrometric analysis. Life Sci Bull 2015c;12:141-8.
9. Hema R, Kumaravel S, Gomathi S, Sivasubramaniam C. Gas chromatography-mass spectroscopic analysis of *Lawsonia inermis* leaves. New York Sci J 2010;3:141-3.
10. Singariya P, Mourya KK, Kumar P. Identification of bioactive components of Isopropyl alcohol extract of Bird wood grass using gas chromatography- mass spectrometry. In: PCBiotechnology in Crop Improvement. Ch. 10. Jaipur: Pointer Publishers; 2016c. p. 118-30.
11. Singariya P, Mourya KK, Kumar P. Gas chromatography-mass spectrometric analysis of acetone extract of *Cenchrus ciliaris* (dhaman grass). Int J Sci Nat 2015b;6:652-61.
12. Singariya P, Kumar P, Mourya KK. Identification of steroid compound using preparative thin layer chromatography, GC-MS and anti-microbial and antioxidant properties of *Cenchrus setigerus* (poaceae). Int J Pharm Life Sci 2012q;3:1909-16.
13. Kaul B, Staba EJ. *Dioscorea* tissue cultures. I. Biosynthesis and isolation of diosgenin from *Dioscorea deltoidea* callus and suspension cells. Lioydia 1968;31:171-9.
14. Meena MC, Patni P. Isolation and identification of flavonoid "Quercetin" from *Citrullus colocynthis* (Linn.) Schrad. Asian J Exp Sci 2008;22:137-42.
15. Heath RL, Packer L. Photo peroxidation in isolated chloroplast. Arch Biochem Biophys 1968;125:189-98.
16. Chance B, Maehly AC. Assay of catalases and peroxidases. Methods Enzymol 1955;2:764-75.
17. Beauchamp C, Fridovich I. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. Anal Biochem 1971;44:276-87.
18. Singleton S, Rossi T. In:, editors.Methodology of Plant Metabolites and Enzymes. Vol. II. London: Academic Press; 1995. p. 245-65.
19. Arnon DI. Copper enzymes in isolated chloroplast: Polyphenol oxidase in *Beta vulgaris*. Plant Physiol 1949;24:1-5.
20. Singariya P, Mourya KK, Kumar P. Gas chromatography-mass spectrometric analyses of acetone extract of Marwar dhaman grass for bio-active compounds. Plant Arch 2015a;15:1065-74.
21. Singariya P, Mourya KK, Kumar P. Identification of some bio-active compounds of iso-propyl alcohol extract of motha dhaman grass by gas chromatography-mass spectrometric analysis. Life Sci Leaf 2016a;72:122-35.
22. Kametani T, Furuyama H. Synthesis of vitamin D3 and related compounds. Med Res Rev 1987;7:147-71.
23. Sundararaman P, Dje-rassi C. A convenient synthesis of progesterone from Stigmasterol. J Org Chem 1977;42:3633-4.
24. Gabay O, Sanchez C, Salvat C, Chevy F, Breton M, Nourissat G, *et al*. Stigmasterol: A phytosterol with potential antiosteoarthritic properties. Am J Clin Nutr 2010;18:106-16.
25. Panda S, Jafri M, Kar A, Meheta BK. Thyroid inhibitory, anti-peroxidative and hypoglycemic effects of stigmasterol isolated from *Butea monosperma*. Fitoterapia 2009;80:123-6.
26. Almeselmani M, Deshmukh RK, Sairam RK, Kushwaha SR, Singh TP. Protective role of antioxidant enzymes under high temperature stress. Plant Sci 2006;171:382-8.
27. Gamble PE, Burke JJ. Effect of water stress on the chloroplast antioxidant system. In: Alteration in glutathione reductase activity. Plant Physiol 1984;76:615-21.
28. Davis K J. Protein damage and degradation by oxygen redicals. In general aspects. J Biol Chem 1987;262:9895-901.
29. Arora A, Sairam RK, Srivastava GC. Oxidative stress and antioxidative system in plants. Curr Sci 2002;82:1227-38.

Source of Support: Nil. **Conflict of Interest:** None declared.