Research Article
CODEN (USA): IJPLCP

[Singariya et al., 3(8): Aug., 2012]
ISSN: 0976-7126

INTERNATIONAL JOURNAL OF PHARMACY & LIFE SCIENCES

Identification of steroid compound using preparative Thin Layer Chromatography, GC-MS and antimicrobial and antioxidant properties of *Cenchrus setigerus* (Poaceae)

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Abstract

The present investigation was carried out to determine the possible bioactive components of *Cenchrus setigerus* using PTLC, IR spectra and GC-MS analysis, *in vitro* antibacterial activity of methanolic extracts and comparative estimation of antioxidant properties (*in-vitro* and *in-vivo*) in terms of POX, CAT, PPO, SOD, LPO activity and carotenoids, total phenolics contents of *C. setigerus* (Poaceae) were done. 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 (MIC) by broth dilution method, against sensitive bacteria and fungi. Result reveals that the most bioactive compound in *C. setigerus* was stigmasterol. The highest activity was exhibited by the root extract against *Bacillus subtilis*. *Klebsiella pnemoniae*, *Proteus merabilis* and *Aspergillus flavus* were the most sensitive pathogens witch show maximum antimicrobial effects. Highest antioxidant properties of *C. setigerus* were found to greater in *in-vitro* then *in-vivo.*

Key-Words: Antibacterial activity, stigmasterol, Minimum Inhibitory Concentration (MIC), GC-MS, PTLC, *Cenchrus* grass.

Introduction

The chemical analysis of methanolic extract of *Cenchrus setigerus* 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. setigerus* was analyzed for the first time using 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.

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Reactive oxygen species (ROS) are inevitable byproducts from the essential aerobic metabolisms (caused oxidative damages). It causes peroxidation of polyunsaturated fatty acids in the membranes (Smirnoff, 1995) and they need to be maintained under sub-lethal 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 (Apel and Hirt, 2004). Multiple antioxidant enzymes systems are involved in the enzymatic scavenging of ROS. Superoxide dismutases (SOD) react with the superoxide radical to produce H_2O_2. Hydrogen peroxide is scavenged by catalases (CAT) and peroxidases (POX).

In the present scenario development of antimicrobial resistance to anti microbial agents has lead to treatment failure and the shift of medical care from orthodox to herbal medicine. Various plant extracts can serve both as potential antimicrobial crude drugs as well as a source of new anti-infective agents (Rios, 2005).

Cenchrus setigerus (C_4 grass) is gaining attention in various field of research, as they are best suited to the present environmental conditions. This grass is more

efficient at gathering Carbon dioxide and utilizing nitrogen from the atmosphere and recycled N in the soil (Bessman, 1956; Singariya, 2009). It has excellent soil binding capacity which helps to conserve soil in desert areas (Sinha *et al*., 1996) and more competitive under the conditions of high temperature, solar radiation and low moisture (Agrawal, 2007).

Material and Methods

Experimental design: [1] Identification of Bioactive Components:

(A) 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.

(B) Separation of active compound from *Cenchrus setigerus* extracts suspension by preparative thin layer chromatography (PTLC): The glass plates (20 x 20 cm) coated with silica gel 'G' (0.4-0.5 mm thick and 45 gm/80 ml distilled water) were dried at room temperature (Meena and Patni, 2008). The dried plates were activated at 100°C for 30 minutes 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 (Stegmasterol).

(C) 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 (Kaul and Staba, 1968).

(D) Spot visualization and identification by TLC and 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. Melting point and IR spectra of each of the isolated compound was taken and a comparison of TLC color reaction was made, which was found to be in accordance with those reported for authentic compounds.

[2] For Antimicrobial Activity: (i) Plant material: *C. setigerus* were collected in the month of August from the CAZRI, Jodhpur (Rajasthan). The collected plant materials were transferred immediately to the laboratory cleaned with water and selected plant parts were separately shade dried until weight has been constant. Shade dried parts were powdered with the help of grinder (Singariya et al., 2012 l).

(ii) Preparation of extracts: Plant samples (root, stem, leaf and seeds) were extracted by the hot extraction technique (Harborne, 1984) by using the soxhlet apparatus for 18 hours at a temperature not exceeding the boiling point of methyl alcohol (65^{o}C) following the method of Subramanian and Nagarjan, (1969). The obtained extracts were filtered by using Whatman No. 1 filter paper and then concentrated at 40^{0}C by using an evaporator. The residual extracts were stored in refrigerator at 4^{0}C in small and sterile glass bottles for antimicrobial activity and the crude extract was dissolved in chloroform before chromatographic examination (Rishi *et al*., 2011).

(iii) Micro-organisms: The organisms used in this study were seven Gram-negative bacteria, two Gram-positive bacteria and three fungi (table 1). Selected microorganisms were procured from IMTECH, Chandigarh, India.

(iv) 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.*, 2012h). AI for each extract was calculated.

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

(v) Serial dilution method: MIC was determined as the least extract concentration which inhibited the growth of the test organisms (Jain and Sharma, 2009; Singariya, *et al.*, 2012g). Bacterial and fungal suspensions were used as negative control, while broth containing standard drug was used as positive control.

(vi)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).

(vii) 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.*, 2012f).

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

[3] For Antioxidant activity: (a) *In vivo* studies: For *in vivo* studies seeds of *C. 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 (Singariya *et al*., 2009).

(b) *In vitro* studies: For *in vitro* studies seeds of *C. setigerus* were first sterilized by treating them with 0.01% $HgCl_2$ solution for five minutes, these sterilized seeds were then germinated in 9cm Petri plates lined with double layer of filter paper in distilled water. These Petri plates were kept in BOD incubator at 28°C±2°C. A cool fluorescent light of 34.1µmol/m^2/s PAR was given to the seeds. Five-day-old seedlings were analyzed for different parameter (Chouhan *et al*., 2007).

(c) Preparation of enzyme extract: All operations for preparing the enzyme extracts were performed at 4°C. Plant material (*in vitro*-seedling, *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.

(d) Estimation of Antioxidant: The level of lipid peroxidation (LPO) was measured in terms of malondialdehyde (MDA) content a product of lipid peroxidation described by Heath and Packer (1968). The activity of catalase (CAT, EC.1.11.1.6), peroxidase (POX, EC.1.11.1.7) and polyphenol oxidase (PPO, EC.1.10.3.1) was assayed after the method of Chance and Maehly (1955) with the some modification. Superoxide dismutase (SOD, EC.1.15.1.1) activity was estimated by the method of Beauchamp and Fridovich (1971). The total phenolics in extracts determined according to Folin-Ciocalteu procedure (Singleton and Rossi, 1995). Arnon's (1949) method was used for calculation of carotenoids content.

Results and Discussion

[1] Identification of Bioactive Components: TLC plate of *Cenchrus setigerus* extracts were visualized under UV lamp one of the spot gave characteristic fluorescence and their Rf values were comparable to their respective standard compound (Stigmasterol-grayish violet, Rf value- 0.83) (Fig.-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 131-132ºC) was also measured and compared with authentic standard compound.

GC- MS analysis: GC – MS was done using the database of National Institute of standard and Technology (NIST) (Fig.-2). The GC-MS studied showed that the retention time and peaks of the isolated Stigmasterol was 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. However, till date there was no report on the presence of sterols from *C. setigerus.* In the present study Stigmasterol has been confirmed in *C. setigerus* of family Poaceae.

[2] Antimicrobial Assay: Antimicrobial assay (assessed in terms of ZOI, AI, MIC, MBC/MFC and TA) (table 2, 3, 4) of the Methanolic extracts of *C. setigerus* against selected microorganisms were recorded. The antibacterial activity of the test samples was determined by measuring the diameter of zone of inhibition expressed in mm. The assay was repeated twice and mean of the three experiments was recorded.

(A) Root: Root extract show highest activity ZOI-12.50±0.64 mm, AI-0.625 followed by ZOI-12.33±0.26 mm, AI-0.617 against *B. subtilis* and *K. pnemoniae* respectively. Lowest MIC valve 0.469 mg/ml and highest total activity value 70.701 were recorded against *Agerobacterium tumefaciens.*

(B) Stem: Stem extract show highest activity ZOI-12.33±0.24 mm, AI-1.028 followed by ZOI-11.67±0.24 mm, AI-0.584 against *P. merabilis* and *K. pnemoniae* respectively. Lowest MIC valve 0.938 mg/ml and highest total activity value 40.533 were recorded against *A. tumefaciens.*

(C) Leaf: Leaf extract show highest activity ZOI-9.50±0.64 mm, AI-0.792 followed by ZOI-9.33±0.25 mm, AI-1.166 against *P. merabilis* and *Aspergillus flavus* respectively. Lowest MIC valves and highest total activity values were recorded against *Escherichia coli*, *B. subtilis*, *K. pnemoniae* and *A. flavus.*

(D) Seed: Seed extract show highest activity ZOI-10.67±0.22 mm, AI-0.889 followed by ZOI-9.67±0.21 mm, AI-0.484 against *P. merabilis* and *K. pnemoniae* respectively. Lowest MIC valve 1.875 mg/ml and highest total activity value 16.107 were recorded against *A. tumefaciens.*

Phyto-chemical estimation: The phyto–chemical estimation for the *C. setigerus* was carried out according to Farnsworth (1966) wherein the consistency was found to be sticky in all the extracts.

The yield of the extracts was also analyzed where in the highest yields were recorded for leaf extract of *C. setigerus* (4.06%) (table 5)

Most susceptible organisms in the investigation were *P. merabilis* and *K. pnemoniae* against which, all the plant extracts showed zone of inhibition. But, according the zone of inhibition *B. subtilis* was the most susceptible organism, which supported the finding that plant extracts are usually more active against Gram positive bacteria than Gram negative (Singariya, *et al.,* 2011a, b, c, d, 2012a; Lin *et al.,* 1999; Palombo and Semple, 2001). The Gram-negative bacterial cell wall outer membrane appears to act as a barrier to many substances including synthetic and natural antibiotics (Tortora *et al.,* 2001). *A. tumefaciens* was the most sensitive bacteria according to lowest MIC valve and highest total activity value (Singariya, *et al.,* 2012b, c). Previous studies have noted alcohols to be reliable and consistent solvents for the extraction of antimicrobial substances from medicinal plants (Ahmad *et al.*, 1998).

[3] Antioxidant activity: In the present study, comparative estimation of antioxidant properties (*In-vitro* and *in-vivo*) of *C. setigerus* in terms of POX, CAT, PPO, SOD, LPO activity and carotenoids, total phenolics contents were done. POX, CAT, SOD, LPO and PPO activity were found to greater in *in-vitro* then *in-vivo* estimation (table 6) but, vise versa in case of carotenoids and total phenolics contents (graph 1). 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 (Almeselmani et al., 2006). Abiotic stress (water, salinity, Heat, Heavy metal) induces generation of active oxygen species (Cadenas, 1989) causing lipid peroxidation and consequently membrane injury, protein degradation, enzymes inactivation, pigment bleaching and disruption of DNA strands (Deshmukh and Dhumal, 2005).

Acknowledgement

Authors are expressing their thanks to UGC for providing the funds for this project under Dr. D. S. Kothari, Post doctoral fellowship scheme.

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Table 1: Name of the tested pathogens

S/No.	Pathogens	Name of Pathogens	G+ve/ G-ve	Specimen no.
1.	Bacteria	*Escherichia coli*	G-ve	MTCC-46
2.		*Staphylococcus aureus*	G+ve	MTCC-3160
3.		*Raoultella planticola*	G-ve	MTCC-530
4.		*Pseudomonas aeruginosa*	G-ve	MTCC-1934
5.		*Bacillus subtilis*	G+ve	MTCC-121
6.		*Enterobactor aerogens*	G-ve	MTCC-111
7.		*Proteus merabilis*	G-ve	MTCC-530
8.		*Klebsiella pnemoniae*	G-ve	MTCC-3310
9.		*Agerobacterium tumefaciens*	G-ve	MTCC-431
10.	Fungi	*Candida albicans*	-	MTCC-183
11.		*Aspergillus flavus*	-	MTCC-277
12.		*Aspergillus niger*	-	MTCC-282

Table 2: Zone of Inhibition (mm)* and Activity index of Methanolic extract *C. setigerus*

Test Pathogens	Bio-activity of different parts of *Cenchrus setigerus* against pathogens							
	Root		Stem		Leaf		Seed	
	ZOI	AI	ZOI	AI	ZOI	AI	ZOI	AI
E. coli	11.17±0.24	0.559	10.67±0.28	0.534	8.50±0.64	0.425	9.33±0.24	0.467
S. aureus	-	-	-	-	-	-	-	-
R. planticola	-	-	-	-	-	-	-	-
P. aeruginosa	-	-	-	-	-	-	-	-
B. subtilis	12.5±0.64	0.625	-	-	9.33±0.24	0.467	-	-
P. merabilis	12.17±0.29	1.014	12.33±0.24	1.028	9.50±0.64	0.792	10.67±0.22	0.889
E. aerogens	-	-	-	-	-	-	-	-
K. Pnemoniae	12.33±0.26	0.617	11.67±0.24	0.584	8.5±0.64	0.425	9.67±0.21	0.484
A. tumefaciens	11.67±0.28	0.729	10.5±0.64	0.656	8.17±0.24	0.511	9.33±0.22	0.583
C. albicans	-	-	-	-	-	-	-	-
A. flavus	8.17±0.26	1.021	-	-	9.33±0.25	1.166	8.33±0.22	1.041
A. niger	-	-	-	-	-	-	-	-

*All values are mean ± SD, n=3, ZOI-Zone of Inhibition, AI-Activity Index

Table 3: MIC and MBC/MFC by methanolic extract of *C. setigerus* against tested pathogens

Test Pathogens	Bio-activity of different parts of *Cenchrus setigerus* against pathogens							
	Root		Stem		Leaf		Seed	
	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MBC/MFC
E. coli	1.875	3.75	3.75	7.5	3.75	7.5	3.75	3.75
S. aureus	-	-	-	-	-	-	-	-
R. planticola	-	-	-	-	-	-	-	-
P. aeruginosa	-	-	-	-	-	-	-	-
B. subtilis	1.875	3.75	-	-	3.75	7.5	-	-
P. merabilis	3.75	7.5	3.75	3.75	7.5	15	3.75	7.5
E. aerogens	-	-	-	-	-	-	-	-
K. Pnemoniae	1.875	1.875	1.875	3.75	3.75	7.5	3.75	3.75
A. tumefaciens	0.469	0.938	0.938	1.875	7.5	7.5	1.875	1.875
C. albicans	-	-	-	-	-	-	-	-
A. flavus	7.5	15	-	-	3.75	7.5	3.75	7.5
A. niger	-	-	-	-	-	-	-	-

Table 4: Total activity of methanolic extract of different parts of *C. setigerus*

S/No.	Test Pathogens	Total activity of different parts			
		Root	Stem	Leaf	Seed
1.	*E. coli*	19.893	10.133	10.827	8.053
2.	*S. aureus*	-	-	-	-
3.	*R. planticola*	-	-	-	-
4.	*P. aeruginosa*	-	-	-	-
5.	*B. subtilis*	19.893	-	10.827	-
6.	*P. merabilis*	9.947	10.133	5.413	8.053
7.	*E. aerogens*	-	-	-	-
8.	*K. Pnemoniae*	19.893	20.267	10.827	8.053
9.	*A. tumefaciens*	79.701	40.533	5.413	16.107
10.	*C. albicans*	-	-	-	-
11.	*A. flavus*	4.973	-	10.827	8.053
12.	*A. niger*	-	-	-	-

Table 5: Phyto-profile of Methanolic extracts of different parts of *C. setigerus*

S/No.	Parts	% Yield	Color	Consistency
1.	Root	3.73	Brown	Sticky
2.	Stem	3.80	Dark brown	Sticky
3.	Leaf	4.06	Dark green	Sticky
4.	Seed	3.02	Brown	Sticky

Table 6: Antioxidants of *Cenchrus setigerus* (*In-vitro* and *In-vivo*)

S/No.	Antioxidants	Parameter	(*In-vitro*)	(*In-vivo*)
1.	POX	(µ mol H_2O_2/min/g F. wt.)	0.742±0.045	0.542±0.035
2.	CAT	(Units/min/g F. wt.)	1.503±0.016	1.34±0.012
3.	PPO	($min^{-1}g^{-1}$F. wt.)	2.92±0.032	2.27±0.032
4.	SOD	(µ mol nitro blue toluene /sec./g D. wt.)	0.487±0.036	0.261±0.021
5.	LPO	(m mol MDA/g F. wt.)	7.283±0.126	6.83±0.16
6.	Carotenoids	(mg/g F. wt.)	0.063 ± 0.007^{NS}	0.159±0.008
7.	Total Phenolics	(mg/g F. wt.)	11.35±0.135	12.125±0.145

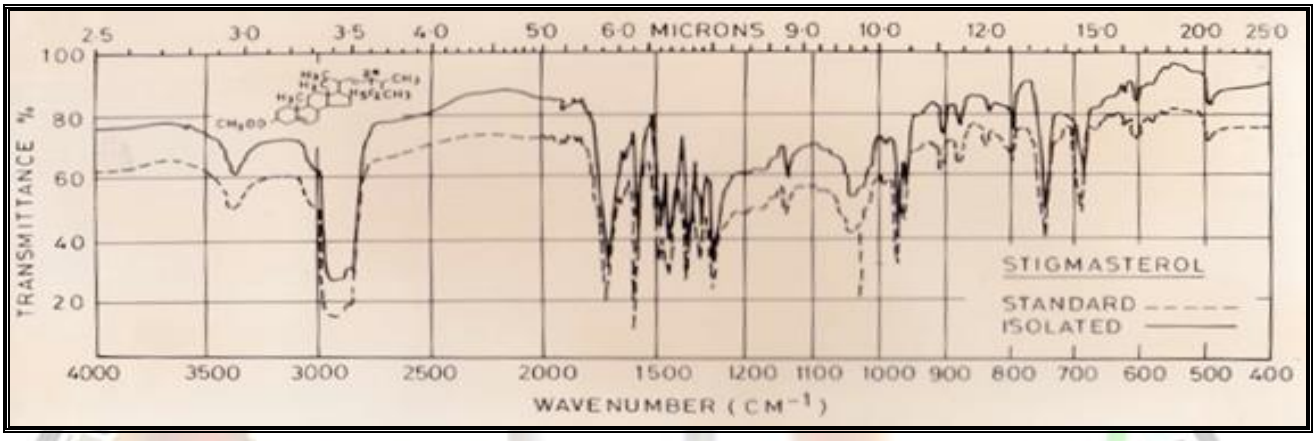


Fig. 1: Superimposed IR spectra of Stigmasterol (standard and isolated) in *C. setigerus*

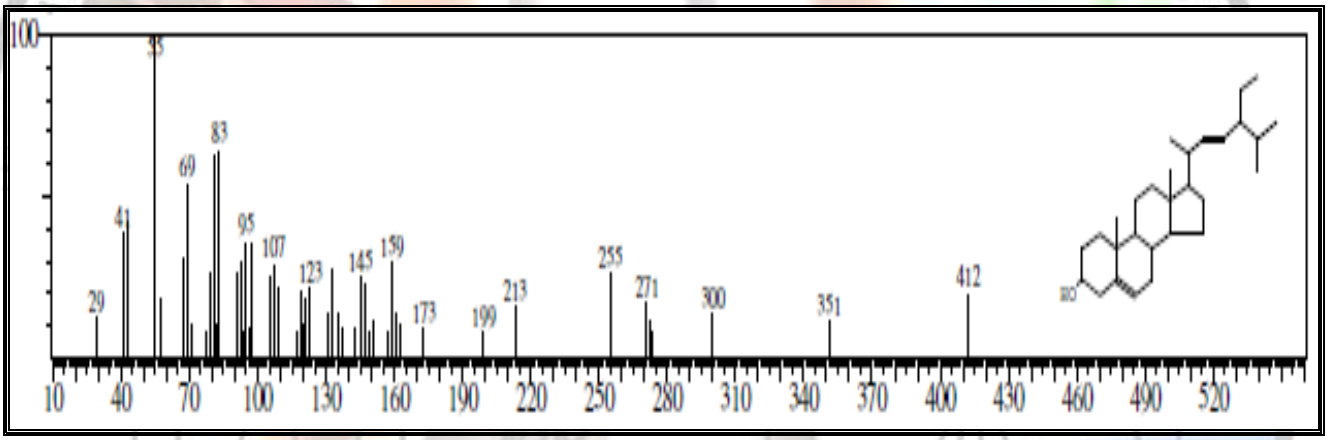
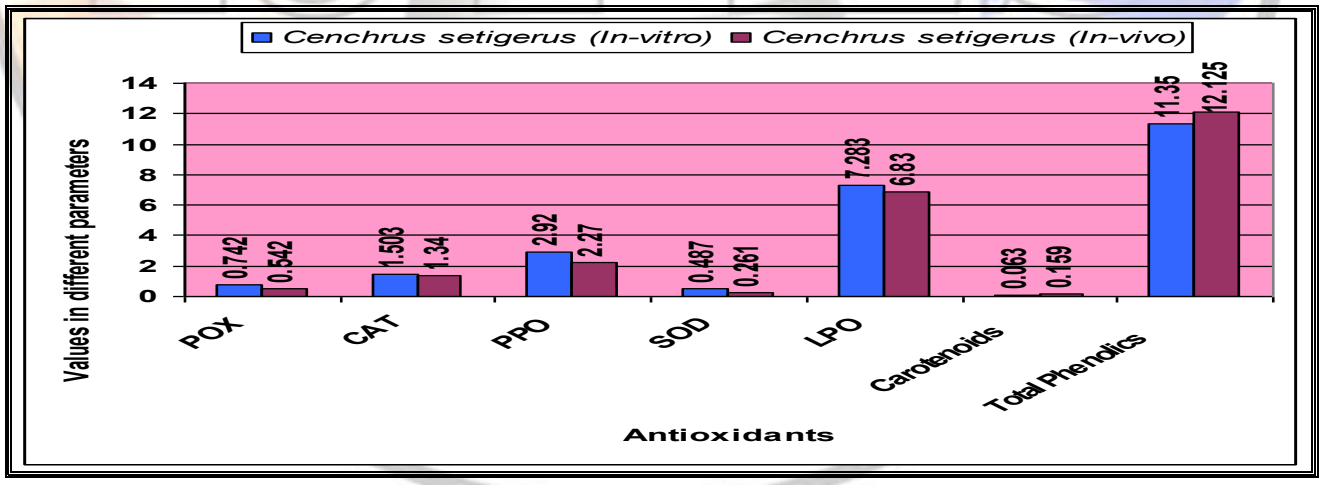


Fig. 2: Mass Spectrum of Stigmasterol (RT: 34.342)



Graph 1: Comparative study of Antioxidants of *Cenchrus setigerus* (*In-vitro* and *In-vivo*)