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GC-MS Profiling and Bioactivity Study of Trans-Himalayan Plant *Centaurea depressa*

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ABSTRACT

The present study was aimed to explore the therapeutic values of Trans-Himalayan plant *Centaurea depressa*. The aerial parts of the plant were extracted using methanol solvent. The methanol extract was found to possess potent biological activities viz. Antidiabetic ($50.64 \pm 0.060\%$ against acarbose), anti-inflammatory ($59.25 \pm 0.065\%$ against indomethacin), antihypertensive ($38.81 \pm 0.045\%$ against captopril), anti-oxidant, while antibacterial activity was observed to its least. Antioxidant assay was done using two methods: 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Total Antioxidant Capacity (TAC). Total Polyphenolic Content (TPC) and Total Flavonoid Content (TFC) were also calculated using gallic acid and rutin trihydrate standards respectively. The hexane extract was analyzed by Gas Chromatography–Mass Spectrometry (GC-MS). The results showed the presence of thirty compounds out of which six were major (Phytol, 1,2-benzenedicarboxylic acid, hexadecanal, stigmast-5-en-3-ol, lupeol, lupeol acetate).

Keywords: *Centaurea depressa*, GC-MS, Antidiabetic, Antihypertensive, Anti-inflammatory

INTRODUCTION

Diabetes Mellitus (DM) is among the leading contributor to the overall disease burden around the globe [1]. India is unfortunately recognized as the capital for the incidence of DM, with 31.7 million reported cases in year 2000 and prediction for such cases by the year 2030 is more than 70 million of urban populations suffering from diabetes [2,3]. It is characterized by hyperglycemia either due to insulin insensitivity and/or compromised insulin secretion. Lowering circulating glucose has been reported as a major therapeutic approach for controlling type-2 diabetes [4]. Commercially available synthetic drugs viz. Acarbose and Miglitol though reduce blood glucose level, but have been associated with severe pharmacological side effects [5]. Moreover, diabetes has been a major risk factor for hypertension [6]. Angiotensin-I Converting Enzyme (ACE-I) which converts angiotensin-I to angiotensin-II, a prominent vasoconstrictor leads to raised blood pressure. Inhibition of ACE is thus considered as a good therapeutic approach in the management of high blood pressure in both diabetes associated and non-associated patients [7,8].

The Trans-Himalayan region is known for their enormous ethno botanical wealth precisely medicinal plants since Vedic periods (5000-1500 BC), the cold desert of Ladakh situated in the northern most part of India, which covers more than 65,000 km² area of trans- Himalayas. The region is characterized by annual precipitation of 20-30 mm rainfall/snowfall with prolonged subzero temperature, the temperature during winters may vary from -30°C to -75°C at different localities of this vast, cold desert has also been reported to rich herbal wealth [9,10]. Plants like *Rhodiola*, *Atrinisia*, *Podophyllum* etc. are extensively studied and it was found that they contain good biologically active compounds which have a profound effect against the diseases like hypertension, stress etc, [11]. The immense therapeutic potential of flora found in Ladakh region is mainly due to the large quantities of precious secondary metabolites present in these plants, which in turn is mainly attributed to tolerate the harsh climatic conditions these plants face in Ladakh.

The genus *Centaurea* L. (Asteraceae) comprised about 500 species distributed predominantly in the Mediterranean region and western Asia. Different species of *Centaurea* has been used as herbal remedies for digestive disorders, fever and in case of diarrhea [12]. Studies conducted on some *Centaurea* species have shown anti-microbial, anti-pyretic, anti-inflammatory and immunomodulatory effects [13]. One of the *Centaurea* sp. namely *Centaurea depressa* locally known as Vashakha is habitat in Ladakh region (Suru & Leh Valley). The whole plant is well known for their medicinal use like fever, headache, and chest pain [14].

However to the best of our knowledge, very less previous studies were conducted to evaluate the therapeutic potential of this species found in Ladakh. This work was aimed to investigate the *in vitro* potential of extracts from *C. depressa* grown wildy in the Trans-Himalayan region at an altitude of 11,500 ft above MSL, over DM associated enzymes (α -Amylase and α -glucosidase) and hypertension associated ACE-I. Also, the quantification of phenolic, flavanoid contents and antioxidant properties of the *C. depressa* extracts were subsequently examined.

MATERIALS AND METHODS

Chemicals

DPPH, ACE-I, 3,5-Dinitrosalicylic acid (DNSA) reagent, 3-5U hyaluronidase, hyaluronic acid, Folin-Ciocalteu phenol reagent were purchased from Sigma-Aldrich. Amylase purchased from HiMedia. The bacterial strains used for antibacterial activity were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh. All other chemicals & solvents used were of analytical grade.

Plant collection

C. depressa was collected from Leh district, Jammu & Kashmir, India (Altitude 11,500 ft above mean sea level) in July 2015. The plant specimen sample was confirmed & Verified by expert ethnobotanist Dr O. P. Chaurasia, DIHAR, DRDO. The aerial parts of the plant were shade dried for 7-10 days and further stored at room temperature for further analysis.

Extract preparation

The shade dried aerial parts of the plant (500 gm) was subjected to methanolic extraction at room temperature in a Soxhlet extractor. The extraction was completed till there was no color coming out of the plant sample in approximately 96 hr. The extract was concentrated using a rotary evaporator (R-215, Buchi-Switzerland); the dried extract was kept at -80°C for 24 h and finally lyophilized using lyophilizer (Labconco 4.5^{Plus} Free Zone) to remove the traces of water remained in the extract. The solvent and water free extract was stored in a refrigerator at -4°C and was used for further experimentation. The plant material was also subjected to hexane extraction by the method same as for methanolic extraction.

Total polyphenolic content

Total phenolic content was estimated using Folin-Ciocalteu reagent method as explained by Stoilova *et al.* [15]. 10 ml of Deionized water, followed by 1 ml of Folin-Ciocalteu phenol reagent was added in 1 ml of extract (1000 µg/ml) and Standard (25-150 µg/ml). Then, 2 ml of 20% sodium carbonate was mixed after incubation for 5 min. The absorbance of the final sample was taken using UV-Visible spectrophotometer (Genesys 10S UV-Vis) at 750 nm. The standards used were Gallic acid and Butylated Hydroxyanisole (BHA) and the results were represented as mg of Gallic acid/ BHA equivalent/g of dry plant extract (DPE). Each experiment was repeated in triplicates.

Total flavonoid content

The total flavonoid content was estimated as given by Zhishen *et al.* [16]. 1 ml of extract (1000 µg/ml) or standard solution was mixed with 4 ml of deionized water followed by the addition of 0.3 ml of 5% sodium nitrite and incubated for 5 min. 0.3 ml of 10% (w/v) aluminium chloride and 2 ml of 1 M sodium hydroxide solution. After that deionized water was added to make the volume 10 ml and absorbance was noted at 510 nm. The standard used was rutin trihydrate and the results were expressed in mg of rutin trihydrate/Quercetin equivalent /gm of DPE as a mean of three observations.

Gas Chromatography–Mass Spectrometry (GC-MS) analysis

The hexane extract of aerial parts of *C. depressa* was subjected to GC-MS analysis. 5 mg of hexane extract was dissolved in 5 ml of the solvent, mixed properly by vigorous shaking and filtered through 0.2 µ syringe filter (Millipore, USA). 1 µl aliquot of the sample solution was injected into the Shimadzu (GC-2010 Plus) GC system coupled with GC-MS-QP 2010 Ultra, Mass spectroscopy. The analysis was performed using the capillary column RTX-5Sil MS (30 m × 0.25 mm × 0.25 mm, Restek USA). The carrier gas used was Helium and flow rate was 1 mL min⁻¹ with the split ratio was 1:5. The temperature of injection port was 280°C. The oven temperature was programmed as: the initial temperature was 100°C (hold time 1 min), then increased at the rate of 4°C min⁻¹ to 250°C (hold time 5 min) and finally increased further at the rate of 5°C min⁻¹ to 280°C (hold time 30 min). Mass spectra were recorded with electron ionization (EI) mode at 70 eV and the spectral range was 40-700 m/z. The interface and ion source temperature were 280 and 200°C, respectively [17]. The identification of various constituents was carried out only by a comparison of their retention time and mass spectral data with that of retention time and mass spectral database of Wiley 8 and NIST11 library. The compounds were quantified on the basis of the area under the peak and results were presented in percentage. Each sample was analyzed in triplicate.

In vitro bioactivity assay

Antidiabetic assay

The Amylase inhibition assay was carried out by using the chromogenic DNSA method. The total assay mixture (2 ml) composed of 0.05 M sodium phosphate buffer (pH 6.9), 50 µl of Amylase and samples with three different concentration i.e., 100, 250 and 500 µg/ml were mixed at 37°C for 15 min. After this, 500 µl of 1% (w/v) starch solution in the above prepared assay mixture buffer was added to each tube and rested at 37°C for 15 min. The reaction was discontinued by adding 1.0 ml DNSA reagent, kept in boiling water bath for 5 min, followed by cooling at the room temperature and the absorbance quantified at 540 nm [18,19]. The control Amylase represented 100% enzyme activity and did not have any sample of the analysis. To get rid of the absorbance showed by *C. depressa*, appropriate extract controls with the extract in the reaction mixture in which the enzyme was added after adding DNS. The released maltose was calculated with the help of the standard maltose curve and activities were deliberated as per the following formula:

$$\text{Activity} = \frac{\text{Conc. of Maltose liberated} \times \text{ml of enzyme used}}{\text{Mol.wt of maltose} \times \text{incubation time (min)}} \times \text{Dilution factor}$$

One unit of enzyme activity is interpreted as the amount of enzyme needed to release one micro mole of maltose per min from starch under the assay conditions. The inhibitory/induction property shown by the *C. depressa* extract was compared with that of control and expressed as percent induction/inhibition. This was estimated according to the formula:

$$\% \text{ Inhibition/Induction} = \frac{\text{Activity in presence of compound}}{\text{Control activity}} \times 100$$

DNS solution: 1 g of DNS dissolved in 2 N NaOH, 30 g of potassium sodium tartarate was added and whole volume was made up to 100 ml.

Testing of acarbose as a standard inhibitor

Acarbose was used as a standard inhibition compound and was tested at above mentioned test sample concentrations. The method was similar to the earlier procedure, rather than plant samples to be tested, acarbose was added. The results were compared to that of *C. depressa*.

In vitro antihypertensive assay

Captopril, a well-known ACE inhibitor was examined in the antihypertensive assay as a standard compound. The methanol extract of *C. depressa* was tested in three different concentrations, 10, 50 and 100 µg/ml, by dissolving 20 µl kidney cortex plasma membranes (ACE enzyme source) and 1 mM Hippuryl-His-Leu as substrate in assay buffer (10 mM HEPES buffer containing 0.3 M NaCl and 10 µM Zinc Sulfate). In short, the compounds were mixed with the enzyme for 10 min at 37°C. After that, the substrate was poured which made a final reaction volume of 50 µl and then again kept at 37°C for a period of 45 min. The reaction was discontinued by the adding 1 M HCl. The yellow colour was observed by the addition of 100 µl of pyridine and 50 µl of benzene sulphonyl chloride. The observed yellow color was measured at 410 nm in an Enzyme-linked Immunosorbent Assay (ELISA) Plate Reader (iMARK, BIORAD). Compounds having an inhibitory potential block the substrate availability to the enzyme and hence cause enzyme inhibition, which results in no formation of yellow color. The inhibition is reported in the form of percentage over control [20,21].

In vitro anti-inflammatory assay

The assay medium was made up of 3-5U hyaluronidase in 100 µl, 20 mM sodium phosphate buffer (pH 7.0) & 77 mM sodium chloride, 0.01% BSA was pre-soaked for the period of 15 min at a temperature of 37°C with different concentrations (10 µg/ml, 50 µg/ml and 100 µg/ml) of the *C. depressa* extract to be tested. The assay was initiated by adding 100 µl hyaluronic acid (0.03% in 300 mM sodium phosphate, pH 5.35) to the soaking mixture and soaked for another 45 min at 37°C. The undigested hyaluronic acid was precipitated with 1 ml acid albumin solution which was made up of 0.1% bovine serum albumin mixed in 24 mM sodium acetate and 79 mM acetic acid, (pH 3.75). After keeping at room temperature for 10 min, the absorbance of the reaction mixture was measured at 600 nm. The absorbance without enzyme was used as a reference value for maximum inhibition. The inhibitory activity of test compound was measured as the ratio in percentage of the absorbance in the presence of compound to be tested, i.e., *C. depressa* vs. absorbance without enzyme. The enzyme activity was acquired by running the control experiment simultaneously, in which the enzyme was pre-soaked with 5 µl DMSO instead, and followed by the above mentioned procedures. The compound was examined with a range of 10-100 µg/ml concentration in the reaction mixture. Indomethacin was used as a reference standard [22].

Antibacterial assay

The anti-bacterial activity was checked by agar diffusion method. For this purpose, two Gram-positive (*Staphylococcus aureus* (MTCC 3160) and *Escherichia coli* (MTCC 723) and two Gram-negative bacteria i.e., *Salmonella typhi* (MTCC 3216) and *Bacillus subtilis* (MTCC 736) were used. The bacterial stock cultures were freshly prepared in broth media (10 g peptone, 10 g NaCl, 5 g yeast extract, and 20 g agar mixed in 1000 ml of distilled water) and grown for a period of 18 h at 37°C. The agar plates of the broth were made and wells were made in the plates. Each plate was treated with 18 h old cultures (100 µl, 10⁴ cfu) and scattered on the plate. After 20 min, the wells were filled with the compound and ciprofloxacin antibiotic at different concentrations. All the plates were incubated at 37°C for 24 h and zone of inhibition was calculated by taking the diameter of inhibition zone [23,24].

Antioxidant activity

DPPH free radical scavenging activity: The free radical scavenging activity of the extract was analyzed by using 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) assay in which DPPH is used to develop the free radical and the sample was analyzed to check its radical scavenging ability. The assay was performed using a methanol extract of aerial parts with the slight modification in the method of Sharma et al. [25]. 3 ml of 0.004% methanol solutions of DPPH was mixed with the 0.2 ml of various extracts and standard at their different concentrations (25-150 µg/ml) followed by an incubation of 30 min. The activity in terms of percentage inhibition (I%) was calculated by the formula:

$$I \% = \left[\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right] \times 100$$

Finally the absorption was read at 517 nm using UV-Visible spectrophotometer. The change in absorbance was measured with respect to control and the IC₅₀ was also calculated. Ascorbic acid was used a standard. The % inhibition values of each sample were represented as a mean of three replicates.

Total Antioxidant Capacity (TAC) assay: The total antioxidant capacity was evaluated according to phosphomolybdenum reducing method used by Sharma et al. [26]. In this assay, 0.3 ml of different extract (1 mg/ml) or standard (25-150 mg/ml) was taken in a test tube. After this, the reagent solution (3 ml; 28 mM sodium phosphate, 4 mM ammonium molybdate and 0.6 M of sulfuric acid) was added to each test tube. All the test tubes were incubated at 95°C for 90 min. Upon cooling to room temperature, the absorbance of the solutions was noted at 695 nm on UV-Visible spectrophotometer. The results were represented in mg of Butylated Hydroxyl Anisole (BHA)/Quercetin equivalent/g of DPE. Again, each experiment was carried out in triplicate.

RESULTS AND DISCUSSION

Total polyphenolic content

Phenolic compounds are known for their medicinal properties like antioxidant, anti-inflammatory, anticarcinogenic, antidiabetic etc., (Huang

WY et al. [27]). Total polyphenolic content was calculated by using the Folin-Ciocalteu reagent method against natural and synthetic standard. The methanol and hexane extract show total polyphenolic content of 2.73 ± 0.05 mg of GAE per g of DPE, 5.85 ± 0.13 mg of BHA/g of DPE and 1.15 ± 0.01 mg of GAE per gm of DPE, 2.22 ± 0.03 mg of BHA/g of DPE, respectively.

Total flavonoid content

Flavonoids are naturally occurring polyphenolic which have been known for their therapeutic potential viz. antioxidant, anti-inflammatory, antidiabetic, anticancer etc. they also help to reduce the risk of major chronic diseases [28]. Total flavonoid content was estimated using two natural standards by the method given by Sharma et al. [17]. The TFC value of methanolic extract was 6.43 ± 0.19 mg of the QE/g of DPE and 0.51 ± 0.01 mg of the RTE/g of DPE, respectively.

GC-MS analysis

The GC/MS analysis of hexane extract of aerial parts of *C. depressa* showed total thirty peaks. The extract revealed the presence of major long chain hydrocarbons and steroids classes of non-polar compounds along with long chain alcohol, fatty acids and their esters. Table 1 shows the list of major constituents (> 1.5%) detected in the analyzed extract while Figure 1 represents the GC chromatogram of hexane extract. The major compounds revealed from GC-MS analysis are given in Figure 2.

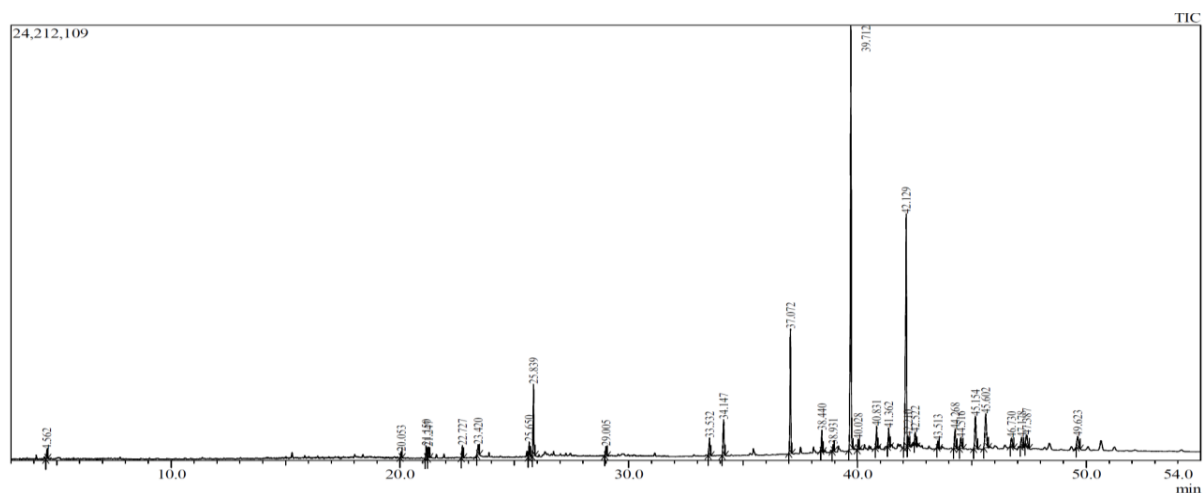


Figure 1: GC chromatogram of n-Hexane extract of *Centaurea depressa*

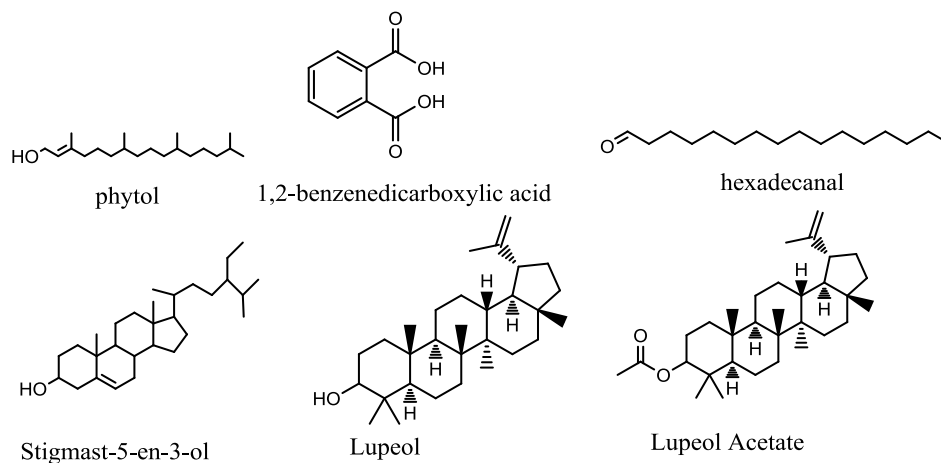


Figure 2: Structure of compounds identified in GC-MS analysis of n-Hexane extract of *Centaurea depressa*

Table 1: List of main constituents detected by GC-MS analysis in bioactive extracts obtained by Soxhlet extraction

S. No.	Name of compound	Retention time	Similarity index	Molecular formula	Molecular weight	Peak area (%)
1	Phytol	25.839	96	C ₂₀ H ₄₀ O	296	5.81
2	Long chain hydrocarbon	33.536	-	-	-	1.5
3	1,2-Benzenedicarboxylic Acid	34.147	95	C ₂₄ H ₃₈ O ₄	390	3.17
4	Long Chain Hydrocarbon	37.072	-	-	-	8.58
5	Long Chain Hydrocarbon	39.712	-	-	-	31.82
6	Long Chain Hydrocarbon	42.129	-	-	-	21
7	Hexadecanal	44.268	90	C ₁₆ H ₃₂ O	240	1.86
8	Long Chain Hydrocarbon	45.154	-	-	-	3.38
9	Stigmast-5-en-3-ol	46.602	87	C ₂₉ H ₅₀ O	414	4.31
10	Lupeol	47.387	84	C ₃₀ H ₅₀ O	426	1.57
11	Lupeol Acetate	49.623	86	C ₃₂ H ₅₂ O ₂	468	1.63

In vitro bioactivity assay*Antidiabetic activity*

The natural flora of trans-Himalayan region is a wide field to search for natural products as remedies for many diseases like diabetes, cancer, headache, stomach infection etc. these natural products are eco-friendly in nature and have no side effects. In view of the above, the methanolic extract of *C. depressa* was examined for antidiabetic activity i.e., α -amylase inhibition assay in three different concentrations 10, 50 and 100 $\mu\text{g/ml}$ against Acarbose standard. In the present examination plant has shown $60.28 \pm 0.065\%$ inhibition for 100 $\mu\text{g/ml}$. The detailed results are shown in Table 2 and Figure 3.

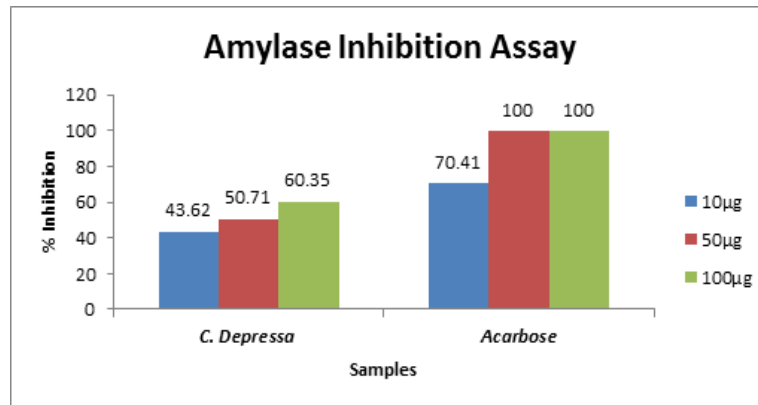


Figure 3: Amylase inhibition activity of *Centaurea depressa* and acarbose

Table 2: % Inhibition of *Centaurea depressa* against standard acarbose

S. No.	Concentration	% Inhibition plant sample (<i>Centaurea depressa</i>)	% Inhibition standard (Acarbose)
1	10 μg	43.55 ± 0.065	70.38 ± 0.036
2	50 μg	50.64 ± 0.060	100
3	100 μg	60.28 ± 0.065	100

Antiinflammatory activity

In the present study, *C. depressa* was examined for another vital activity that is anti-inflammatory analysis also called as hyaluronidase inhibition activity of three different concentrations 10, 50 and 100 $\mu\text{g/ml}$ against indomethacin used as a standard. The plant has shown $59.25 \pm 0.065\%$ inhibition of 100 $\mu\text{g/ml}$ conc. The detail findings of the above test are shown as % inhibition in the Table 3 and Figure 4.

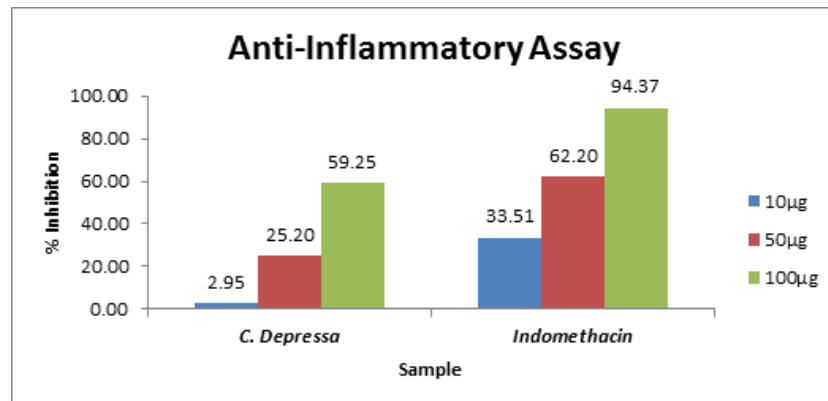


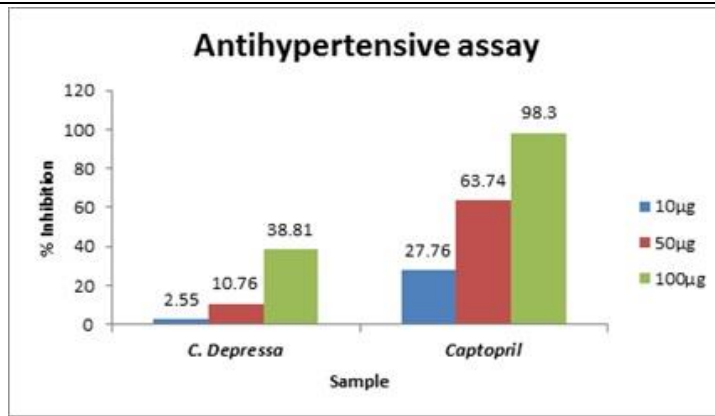
Figure 4: Antiinflammatory activity of *C. depressa* and indomethacin

Table 3: % Inhibition of *C. depressa* against standard indomethacin

S. No	Concentration	% Inhibition plant sample (<i>Centaurea depressa</i>)	% Inhibition standard (Indomethacin)
1	10 μg	2.95 ± 0.060	33.51 ± 0.080
2	50 μg	25.20 ± 0.070	62.20 ± 0.060
3	100 μg	59.25 ± 0.065	94.37 ± 0.040

Antihypertensive activity

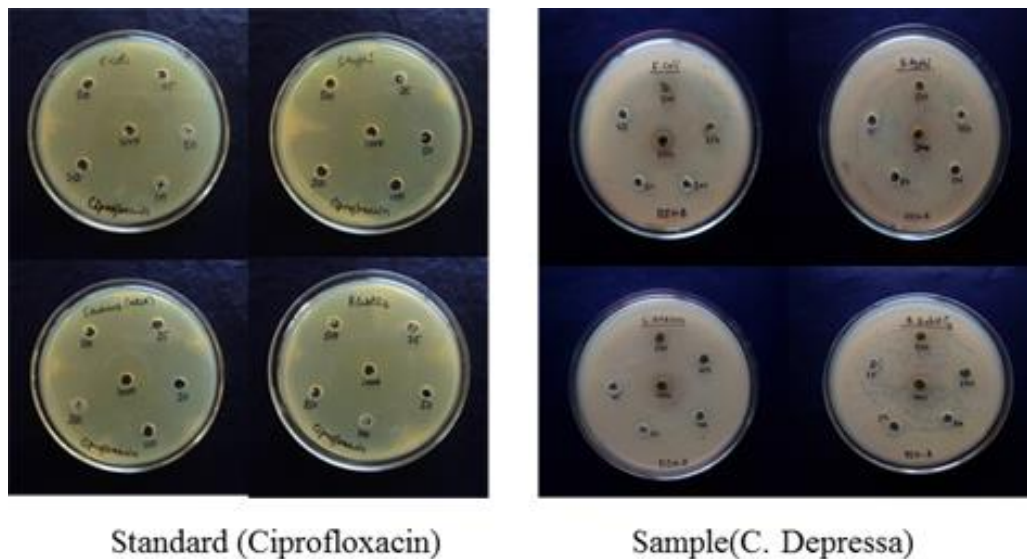
There are many synthetic drugs in market for hypertension, but they have many side effects as dizziness, nausea, stomach problems, impotence, fatigue etc, [29]. In the present study, *C. depressa* was examined for antihypertensive activity. The experiment was carried out in the different concentrations 10 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$ against captopril which was used as a standard. The readings of the test are mentioned as % inhibition in Table 4 and Figure 5.

Figure 5: Antihypertensive activity of *Centaurea depressa* and captoprilTable 4: % Inhibition of *Centaurea depressa* against standard captopril

S. No.	Concentration	% Inhibition Plant Sample (<i>Centaurea depressa</i>)	% Inhibition Standard (Captopril)
1	10 µg	2.55 ± 0.065	27.76 ± 0.062
2	50 µg	10.76 ± 0.052	63.74 ± 0.075
3	100 µg	38.81 ± 0.045	98.30 ± 0.081

Antibacterial activity

In the present study, *C. depressa* was also examined for antibacterial analysis against two Gram-positive bacteria (*S. aureus* (MTCC 3160) and *E. coli* (MTCC 723)) and two Gram-negative bacteria i.e., *S. typhi* (MTCC 3216) and *B. subtilis* (MTCC 736) by agar diffusion method in the under mentioned concentrations of 25, 50, 100, 250, 500 and 1000 µg/ml, ciprofloxacin antibiotic was used as a standard for comparison. The result of the test shows that methanolic extract of *C. depressa* species do not show any or less antibacterial activity in comparison to the ciprofloxacin antibiotic which shows 100% inhibition. Activity comparison is shown in Figure 6.

Figure 6: Antibacterial Activity of *Centaurea depressa* & indomethacin for two Gram-positive and two Gram-negative bacteria

Antioxidant activity assay

DPPH free radical scavenging activity: The present assay shows 28.95 ± 1.10 and $1.96 \pm 1.00\%$ inhibition of DPPH free radical by methanolic and hexane extract, respectively in comparison to $71.75 \pm 1.31\%$ inhibition shown by BHA at 1 mg/ml concentration.

Total antioxidant capacity assay: In this assay, a green color complex was obtained by the reduction of Mo (VI) to Mo (V) which shows the maximum optical density at 695 nm. The TAC results of Methanolic extract was 4.50 ± 0.40 mg/g of QE/g of DPE and 4.91 ± 0.46 mg/g of BHA/g of DPE. On the other hand hexane extract shows TAC value 4.72 ± 0.36 mg/g Quercetin equivalent of extract and 5.16 ± 0.41 mg/g of BHA of extract.

CONCLUSION

To the best of our knowledge, the GC-MS analysis, *in vitro* bioactivity assay i.e., Amylase inhibition assay (Antidiabetic), hyaluronidase inhibition assay (Anti-inflammatory), angiotensin-I inhibition assay (Antihypertensive), antioxidant activity of *C. depressa* species found in the Trans-Himalaya Ladakh region has never been reported earlier, hence our findings can be admitted as the first report about the biological and

chemical properties of mentioned species. In the present study, the GC-MS analysis of hexane extract of *C. depressa* reported to have 30 compounds out of which phytol, 1,2-benzenedicarboxylic acid, Hexadecanal, Stigmast-5-en-3-ol, lupeol and lupeol acetate were found in good concentration. The methanolic extract of *C. depressa* exerted remarkable anti-inflammatory & Amylase inhibition activity, but a moderate level of anti-hypertensive activity; also the TPC and TFC values were considerable. The study suggests that the plant has a potential against diseases like, diabetes and hypertension and can be used in the formulation or alone. Moreover the results of the present study allow us to conclude that there is a positive possibility that the plant have potential bioactive compounds which can be isolated in further studies.

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