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EFFECT OF ABIOTIC STRESSES ON THE MARKER CONTENT AND THE ACTIVITY OF *CENTELLA ASIATICA*

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Keywords:

Abiotic stress, Asiatic acid, *Centella asiatica*, Marker content, Antioxidant activity

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ABSTRACT: *Centella asiatica* (CA; family Apiaceae) is extensively used in traditional medicine. A regular supply of this plant is required by the herbal drug industry. Hence, the cultivation of this plant is required. The emphasis of the available cultivation practices is on biomass yield. The activity of the plant, however, depends on the amount of bioactive constituents. The production of these secondary metabolites is influenced by biotic as well as abiotic factors. This study was carried out to determine the abiotic factors that enhance the production of secondary metabolites in CA. CA plants were grown in two seasons (*i.e.*, season 1: August to December and season 2: February to June) on different soil types (Clay loamy, Red, Black), and various stresses were applied to the plants. At the end of each season, plant yield, extract yield, asiatic acid content, antioxidant, and acetylcholinesterase inhibition activities were determined. Asiatic acid content was determined by an HPLC method. The results show the varied response of CA plants to seasons, soil, and stresses. CA plants grown in season 2, on black soil under salt stress have the highest asiatic acid content and AChE inhibition. Hence, these conditions may be recommended for incorporation in the cultivation practices of this valuable medicinal plant. This would ensure the commercial supply of plants with higher asiatic acid content and better activity.

INTRODUCTION: Medicinal plants have an important role in the health care system and have immense potential in domestic and international market [1, 61]. Approximately 70-80% of the world population uses herbal medicines for health management because of better acceptability and lesser side effects [2, 52]. The demand for medicinal plants for use in herbal formulations has been increasing day- by- day; thus, a sustainable supply of medicinal plants to meet the market demand is of prime importance, which can be fulfilled by developing optimum cultivation conditions.

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The therapeutic potential of herbal drugs has been attributed to the presence of various chemical constituents. It is well documented that different environmental factors particularly, abiotic factors including temperature, shade, minerals, drought, flooding, and salinity, affect plant growth, quality, and quantity of phytoconstituents [3, 4, 53, 54]. Thus, manipulation of environmental factors may lead to enhanced secondary metabolite biosynthesis and thus increasing the medicinal value of plants [5, 62].

Centella asiatica L. Urban (CA) (family Apiaceae) is a perennial herbaceous, creeper, and hardy medicinal plant used in different traditional medicinal systems since ancient times [6, 55]. In Ayurveda, CA (commonly known as Mandukparni or Gotu Kola) is documented as a “miracle elixir of life” [7, 63]. It is described as a psychoactive medicinal plant that has been used as a brain tonic, memory enhancer, neuroprotective, anxiolytic,

anti-alzheimer and anti-depressant [8, 9, 64]. Scientific studies showed numerous pharmacological activities of CA, including memory enhancing and learning, neuroprotective, anxiolytic, anti-inflammatory, wound healing, cardiovascular protective and anti-cancer, thereby validating its traditional claims [7, 10, 11, 56, 63]. The plant contains various phytoconstituents *viz.* triterpene (asiatic acid, madecassic acid, brahmic acid, isobrahmic acid, betulic acid), saponins (asiaticosides, medacassoside, madasiatic acid), flavonoids (kaempferol, castiliferol, castilicetin), glycosides (asiaticoside A, asiaticoside B, madecassoside, centelloside) [12, 13, 14, 15, 16, 65, 68]. The memory enhancing and neuroprotective effects of CA have been attributed to the presence of asiatic acid [7, 13, 17, 18 ,19, 20, 65]. The cultivation practices for CA are well documented, but the emphasis has been on plant growth rather than bioactive constituents and related biological activities. Considering the medicinal importance of CA, it is worthwhile to optimize the environmental factors that enhance asiatic acid production and biological activities. Thus, the present study was designed to understand the effect of abiotic factors (seasons, soil, salinity, flooding, drought, fertilizer, shade stress) on the production of marker compounds (asiatic acid) and antioxidants and anti-acetylcholinesterase activities of CA extracts.

MATERIALS AND METHODS: The field experiment was conducted at Medicinal Plant Garden of Department of Pharmaceutical Sciences and Drug Research, Punjabi University, Patiala, India. The fresh plantlets of *Centella asiatica* were procured from Devi Lal Herbal Park, Yamunanagar, Haryana, India, at the start of the respective season and cultivated under different conditions.

The plant was authenticated by Haryana Forest Department, Chuhadpur Klan (Yamunanagar), with Specimen number: 27/1/2016:4; 32. **Fig. 1** summarizes the plan of work of this investigation.

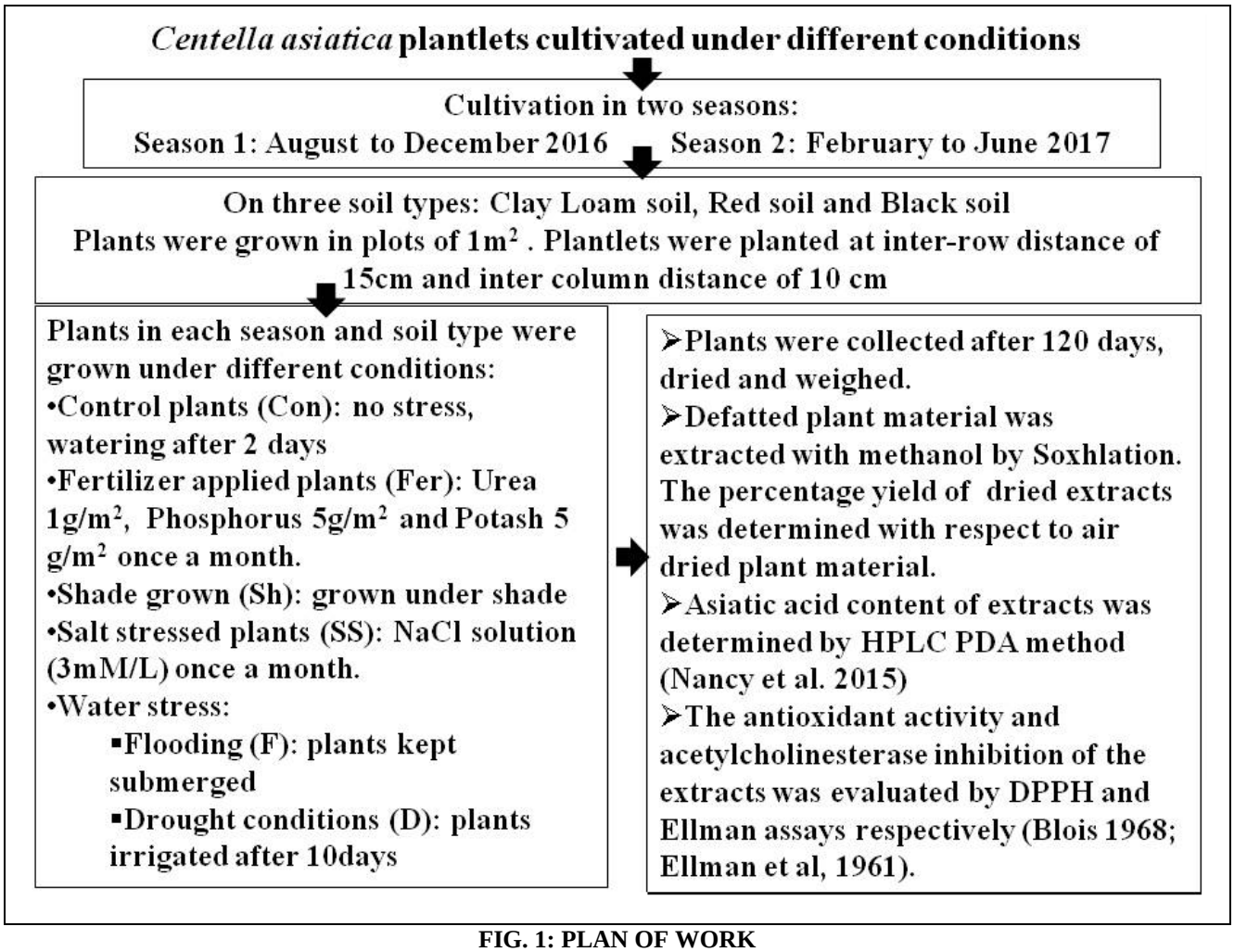

FIG. 1: PLAN OF WORK

Estimation of Asiatic Acid Content in all Prepared Extracts using Validated HPLC Method: Content of asiatic acid, taken as a marker compound, in different extracts was determined by an HPLC PDA method [21].

Standard solution of asiatic acid (0.1 mg/ml) in methanol was prepared and separated on a NUCLEODUR C18 (250 mm × 4.6 mm) column fitted to HPLC PDA (Waters, Milford, MA, USA) system.

A mixture of acetonitrile and phosphate buffer (20 mM, pH 3.5) (55:45% v/v) was used as a mobile phase, and detection was carried out at 206 nm. The mobile phase was filtered through a 0.45-micron membrane filter and degassed. The injection volume was 20 µl, and the flow rate was maintained at 1 ml/min.

Run time for Standard and sample was 45 min. Data acquisition was done using empower 3 software compliantly. A linear calibration plot was used for the quantification of asiatic acid in all samples, as described by Nancy *et al.,* (2015).

LC-PDA detector analyses of standard asiatic acid of all products were carried out to establish the purity of asiatic acid peaks. Readings for each extract were taken in triplicate.

Evaluation of *in-vitro* Biological Activities of the Extracts:

***In-vitro* Antioxidant Activity:** The antioxidant activity of various plant extracts was determined by the DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay [22]. The IC_{50} value of each extract was calculated by using linear regression analysis and expressed in µg/ml. All readings were taken in triplicate.

***In-vitro* Acetylcholinesterase (AChE) Inhibitory Activity:** The AChE inhibitory effect of each test extract was evaluated using method of Ellman *et al*, (1961) [23]. The enzyme acetylcholinestrase hydrolyzes the substrate acetylcholine which results in production of thiocholine. The later reacts with 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB), thereby producing 2-nitrobenzoate-5-mercaptothiocholine and %-thio-2-nitrobenzoate, which can be detected at 412 nm. All the readings were taken in triplicate.

RESULTS:

Effect of Abiotic Stresses on Plant Growth: CA is a prostrate, creeper. This can easily be propagated by stem cuttings or runners or seeds [24, 25]. In this study, propagation was done by plantlets that spread fast. The plants grown under different conditions were collected, shade dried and weighed separately. **Table 2** summarizes the amount of plant collected per unit area expressed as g/m^2.

TABLE 2: AMOUNT OF *CENTELLA ASIATICA* PLANTS PER UNIT AREA GROWN UNDER DIFFERENT ABIOTIC STRESSES

Season	Soil	Yield of plants collected per unit area in both seasons expressed as (g/m^2)					
1	Clay loamy	Con. 28.5	Dro. 17.8	Fer. 42.4	Sh. 13.45	S.S 7.0	F 9.6
	Red	Con. 15.2	Dro. 27.5	Fer. 22.0	Sh. 36.9	S.S N.A	F 6.97
	Black	Con. 11.3	Dro. 12.4	Fer. 14.0	Sh. N.A	S.S N.A	F N.A
2	Clay loamy	Con. 17.8	Dro. 10.0	Fer. 20.0	Sh. 16.0	S.S N.A	F 8.4
	Red	Con. 20.0	Dro. 20.0	Fer. 15.0	Sh. 10.0	S.S N.A	F 5.2
	Black	Con. 14.2	Dro. N.A	Fer. 16.0	Sh. 5.0	S.S 8.0	F 8.0

Con.-Control, Dro.- Drought, Fer.- Fertilizer, Sh.- Shade, S.S- Salt stress, F- Flooding; N.G - not applicable as no growth of plants had been observed

As is seen in the results, there is no uniform pattern in terms of season or soil. In the control plants, the yield was highest in the plants grown in Season 1 in Clay loamy soil. The highest yield was observed in the group treated with fertilizers and grown in Season 1, loamy clay soil.

Some reports state that sandy loam soil is better than clay [26]. According to the NMPB this plant grows over moist, fertile, loose, sandy loam and clayey soil. It grows best in monsoon periods [24].

The results of our study are similar since good growth was seen in clay loam soil during season 1, *i.e.,* during the rainy season.

Effect of Abiotic Stresses on Centella Asiatica Extract Yield in Two Seasons: Various methods such as maceration, Soxhlet extraction, microwave-assisted, etc. are reported for the preparation of extracts from CA [27, 28, 66, 67, 68]. Alcoholic or aqueous extracts of CA are generally prepared since these contain higher quantities of asiaticoside

and the aglycones i.e., asiatic acid and madecassic acid [29]. Thus, based on literature in our study, methanol extracts of CA were prepared by Soxhlet extraction. **Table 3** summarizes the yield (% w/w, dry weight basis) of methanol extract prepared from plants grown under different conditions.

TABLE 3: YIELD OF METHANOL EXTRACTS OF *CENTELLA ASIATICA* PLANTS GROWN UNDER DIFFERENT ABIOTIC STRESSES

Season	Soil	Yield of methanol extracts of plants grown under different abiotic stresses in both seasons expressed as % w/w, (dry weight basis)					
1	Clay loamy	Con. 9.0	Dro. 5.5	Fer. 4.7	Sh. 5.8	S.S 5.0	F 6.7
	Red	Con. 10.6	Dro. 7.0	Fer. 14.0	Sh. 6.0	S.S N.A	F 3.2
	Black	Con. 4.0	Dro 5.5	Fer. 14	Sh. N.A	S.S N.A	F N.A
2	Clay loamy	Con. 7.0	Dro. 4.1	Fer. 3.2	Sh. 8.2	S.S N.A	F 7.4
	Red	Con. 13.2	Dro. 6.0	Fer. 11.0	Sh. 4.8	S.S N.A	F 4.8
	Black	Con. 5.0	Dro. N.A	Fer. 16.0	Sh. 3.5	S.S 5.0	F 6.4

Con.-Control, Dro.- Drought, Fer.- Fertilizer, Sh.- Shade, S.S- Salt stress, F- Flooding; N.A- not applicable as no growth of plants had been observed

Phytochemical Screening of Methanol Extract of *C. asiatica*: The results revealed the presence of carbohydrates, tannins, flavonoids, phenols, and triterpenoids in the tested extracts.

Estimation of Asiatic Acid Content in Extracts of Different Stress Effected Plants of *Centella asiatica* using HPLC Method: CA builds up large quantities of pentacyclic triterpenoid saponins that include asiaticoside, centelloside, madecassoside, brahmoside, brahminoside, thankuniside, scef-foleoside, centellose, asiatic, brahmic-, centellic- and madecassic acids [30, 68]. Asiaticoside and madecassoside and the sapogenin –asiatic acid are generally employed as the bioactive markers for standardization of the plant [31, 69].

The extracts of different stress effected plants of *Centella asiatica* of both seasons were standardized for asiatic acid content by an HPLC method as [21, 70, 71]. Using the standard plot of marker compound asiatic acid **Fig. 2**, the asiatic acid content in various extracts was determined in **Table 4**.

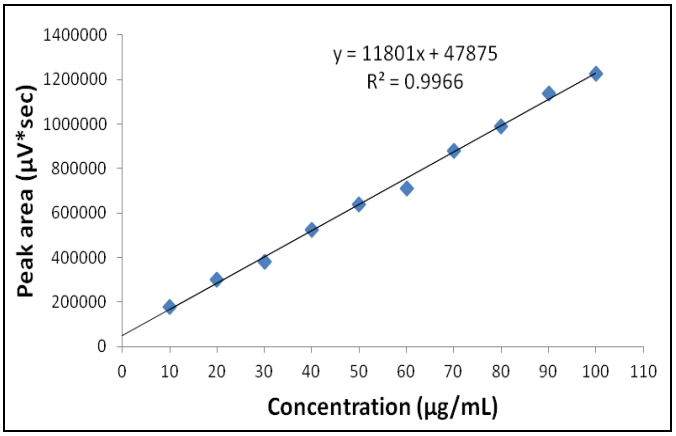

FIG. 2: STANDARD PLOT OF ASIATIC ACID EMPLOYING AN HPLC ANALYSIS

TABLE 4: ASIATIC ACID CONTENT IN EXTRACTS OF DIFFERENT STRESS EFFECTED PLANTS OF *CENTELLA ASIATICA* USING HPLC METHOD

Season	Soil	Asiatic acid content in methanol extracts of different stress effected plants (µg/ml) (Mean^n ± S.D.)					
1	Clay	Con.	Dro.	Fer.	Sh.	S.S	F 8.74 ± 0.05
	loamy	19.18 ± 0.78	7.29 ± 0.13	8.44 ± 0.42	12.16 ± 0.20	14.11 ± 0.18	
	Red	Con.	Dro.	Fer.	Sh.	S.S	F
		15.77 ± 0.48	12.49 ± 0.18	2.30 ± 0.07	9.62 ± 0.21	NA	NA
	Black	Con.	Dro.	Fer.	Sh.	S.S	F
		15.13 ± 0.19	11.53 ± 0.79	11.09 ± 0.53	N.A	N.A	N.A
2	Clay	Con.	Dro.	Fer.	Sh.	S.S	F
	loamy	16.06 ± 0.9*	8.57 ± 0.91	8.32 ± 0.66	18.64 ± 0.8*	N.A*	2.81 ± 0.5*
	Red	Con.	Dro.	Fer.	Sh.	S.S	F
		8.51 ± 0.54*	NG*	0.17 ± 0.13*	7.92 ± 0.83	N.A	56.94 ± 0.11*
	Black	Con.	Dro.	Fer.	Sh.	S.S	F
		21.06 ± 0.9*	NA*	21.92 ± 0.99*	39.29 ± 0.96*	66.76 ± 0.99*	45.85 ± 0.50*

The data was expressed as mean ± S.D and analyzed by student t-test of independent samples,*= $p<0.05$ vs.S1, n = 3, Con.- Control, Dro.- Drought, Fer.- Fertilizer, Sh.- Shade, S.S- Salt stress, F- Flooding; N.A- not applicable as no growth of plants had been observed

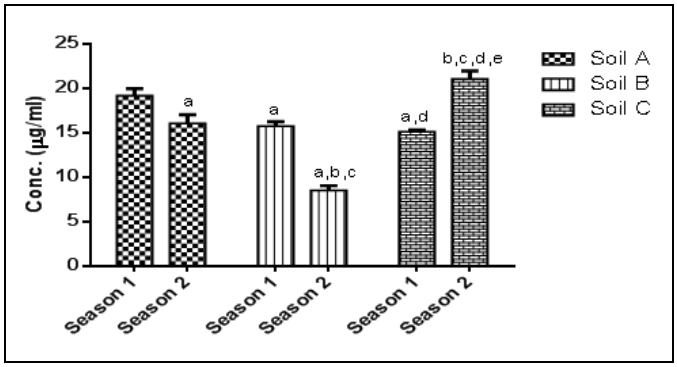

FIG. 3: COMPARISON OF ASIATIC ACID CONTENT OF CONTROL PLANTS GROWN IN SOIL A, B AND C IN BOTH SEASONS. The data is expressed as Meann ± S.D. (n=3) and analyzed by One way ANOVA followed by Tukey's post hoc analysis. ap<0.05 *vs.* soil A season 1; bp<0.05 *vs.* soil A season 2; c p<0.05 *vs.* soil B season 1; dp<0.05 *vs.* soil B season 2; ep<0.05 *vs.* soil C season 1.

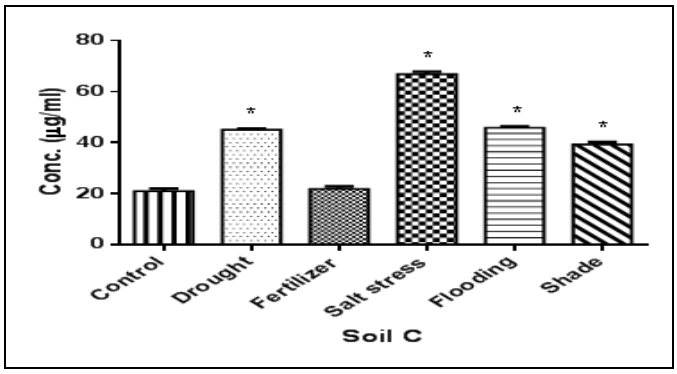

FIG. 4: ASIATIC ACID CONTENT OF PLANTS GROWN UNDER DIFFERENT STRESSES IN SEASON 2 AND SOIL C. The data is expressed as Meann ± S.D. (n=3) and analyzed by one way ANOVA followed by Tukey's post hoc analysis. ap<0.05 *vs.* control.

In season 2, plants grown under different abiotic stresses showed statistically significant variation in asiatic acid content in comparison to control plants **Fig. 4**. The methanol extract prepared from salinity effected plants grown in soil C in season 2 contains the highest asiatic acid content.

Evaluation of Bioactivities:

***In-vitro* DPPH Free Radical Scavenging Activity of Extracts of Different Stress Effected Plants of Both the Seasons:** The antioxidant activity of extracts of different stress effected plants of *Centella asiatica* of both the seasons were evaluated using *in-vitro* DPPH assay. The IC_{50} values are reported in **Table 5**.

The antioxidant activity of extracts prepared from different stress effected plants of both seasons is significantly different from control plants. The plants grown in Season 2 in all soil types had higher antioxidant activity than the plants grown in Season 1.

In season 1, most significant activities was observed in extracts of plants grown under controlled conditions and under salt stress in clay loamy soil (*i.e.* soil A). The plants grown in fertilized red soil show strong antioxidant activity in season 2. Salinity effected plants in season 1 have the highest antioxidant potential.

TABLE 5: THE IC_{50} VALUE IN DPPH ASSAY OF THE PLANTS OF BOTH THE SEASONS EFFECTED BY DIFFERENT STRESSES

Soil	Sample	IC_{50} value (µg/ml) ($Mean^n$ ± S.D) Season 1	IC_{50} value (µg/ml) ($Mean^n$ ± S.D) Season 2
	Ascorbic acid (Standard)	5.2 ± 0.6	5.2 ± 0.6
	Control plant	48.68 ± 0.06	48.63 ± 0.05
A	Drought stress	109.00 ± 0.15	109.16 ± 0.15
	Fertilization stress	78.63 ± 0.12	53.23 ± 0.15
	Salt stress	42.03 ± 0.42	N.A
	Flooding stress	60.75 ± 0.26	60.72 ± 0.23
	Shade stress	78.56 ± 0.05	58.08 ± 0.30
	Control plant	51.6 ± 0.4	71.53 ± 0.11
	Drought stress	73.14 ± 0.27	73.11 ± 0.27
B	Fertilization stress	70.94 ± 0.36	53.36 ± 0.07
	Salt stress	N.A	N.A
	Flooding stress	N.A	69.59 ± 0.25
	Shade stress	106.06 ± 0.58	105.4 ± 0.85
	Control plant	76.65 ± 0.42	76.65 ± 0.44
	Drought stress	53.46 ± 0.13	N.A
C	Fertilization stress	69.16 ± 0.07	69.13 ± 0.05
	Salt stress	N.A	57.01 ± 0.31
	Flooding stress	N.A	59.1 ± 0.38
	Shade stress	N.A	69.09 ± 0.59

N.A- Not applicable as no growth of plants had been observed

TABLE 6: THE IC_{50} VALUE OF ACETYLCHOLINESTRASE INHIBITORY ASSAY OF THE PLANTS OF BOTH THE SEASONS

Soil	Sample	IC_{50} value (mg/ml) (Mean^n ± S.D) Season 1	IC_{50} value (mg/ml) (Mean^n ± S.D) Season 2
	Donepezil (Standard)	0.65 ± 0.003	0.65 ± 0.003
	A Control plant	519.7 ± 0.60	368.00 ± 0.76*
	A Drought plant	431.30 ± 0.79	380.00 ± 0.69*
A	A Fertilized plant	369.9 ± 0.76	353.00 ± 0.50*
	A Salinity plant	504.3 ± 0.08 *	N.A
	A Flooding plant	445.3 ± 0.85	252.00 ± 0.31*
	A Shade plant	454.3 ± 0.96	257.01 ± 0.46*
	B Control plant	306.2 ± 0.10	335.00 ± 0.37 *
	B Drought plant	431.3 ± 0.79	498.01 ± 0.64*
B	B Fertilized plant	572.2 ± 0.15	410.01 ± 0.85 *
	B Salinity plant	N.A	N.A
	B Flooding plant	N.A	175.01 ± 0.98*
	B Shade plant	529.1 ± 0.09	285.01± 0.72*
C	C Control plant	361.8 ± 0.60	267.01 ± 0.85*
	C Drought plant	462.6 ± 0.94*	N.A
	C Fertilized plant	371.7 ± 0.69	193.00 ± 0.91*
	C Salinity plant	N.A	140.01 ± 0.43*
	C Flooding plant	N.A	204.01 ± 0.75*
	C Shade plant	N.A	163.00 ± 0.50*

The data was expressed as mean ± S.D and analyzed by t-test of independent samples,*= $p<0.05$ *vs.* S1, n = 3 Acetylcholinesterase inhibitory activity in season 2 > season 1

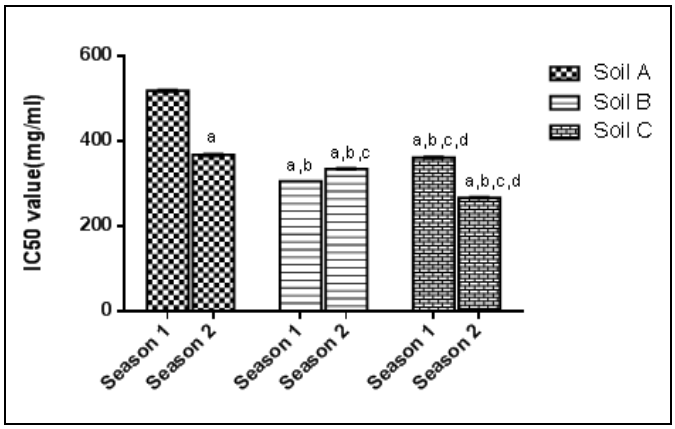

FIG. 5: COMPARISON OF ACETYLCHOLINESTERASE INHIBITORY ACTIVITY OF CONTROL PLANTS GROWN IN SOIL A, B AND C IN BOTH SEASONS. The data is expressed as mean ± S.D. (n = 3) and analyzed by one way anova followed by newman keul's post hoc analysis. ap<0.05 *vs.* soil a season 1; bp<0.05 *vs.* soil a season 2; c p<0.05 *vs.* soil b season 1; dp<0.05 *vs.* soil b season 2

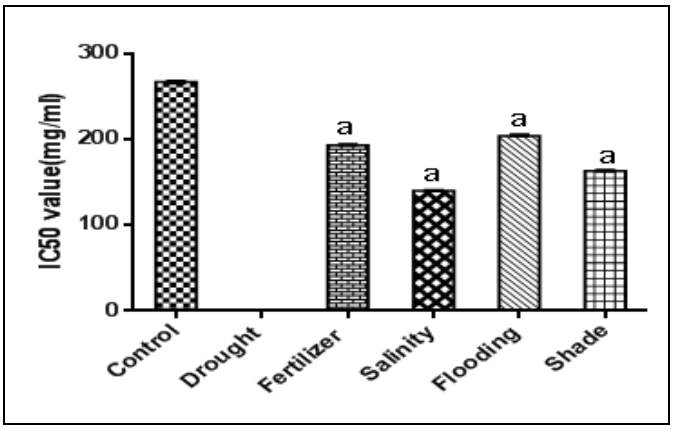

FIG. 6: COMPARISON OF ACETYLCHOLINESTERASE INHIBITORY ACTIVITY OF PLANTS GROWN UNDER VARIOUS STRESSES IN SEASON 2 AND SOIL C. The data is expressed as Mean ± S.D. (n = 3) and analyzed by one way ANOVA followed by followed by Newman Keul's test. ap<0.05 *vs.* control. The salinity effected plants of soil C statistically produce better acetylcholinesterase inhibitory activity in season 2

Acetylcholinestrase Inhibitory Activity: The acetylcholinestrase inhibitory activity of extracts of different stress effected plant of *Centella asiatica* of both the seasons were evaluated. The IC_{50} values are reported in **Table 6**.

The results show that the acetylcholinestrase inhibitory activity of different stress effected plants of both seasons is significantly different from control plants. Similar to acetylcholinestrase inhibitory potential than the plants grown in season 1. The different stress effected plants grown in black soil (*i.e.* soil C) had better acetylcholinestrase inhibitory potential than the plants of red and clay loamy soil in the season 2 **Fig. 5**. The salinity effected plant has the highest acetylcholinestrase inhibitory potential among all the stress effected plants in the season 2 **Fig. 6**.

DISCUSSION: Abiotic and biotic factors greatly effect the growth of plants and production of secondary metabolites. It is well documented that

change in season, soil type *(e.g.* clay loamy, red, black) or variation in abiotic factors that the (*i.e.* light, temperature, drought, flooding, shade, salinity, fertilizer *etc.*) significantly influence the growth and yield of the plants [32, 72]. Alteration in abiotic and biotic stresses are also responsible for change in quantity and quality of secondary metabolites. For example in Alfaalfa plants the proline content in roots is rapidly doubled under salt stress [33]. Anthocyanines are reported to increase in response to salt stress [34, 57]. In case of red peppers the total phenol content is increased with moderately saline level [35]. This may be happen because salt stress often creates both ionic as well as osmotic stress in plants, resulting in accumulation or decrease of specific secondary metabolites in plants [36]. Water deficit or drought often causes oxidative stress and was reported to show increase in the amounts of flavonoids and phenolic acids in willow leaves. Drought increased hyperforin and decreased hypericin and pseudo hypericin content in Hypericum perforatum [37]. Water stress (flooding) produce changes in endogenous jasmonates in Pinus pinaster [38]. Shading or low light intensity decreased the linalool and eugenol quantity and increased the methyl eugenol content in the oil of Ocimum basilicum [39]. The flavonoid and phyllanthin content increased in *Phyllanthus niruri* due to fertilization of plant with nitrogen and magnesium [40]. Compotothecin content is varied in *Camptotheca acuminata* along with season [41]. Several studies reported that environmental factors and abiotic stress can alter plant growth, production of secondary metabolites and hence the activity of plant also effected [7, 13, 42, 43, 59]. So, the study of the effect of abiotic stresses may help in increasing production of valuable secondary metabolites in medicinal plants.

CA is known as miracle elixir of life because of its vital uses from prehistoric time to the modern system of medicine. *C. asiatica* is cherished for its memory enhancing effect traditionally and acetylcholine esterase inhibitory activity, which is attributed to the asiatic acid present in the plant [7, 13, 17, 18, 20, 44]. In the present study, the effect of abiotic stresses, seasons and different soils on the asiatic acid content and plant's antioxidant and acetylcholinesterase inhibitory activities were evaluated with a view to understand which soil to use, which season to prefer and what environmental conditions to use during cultivation to increase the production of asiatic acid in the cultivated plants and hence enhance its antioxidant and acetylcholinesterase inhibitory activity. To assess the suitability of soil for better growth of *C. asiatica*, the plants were grown in three different types of soils *viz.* clay loamy soil, red soil, black soil. It was seen that the performance with respect to growth and content due to various soil types used in the experiment differed significantly. The present data showed variation of growth in the three different soils. Plants grown in clay loamy soil have better yield with respect to biomass yield (*i.e.* plant/unit area). Secondary metabolite production is also influenced by different soils. For example, a study shows that the sennoside content altered among the sandy soil and black soil grown plants, the sandy soil was reported to have higher sennoside content than black soil [45].

Asiatic acid is the marker compound mainly responsible for the activity of *C. asiatica* [7, 13, 17, 18, 19, 20, 60]. This was used as the standard in HPLC studies. The technique has been used for the determination of Asiatic acid content in plants grown under different stress parameters, in different soils and under both season on the production of the marker compound. Asiatic acid content in the control plants grown in clay loam soil was higher than the plant grows in red and black soils in seasons 1. Plants grown in black soil under the stress of salinity was found to have the better acetylcholinestrase inhibitory activity than the plants grown in clay loam soil and red soil.

Different seasons not only effect the growth but also the production of secondary metabolites in the plants [46]. For example seasonal variation is responsible for alteration in essential oil yield in *Ocimum basilicum* [47]. The growth was found to be higher in season 1 (August to December) than season 2 (February to June). The results of present investigation showed that the plants grown in season 2 have higher content of asiatic acid as compared to plants grown in season 1. The results revealed that the acetylcholinestrase inhibitory activity of all stress effected and control plants were better in season 2 than the season 1 plants. Plant growth is very much susceptible to various abiotic stresses. For instance, decrease in growth

with increase in salinity [48] and water stress is well documented [49]. The present study demonstrated that the plant growth was lowest in plants subjected to water stress (flooding), salinity, shade in both the seasons. *C. asiatica* is reported to have healthy growth in moist, swampy and rocky areas 7, thus excessive water during growth can effect the growth of plant. The present examination shows that the salt stress effected plants grown in black soil (Soil C) have highest content of asiatic acid (*i.e.* 39.29 µg/ml) as compared to other stress effected and control plants in season 2. Plants grown in black soil under the stress of salinity was found to have the better acetylcholinestrase inhibitory activity than the plants grown in clay loam soil and red soil. Hence, it may be concluded that it is beneficial to grow the plant *C. asiatica* under salt stress in black soil in season of January to June to produce higher quantities of asiatic acid in the plant.

Pharmacological investigations have reported that *C. asiatica* is uesd for improving cognition and relieving anxiety [7, 50, 51, 58, 60]. In the present investigation the effect of abiotic factors on acetylcholinestrase inhibitory potential of plant was estimated by modifying the method proposed by Ellman *et al.,* 1961 [23, 73]. The results revealed that the acetylcholinestrase inhibitory activity of all stress effected and control plants were better in season 2 than the season 1 plants. This may be due effect of seasonal variation. Plants grown in black soil under the stress of salinity was found to have the better acetylcholinestrase inhibitory activity than the plants grown in clay loam soil and red soil.

SUMMARY AND CONCLUSION: Differences in terms of plant yield, methanolic extract yield, asiatic acid content, antioxidant and acetyl-cholinesterase inhibitory activity was observed in plants grown in both seasons. HPLC studies showed that the plants of season 2 have higher asiatic acid content. *In-vitro* studies also showed that the plants grown in season 2 have better antioxidant and acetylcholinestrase inhibitory activity. Salinity effected plants of soil C have highest asiatic acid content, as well as acetylcholinesterase inhibitory activity among all other stress effected plants in season 2. From the results of this investigation, it may be concluded that for commercial cultivation of the plant for medicinal purposes the period from January to June is preferable. For maximum asiatic acid content and better acetylcholinesterase inhibition activity plants should be grown in black soil and given salt stress.

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