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Organic and inorganic fertilizers influence biomass production and esculin content in *Cichorium intybus* L.

Zahid Ali[1], Showkat Hussain Ganie[1, 2], Alka Narula[1], Malik Zainul Abdin[1] and Prem Shankar Srivastava[1]*

[1]Department of Biotechnology, Jamia Hamdard, Hamdard Nagar, New Delhi, 110062, India
[2]Department of Botany, Jamia Hamdard, Hamdard Nagar New Delhi, 110062, India

Abstract
Cichorium intybus L. is a promising and potent industrial medicinal herb but the relatively low content of esculin and unavailability of mass scale propagation protocol are major impediments for the commercial production of the drug. The enhancement in the yield of esculin is hence, highly desirable, which can be achieved by adequate and judicious supply of plants nutrients or by adopting more expensive and complex protocol of biotechnology. The field experiment was designed in random plots to study the effect of organic manure (20 tonnes ha^{-1}) and chemical fertilizers (N_{240}, P_{120}, K_{100}, S_{80}, kg ha^{-1}; Nitrogen, Phosphorus, Potassium and Sulphur, respectively) on the accumulation of esculin and biomass in various plant parts through the various developmental stages, rosette, bolting, pre-flowering, flowering and post-flowering of *C. intybus*. Esculin yield from dried leaves increased significantly at flowering stage in the plants treated with $N_{80+100+60} + P_{120} + K_{100} + S_{20+40+20}$ (31.71% and 111.22 %) when compared with control. Yield of dry leaf biomass ranged between 628.15 to 986.07 kg ha^{-1} at flowering stage in various treatments.

Keywords: *Cichorium intybus* L**.** Organic manure**,** Inorganic fertilizers**,** Biomass, Esculin

INTRODUCTION

Due to the rapid increase in the use of medicinal plants and herbal remedies the world over; demand for these plants has significantly increased in recent years. A majority of new drugs have been generated from natural products (secondary metabolites) and from compounds derived from natural products [1]. The use of mineral fertilizers has been the most acceptable means of increasing the crop yield throughout, accounting for as much as 50% of yield [2]. The biosynthesis of secondary metabolites in medicinal plants is strongly influenced by nutrients and environmental factors [3]. These conditions affect the fresh and dry weight as well as active component/s [4]. Judicious use of organic and inorganic fertilizers therefore be applied for increase the yield of essensial oil, secondary metabolites, the main components of medicinal plants [5-8].

C. intybus, a perennial herb of the Asteraceae, with blue flowers, is also known as blue sailors, endive succory, coffee weed and kashen's or kasini. It is native to the temperate part of the Old World. It is cultivated in Bihar, Gujarat, Himachal Pradesh and Tamil Nadu [9]. Historically, chicory was grown by the ancient Egyptians, as a medicinal plant, coffee substitute, vegetable crop, and occasionally for animal forage [10-11]. For its special chemical composition and physiological bioactivities, natural products extracted from chicory have been used for developing natural medicine [12-13]. The whole plant (root, seed and leaf) is medicinally used traditionally to cure various ailments. The root, leaf and seeds contain a number of medicinally important compounds such as inulin, sesquiterpene lactones, coumarins, flavonoids and vitamins. The plant is used as antihepatotoxic, antiulcerogenic, anti-inflammatory, appetizer, digestive stomachic, depurative and diuretic [14]. Pharmacological investigations also reveals anticancer properties [15]. Inulin is a reserve carbohydrate, present in higher amounts in the roots of chicory which is reported to be useful for fat and sugar replacement [16]. The plant has immunomodulator properties and preventive effect against colon cancer [17].

A major component esculin, otherwise obtained from horse-chestnut (*Aesculus hippocastanum*) has also been reported in the leaf of chicory. Esculin is an important component used in pharmaceutical industry [18]. It is a glycosidic coumarin (monosaccharide of esculetin) belonging to a group of phenolic compounds distributed in natural plants [19].

Esculin is a well known UV-B protective agent [20] and one of the phytomedicines in practice for the treatment of various peripheral vascular disorders [21]. It is an elasticity giving and sealing material for blood vascular system, vitamin P activity, protects cells against DNA damage and inhibits carcinogenesis [22]. The hydrolysis of esculin is an important marker in the microbial medium for the detection of several bacteria [23].

Earlier, in vitro hairy root culture has been used to increase the esculin yield in *C. intybus* [23]. The root cultures (transformed and untransformed) were subjected to different growth regulators in bioreactors [24-25]. Leaf derived plants [26] and *in vitro* regenerated roots have also been recorded [27].

There are reports dealing on *C. intybus* with the influence of fertilizers, notably nitrogen on the yield of biomass, essential amino acids, proteins etc [28-32]. However, no comprehensive data is available comparing the application of N and P [33], including organic and inorganic fertilizers [28] on growth and secondary metabolites accumulation in medicinal plants [8]. Secondary

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*Corresponding Author
Prem Shankar Srivastava
Department of Biotechnology, Jamia Hamdard, Hamdard Nagar, New Delhi, 110062, India

Tel: +91-9818135178;
Email: pss410@rediffmail.com

metabolite production in plants basically depends on production of dry matter, proportion of plant tissue in desired plant organ, and accumulation levels in the plant tissue. Indeed increased yield of secondary metabolite in medicinal plants by increasing dry-matter has proven to be two to three times more effective than other methods [34].

The present study reports the effect of organic manure and chemical fertilizers (N, P, K and S) on the yield of esculin content in *C. intybus*. It appears to be a feasible approach for enhancing the esculin to cost effective commercial level.

MATERIALS AND METHODS

Field trials

Field trials were carried out at the experimental garden at Hamdard University, New Delhi, India, 28.3´N, 77.11´E and 228 m altitude. The soil in the experimental field is sandy loam in texture having pH 7.3, electrical conductivity 0.33 dsm^{-1}, nitrogen 150 kg ha^{-1}, phosphorus 4 kg ha^{-1}, potash 104 kg ha^{-1} and deficient in sulphur.

Seed sowing

Seeds of *C. intybus* L. collected from the Herbal Garden, Hamdard University were sown in the month of November. The plot size was 2.25 m^2 (1.5m×1.5m) and row to row and plant to plant distance was 30 cm. The plants were irrigated twice a week till the bolting and thereafter three times week till flowering depending upon weather conditions.

Treatments

T_1= control (without manure and fertilizer); T_2 = organic manure (@20 tonnes ha^{-1}); T_3 =N_{240}+P_{120}+K_{100}+S_{80} kg ha^{-1}; T_4 =$N_{80+100+60}$ + P_{120} + K_{100} + $S_{20+40+20}$ kg ha^{-1}. Urea, diammonium phosphate, murate of potash and gypsum served as source of nitrogen, phosphorus, potassium and sulphur, respectively. Phosphorus (120 kg ha^{-1}) and potassium fertilizers (100 kg ha^{-1}), each were applied at the time of sowing in bothT_3 and T_4 treatments, while nitrogen (80, 100, 60 kg ha^{-1}) and sulphur(20, 40, 20 kg ha^{-1}) were applied in three split doses, at the time of sowing, bolting and pre-flowering, respectively. Organic manure (20 tonnes ha^{-1}) was also applied at the time of sowing. The experimental procedure was arranged in a completely randomized block design. Samples were harvested at five phenological stages (rosette, bolting, pre-flowering, flowering and post flowering).

Biomass of the Plant

Leaves and stem, were separated manually, oven dried at 70 ◦C and weighed. Dried samples were kept in plastic bags at room temperature until analysis for esculin.

Estimation of esculin by high performance liquid chromatography (HPLC)

1 g dry powdered leaf was used for the estimation of esculin. 20 ml methanol was added to fine powdered plant material and extracted on soxlet for one hour at 70 ºC. Solvent was concentrated till two ml was left. All the samples and standard were filtered through 0.45µm pore size syringe before HPLC analysis was carried out. RP-HPLC analysis was performed on Waters HPLC system (Binary Pump 600 controller), Waters PDA detector (996), Waters auto sampler (2707) and C_{18} column (125×4). Empower software was used for the data collection. The mobile phase consisted of methanol and water (70:30 v/v) and pumped at flow rate of 1.0 ml/min; the detection was carried out at 340 nm. 20 µl of samples were injected by auto sampler. Esculin was quantified with the help of HPLC using different concentrations of standard esculin (M/S Sigma-Aldrich USA). Esculin content was expressed as percentage dry weight basis.

RESULTS

Effect of organic manure and inorganic fertilizers on biomass yields.

Table1 shows the amount of increased dry leaf yield. The maximum reached at flowering stage.T_4 (56.98%) treatment supported best increase in dry wt followed by T_3 (36.83%) and T_2 (8.19%). The dry wt of stem increased with the developmental stage with no, effect of treatments (Table2). T_4 thus proved best for dry wt yield (33.32%) by T_3 (22.58%) and T_2 (5.08%).

Table 1. Effect of organic manure and inorganic fertilizers on dry wt of leaves (kg ha^{-1}) at various phenological stages of *C.intybus* L.

Treatments	Rosette	Bolting	Pre- Flowering	Flowering	Post Flowering
T_1	250.79±7.54	389.14±5.36	541.47±8.82	628.15±2.43	259.79±6.19
T_2	280.41±6.66	469.81±6.47	599.47±8.43	666.94±5.22	264.45±4.19
T_3	338.64±10.72	608.35±8.83	760.71±11.44	859.50±8.53	409.28±4.86
T_4	352.24±5.86	674.47±11.31	913.94±9.38	981.07±7.88	466.73±61.0
LSD at 5%					
Stages(S)				14.29	
Treatment(T)				12.78	
S×T				28.59	

Table 2. Effect of organic manure and inorganic fertilizers on dry wt of stem (kg ha^{-1}) at various phenological stages of *C.intybus* L.

Treatments	Bolting	Pre- Flowering	Flowering	Post Flowering
T_1	359.60±9.63	1370.06±18.15	1601.90±21.69	1362.57±8.62
T_2	373.65±19.11	1497.36±32.18	1683.28±17.37	1392.51±10.51
T_3	408.35±6.60	1729.25±16.87	1963.54±31.05	1744.12±10.63
T_4	419.79±5.17	1830.56±14.02	2135.70±15.54	1910.08±13.94
LSD at 5%				
Stages(S)			24.90	
Treatment(T)			24.90	
S×T			49.80	

Effect of organic manure and inorganic fertilizers on leaf stem ratio at various phenological stages of C. *intybus* L. plants.

The results presented in Table 3 revealed that the maximum leaf stem ratio was observed in T4 (1.607, 0.499, 0.462, and 0.244) followed by T3 (1.494, 0.440, 0.439 and 0.235) and T2 (1.291, 0.401, 0.397 and 0.190) when compared with control (1.089, 0.397and 0.393 and 0.179) at bolting, pre-flowering and flowering and post-flowering stages, respectively.

Effect of organic manure and inorganic fertilizers on esculin content

Esculin content in leaf increased progressively irrespective of treatments over control and reached to maximum at flowering stage (0.47–0.64%). The maximum increase in esculin content was recorded in T_4 (31.71%) followed by T_3 (18.68%) and T_2 (9.05%) over control, at flowering stage (Table 4).

Effect of organic manure and inorganic fertilizers on esculin yield

The results show that various treatments had significant effect on esculin yield at flowering stage when compared among the treatments. The esculin yield from leaf however, was ranged from 2.95 to 6.22 kg ha^{-1} at flowering stage. The highest esculin yield was recorded with treatment T4 (111.22%) followed by T3 (77.55%) and T2 (14.28%) over control at flowering stage (Table 4).

Table 3. Effect of organic manure and chemical fertilizers on leaf stem ratio at various phenological stages of *C. intybus* L. plants

Treatments	Bolting	Pre -Flowering	Flowering	Post Flowering
T_1	1.089±0.04	0.397±0.02	0.392±0.013	0.179±0.004
T_2	1.295±0.086	0.415±0.012	0.396±0.008	0.191±0.003
T_3	1.493±0.041	0.440±0.006	0.439±0.013	0.235±0.018
T_4	1.608±0.029	0.499±0.003	0.462±0.008	0.245±0.012
LSD at 5%				
Stages(S)			0.04	
Treatment(T)			0.04	
S×T			0.08	

Table 4. Percent content and Esculin yield (kg ha^{-1}) in leaf influenced by organic manure and chemical fertilizers at various phenological stages

Treatment	Rosette		Bolting		Pre-Flowering		Flowering		Post Flowering	
	%	yield	%	yield	%	Yield	%	Yield	%	Yield
T_1	0.32±0.004	0.75±0.01	0.39±0.005	1.56±0.02	0.44±0.004	2.14±0.02	0.47±0.007	2.95±0.05	0.31±0.005	0.81±0.81
T_2	0.34±0.006	0.87±0.01	0.40±0.008	1.80±0.03	0.49±0.014	2.81±0.08	0.54±0.005	3.36±0.03	0.35±0.008	0.86±0.86
T_3	0.37±0.006	1.34±0.03	0.45±0.006	2.58±0.03	0.54±0.004	3.84±0.05	0.59±0.011	5.22±0.05	0.43±0.002	1.68±0.08
T_4	0.37±0.003	1.37±0.01	0.47±0.004	3.03±0.02	0.89±0.004	4.18±0.05	0.64±0.003	6.22±0.04	0.45±0.007	2.22±0.03
LSD at 5%										
Stages(S)							0.009	0.045		
Treatment(T)							0.008	0.40		
S×T							0.018	0.091		

DISCUSSION

Soil fertility is an environmental variable associated with biomass production and accumulation of compounds originated through secondary metabolism. Environmental factors, responsible for complex growth and development interactions in plants, may also influence the production and accumulation of secondary metabolites [34]. It has been shown earlier that the addition of organic matter improves soil properties such as aggregation, water holding capacity, hydraulic conductivity, bulk density, the degree of compaction, fertility and resistance to water and soil erosion. These soil factors positively affect the growth and development of plants [35-36] and also metabolites.

Our results showed that application of organic (T_2) and inorganic nutrients (T_3 and T_4) increase the dry biomass in leaves and stem peaks at flowering stage. Among the various treatments, maximum biomass yield in leaves was recorded in plants receiving nitrogen and sulphur in three split doses, along with the basal dose of phosphorus and potassium (T_4).

Addition of green compost derived humic acids to a sandy soil has consistently shown positive effects on plant biomass productivity in *C.intybus* [37]. Chicory production increased when N fertilizer was applied in split doses [38]. According to [39] the highest yield of radicchio heads was obtained when a dose of 200 kg N ha^{-1} was supplied in split treatment (100+100 kg N ha^{-1}). [40] noticed that the highest yield occurred in treatments with a total dose of 150 kg ha^{-1} divided in two rates: pre-plant 50kg N ha^{-1} and supplementary top dressing of 100 kg N ha^{-1}.

Our results on positive effects of organic manure and chemical fertilizers on dry matter yields are in agreement with previous reports on other medicinal plants, such as, *Catharanthus roseus* [41], *Cymbopogon winterianus* [42], *Pogostemon cablin* [43], *Plantago ovata* [44] *Psoralea corylifolia* [45], *P. cablin (Blanco) Benth* [46], *Artemisia annua* [8] and others. The leaf stem ratio decreased with progressive developmental stages. The leaf to shoot (l/S) ratio was significantly greater in the T4 treatment compared to the others.

Esculin has been reported to accumulate in *in vivo* plants during different phenological stages (rosset, bolting, pre-flowering and flowering stages in wild plant) thus indicating that in *C. intybus* also it was largely influenced by the developmental stages of the plant. The esculin content reached its maximum at flowering stage [26].

Factors that increase dry matter production may also influence the interrelationship between primary and secondary metabolism, leading to increased biosynthesis of secondary products [47]. The enhanced concentration of esculin by organic and inorganic nutrients may be attributed to the improved growth and nutrient status of the plants. The maximum content was in plants receiving nitrogen and sulphur in three split doses along with basal dose of phosphorus and potassium (T4). It is well documented that approximately 60% of nitrogen in plant leaf is invested in photosynthetic components. Thus, nitrogen nutrition plays a crucial role in determining the photosynthetic productivity of plants in both natural and agricultural environment [48]. While photosynthetic efficiency is directly affected by nitrogen, sulphur does so indirectly by improving the nitrogen utilization efficiency [49]). Consequently, adequate sulphur supply increases nitrogen utilization into protein (Rubisco) synthesis, thereby enhancing the photosynthetic rate and increased biomass.

Because of highest biomass the maximum esculin yield was obtained from the leaves of the plants receiving T_4 treatment at flowering stage. The positive effects of nutrient application on secondary metabolites are in agreement with the previous reports on other medicinal plants, such as, mint [50], palmarosa [51], basil [52], fennel [53] and Artemisia [8]. One explanation for an outcome is that nitrogen is a major constituent of several precursors [50] while sulphur plays a positive role in nitrogen use efficiency by regulating nitrate reductase, an enzyme that catalyses the rate limiting step in nitrogen assimilatory pathway [54].

CONCLUSIONS

C. intybus L is a medicinal plant used in various diseases in folk medicine. Our results show that organic and inorganic nutrients increase dry weight of leaf, stem and esculin content in leaves and stems. The use of nutrients in split dose proved best for the both. S and N should be applied in split dose to optimize esculin yield in *C. intybus.* Also application of S and N before flowering is beneficial in improving the yield.

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