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Effect of variation of seasonsat the transcriptional level of PAL gene and study of alteration in synthesis of essential oil in Ocimum tenuiflorum.

Rahul Kumar Yadav*, Prof Dr. Kunal Mukhopadhyay**
*Btech,Mtech,CSIRJRF
*/** Professor at BIT Mesra Ranchi. Department of Biotechnology, Birla Institute of Technology, Mesra, Ranchi-835215, Jharkhand, India.

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ABSTRACT:
Background: Human societies constantly endure in contact with their environment since the beginning of an era, which makes us depended on plants source to obtain food and medicine. Medicinal plants are beheld as a cure for innumerable diseases in almost all cultures. Safeguarding the safety, quality and efficiency of herbal plants very recently became a hotspot in the developing countries. Phytotherapy has long been a foundation of medicinal products and over the years there have been numerous attempts to use herbal medicines. Ocimum tenuiflorum as Ocimum tenuiflorum L has various therapeutic bioactive components which are have various Ethan pharmaceutical properties such as Diabetes, Hypertension, Metabolic Syndrome, and bundles of more
Methods: In our study we divided two set of groups. In Group one fresh mature 10-15 leaves were plucked from plant and store in 2ml Eppendorf for GC-MS and another set in which again 10-15 leaves were plucked in store in 2ml Eppendorf with RNA later solution.This was repeated monthly on specific date of months from July 2014- May 2015 continuously. At end Realtime analysis was performed to know PAL Gene mRNA Expression and GC-MS performed to know the essential oil secretion in specific months
Result: our results show upregulation of PAL gene expression Profile in winter season specifically from January to March. Similarly, essential oil Eugenol shows high secretion compared to other essential oil in winter season.

Conclusion: Winter season is state of stressful condition for plant which makes increase level of flavonoids secretion may be as a method of defensive mechanism

Key word: Ethan pharma, hepato-protective, radioprotective,immunomodulatory effects.

I. INTRODUCTION:

As evolution materializes plant have advanced various defensive mechanism with the environmental stressful condition,dating back to Mesozoic era[1]. These diverse ecological habitats which expose them to numerous biotic and abiotic stresses have made them to developed diverse chemical defense systems as a need for their survival strategy [2]. Core pathway for development of these chemical and biological adaption passes through a variety of Phenolic compounds such as flavonoids, lignin etc. The precursors for several of these phenolic defense complexes are synthesized through the phenylpropanoid pathway [3]. Phenylpropanoid pathway is initiated by Phenylalanine ammonia lyase (PAL) gene family. PAL is a key enzyme of the phenylpropanoid pathway that catalyzes the deamination of phenylalanine to trans-cinnamic acid, which is a precursor for the flavonoid and ligninbiosynthetic pathways. It is responsible for the production of wide range of products,as lignin, flavonoids, isoflavonoids, coumarins, hydroxycinnamic acids, and many more type of different phenolic compounds. These metabolites play vital functionsin plants, including guarding against biotic andabiotic stresses, UV protection, cellular signaling [4].

Phenylalanine ammonia lyase (PAL; E.C 4.3.1.5), thekey enzyme linkingprimary metabolism of aromaticamino acids with secondary metabolic products inplants, has been extensively studied since its discoveryby Koukal and Conn [5].

Ocimum tenuiflorum:

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Ocimum tenuiflorum L. or Ocimum sanctum L., commonly known as the Holy Basil inEnglish or Tulsi in the various significant Indian languages, is significant medicinalplant in the various traditional medicine system in Southeast Asia.

Chemical constituents:

Vitamins like C and A, minerals such as calcium, zinc, and iron, as well as chlorophyll and many other phytonutrients. Protein content: 30 Kcal, 4.2 g; Fat: 0.5 g; Carbohydrate 2.3 g; Calcium: 25 mg; Phosphorus 287 mg; Iron: 15.1 mg and Edible portion 25 mg vitamin C per 100 g. [6] The leaf contains volatile [7] essential oil eugenol, Isoeugenol , urosolic acid methylcharicol, limatrol and caryophylline [8] and these are a bioactive component which preserves pharmacological properties.

Pharmaceutical Properties:

The plant has been considered ethnobotanically significant because of its usage in the traditional health care system [9]. The synergistic interactions of several different bioactive phytochemicals indicate to varied pharmacological properties of the whole herb in its native form. Subsequently, the overall properties of Ocimum tenuiflorum L cannot be fully reproduced with isolated compounds or extracts due to its inherent botanical and biochemical complexity, Ocimum tenuiflorum L standardization has, so far, eluded modern science.

Brief properties of Ocimum tenuiflorum L plant are as below:

Leaves of Ocimum tenuiflorum L are diaphoretic, antiperiodic, used in bronchitis, gastric and hepatic disorders, etc. Leaves of Ocimum tenuiflorum L is generally used in coughs, colds, mild indigestion, diminished appetite, and malaiseanti-helmintic, anti-inflammatory deodorant, cardiotonic and blood purifier, used in skin diseases and as an antipyretic, particularly in malarial fevers. It is advantageous in abdomen pain, nausea, cough, worms, allergic rhinitis, and respiratory disorders. It is externally applied to chronic nonhealing ulcers, inflammation, skin disorders. The seeds contain oil composed of fatty acid and sitosterol. Chinese medicine uses basil for kidney conditions, stomach spasms, promote blood circulation, and to treat snake and insect bites [10]. Ocimum tenuiflorum L needs a comprehensive evaluation for it has immense therapeutic possibilities [11].

Flavonoids

Flavonoids are adherents of hefty group of phenol derivatives whose molecules entails of a basic C6-C3-C6 phenyl benzopyran backbone. They commonly occur in plants as glycosylated derivatives and theirpsychological roles in ecology of plants are miscellaneous. Curiosity in this group ofcompound ascends because of extensivelyshown pharmaceutical interested biological actions, wide distribution in natureand their minor or absent toxicity.

Metabolite contents of the plant Ocimum tenuiflorum varies with seasonal changes (12)The intention of the study is to characterize (PAL) Phenylalanine Ammonia Lyase, gene involved in the biosynthetic pathway and study of its alteration in expression profile due to stressful condition which was provide due to alteration in seasons. This profile will be useful during the extraction of bioactive components for pharmaceutical uses. Though the fact can't be denied that only one factor i.e. stress is responsible in alteration of expression profile of PAL gene m-RNA and secretion of Essential oil such as eugenol, Isoeugenol, Methyl eugenol, Chavinol which serves high bioactive properties.

II. MATERIAL AND METHODS:

Sample collection:

Approximately 300gm Leaves of Ocimum tenuiflorum were collected from Medicinal Plant Garden of Birla Institute of Technology, Mesra, Ranchi, Jharkhand, India from the period July 2014 and May 2015.Green Ocimum tenuiflorum Lsaplings were collected and grown in Department of Bio Engineering, BIT Mesra, Ranchi. 15-16 leaves of the plant were plucked out and store in Eppendorf tube filled with RNA later and other set was kept in another Eppendorf Tube for GC-MS study.

Extraction of Sample:

RNA later dipped sample were collected in 2 duplicatesfor transcriptional analysis of PAL gene. The set of samples for transcriptional analysis of PAL gene were stored in RNA later (a solution to prevent RNA from degrading) at -70 ^{0}C until further utilization.

- RNA isolation:

300 mg of green Ocimum tenuiflorum L leaves were washed withDEPC water and grinded with liquid nitrogen and RNA isolation was performed with 2ml of TRIzol reagent according to

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TRIzol RNA isolation protocol.

- **cDNA preparation**:

RNA concentration and purity were estimated by spectrophotometry at 260 and 280 nm. The RNA sample with 260/280 ratio upper 1.9 approx. were used for cDNA synthesis. cDNA was synthesis from 2µg of total RNA for each sample using Revertaid first strand cDNA synthesis kit (Fermentas) in a final volume of 20 µL, as per manufacturer's instruction with help of conventional PCR.

- **Real time PCR:**

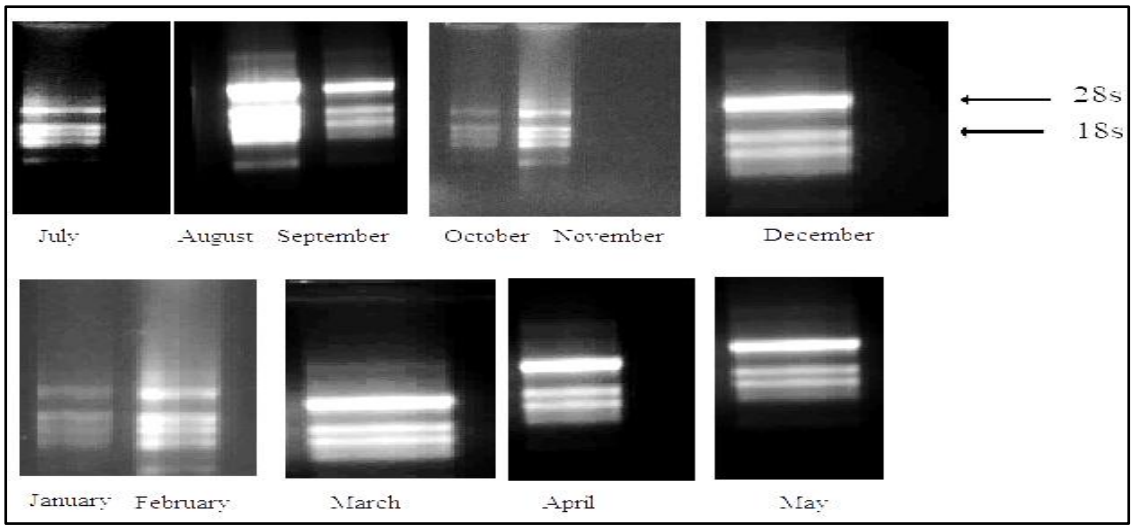
The cDNA samples obtained were analyzed in duplicate using real time PCR. Finally, replicated sample were evaluated for each month.Quantitative RT-PCR was performed with Applied Biosystems 7500 Real Time PCR using SYBR Green detection chemistry with First strand cDNA synthesis. The reaction for real time PCR was prepared in total volume of 15 µL having 1 µL diluted cDNA sample (corresponding to 100 ng of initial total cDNA) using Actin primer as cofactor. The standard thermal profile was considered for all PCR reaction: polymerase activation at 95^0C for 10 min, followed by amplification and quantification cycle repeated 50 times (95^0C for 15 second, and 60^0C for 1 min).

III. RESULTS:

RNA isolation:

The isolation of RNA from ocimum tenuiflorum by using TRIzol reagent presented the subsequent results for different months

Figure: 01These images of gel shows the presence of RNA with these stranded as lower band showing



(LB) 5s,middle band (MB) 18s and Top Band (TB) 28s

Concentration of RNA isolated:

Sample: month and year	Concentration (µg/ml)	A_{230}	A_{260}	A_{280}	A_{320}	$A_{260/280}$	$A_{260/230}$
(July 2014)	302.80	1.810	0.430	0.285	0.200	1.33	0.35
(August 2014)	500.85	1.650	0.401	0.348	0.100	1.65	0.48
(September 2014)	274.52	1.412	0.604	0.312	0.158	1.95	0.82
(October) -2014	304.50	0.910	0.305	0.153	0.001	1.86	0.26
(November) -2014	517.0	1.997	0.517	0.509	0.490	1.82	0.47
(December) -2014	456.9	1.078	0.405	0.246	0.105	1.89	0.58

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(January) -2015	601.8	1.502	0.802	0.514	0.205	1.89	0.51
(February) -2015	589.1	1.380	0.508	0.329	0.108	1.90	0.30
(March) -2015	607.5l	1.512	0.501	0.389	0.204	1.95	0.89
(April) -2015	402.5	1.407	0.400	0.200	0.056	1.81	0.76
(May) 2015)	578.85	1.652	0.754	0.451	0.254	1.75	0.55

Table: 01 O.tenuiflorum was subjected to RNA isolation by TRIzole method. This table shows the quantification and absorbance value of RNA observed in spectrophotometer.

c-DNA quantification:

Sample: Month and year	Concentration (µg/ml)	A_{230}	A_{260}	A_{280}	A_{320}	$A_{260/280}$	$A_{260/230}$
(July 2014)	2048	0.551	1.221	0.658	0.055	1.74	1.88
(August 2014)	3856	0.695	1.854	0.774	0.045	1.92	1.55
(September 2014)	4512	0.684	1.512	0.881	0.074	1.87	1.99
(October) -2014	3458	0.703	1.383	0.804	0.092	1.72	1.97
(November) -2014	3078	0.638	1.482	0.821	0.042	1.69	1.70
(December) -2014	4118	0.724	1.605	0.710	0.029	1.88	1.83
(January) -2015	3045	0.524	1.400	0.689	0.088	1.71	1.20
(February) -2015	3951	0.510	1.180	0.587	0.022	1.61	1.40
(March) -2015	4088	0.910	1.820	1.011	0.521	1.84	1.76
(April) -2015	3852	0.751	1.952	0.648	0.458	1.74	1.66
(May) 2015)	4315	0.845	1.562	0.455	0.478	1.98	1.87

Table:02 O.tenuiflorum was subjected to cDNA conversion. This table shows the quantification and absorbance value of cDNA observed in spectrophotometer.

Real time analysis:

Representation of Real time PCR analysis data.

Sample: Green Ocimum tenuiflorum L	Actin	**Sample**	**ΔCt**	ΔΔCt	R.Q
July 2014	29.451	24.652	-4.79	-0.06	1.04
August 2014	28.652	22.541	-6.11	-1.38	2.60
September 2014	30.589	24.832	-5.75	-1.02	2.02
October- 2014	28.629	23.892	-4.73	0	1
November-2014	34.479	29.049	-5.43	-0.7	1.6
December-2014	29.234	23.344	-5.89	-1.158	2.22

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January-2015	28.917	22.619	-6.297	+1.56	2.94
February-2015	30.156	23.787	-6.369	-2.15	4.4
March-2015	30.775	24.421	-6.354	-1.95	3.8
April -2015	29.225	23.121	-6.104	-1.70	3.24

Table:03 cDNA was subjected to Realtime PCR. This table shows Relative expression level observed of PAL Gene expression

Transcriptional Analysis:**By using the results of real time PCR, a graph representing relative quantification Vs expression in months was depicted below.**

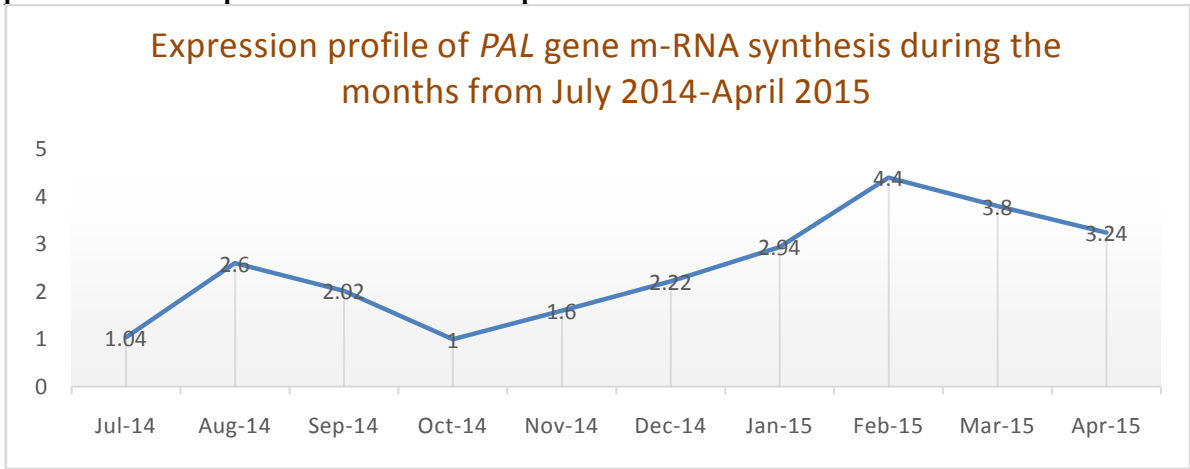

Figure:02 Relative Expressional Profile of PAL gene along with variation in Months.

GC-MS (gas chromatography-mass spectroscopy) analysis

The GC–MS analysis of the samples of Ocimum tenuiflorum was performed. Analyses of the extracted samples were performed in a Clarus 500 gas chromatograph, using Elite5MS fused silica capillary column. Mass range was recorded from 100 to 600 m/z with electron energy of 70 eV. The instrument was operated using Turbomass v 5.4.2.1617 software and identification of the major compounds was done by Wiley Mass Spectral Browser ver. 3.2.3 and NIST 2005 GC–MS Mass Spectral library ver 2.0.

Authentic standards of eugenol, chavicol, methyleugenol,chavicol,isoeugenol and chalcone were used for quantification

The preliminary analysis of the extracts using GC– MS provided a snapshot of the several volatile compounds present among samples in selected the months in Ocimum tenuiflorum plants. The essential oil as eugenol, ethlyeugenol, isoeugenol, chavicol were taken into consideration and with respect to their retention time, their area was estimated**.**

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Table:04 Showing the value of essential oil in respect of their retention time and m/z ratio which was compared to their standards. From Months October 2014 to April 2015

Compound	Retention time	m/z ratio	October	November	December	January	February	March	April
Eugenol	9.88	164.28	1569692	2318073	295085	795010	483320	2176431	4585058
Isoeugenol	9.74	164.08	8285981	929832	780592	92058	50838	985061	1063987
Methyeugenol	10.48	178.09	61487560	50927816	35930616	20417322	83440880	47332092	84661808
Chavicol	10.83	134.18	12608938	13158378	11140470	5487566	22319134	13343868	28399534
Chalcone	11.90	208.26	5368947	6317672	4858067	4640524	5099718	5170544	5460119

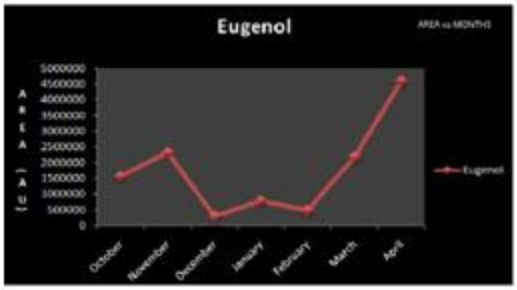

Figure: 03 Eugenol Level

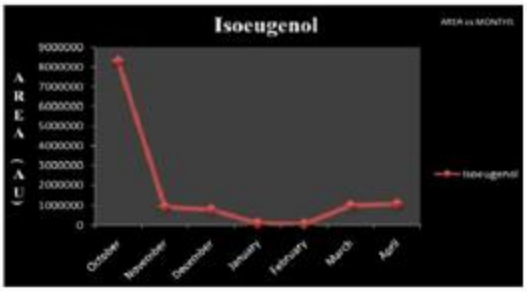

Figure: 04 Isoeugenol Level

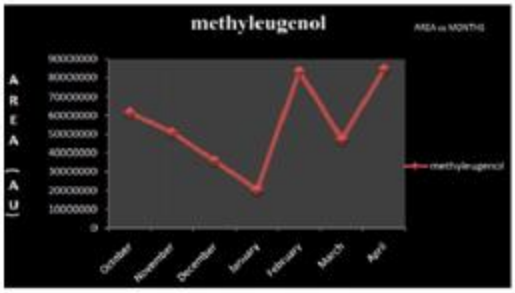

Figure: 05 Methyleugenol Level

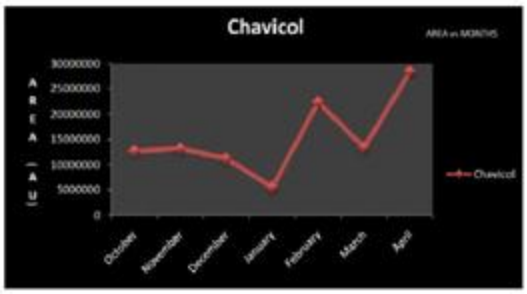

Figure: 06 Chavicol Level

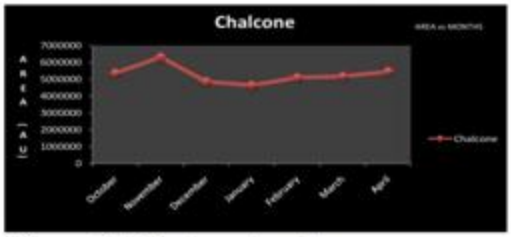

Figure: 07 Chalcone Level

Figure: 08- Essential oil which were secreted from Plant were subjected to GC-MS and based on their standard and retention time the essential oils were detected and their value to their Area was plotted on graphical Patten

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with their Months from October 2014 to April 2015.

IV. DISCUSSION:

In a realtime PCR assay a positive reaction is detected by accumulation of a fluorescent signal. The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level). Ct levels are inversely proportional to the amount of target nucleic acid in the sample (i.e. the lower the Ct level the greater the amount of target nucleic acid in thesample). Cts< 29 are strong positive reactions indicative of abundant target nucleic acid in the sample Cts of 30-37 are positive reactions indicative of moderate amounts of target nucleic acid Cts of 38-40 are weak reactions indicative of minimal amounts of target nucleic acid which couldrepresent an infection state or environmental contamination

There are few review papers regarding the variation of flavonoids secretion with changing seasons but the comparative study of secretion of flavonoids was not identified relative to the months.The transcriptional study of PAL gene throws indirect light to difference in flavonoids secretion.

RNA isolation was done showing 28s, 18s, 5s RNA and c-DNA conversion then Real-time analysis shows the results. In Transcriptional analysis plotted graph represented by Figure above it is very well observed that the PAL gene expression depends on the stress condition factor especially seasonal change from summer and winter seasons. In our case, stress factor caused flavonoids secretion to increase that factor is winter season. The transcriptional analysis has shown firstly low PAL expression in months from July to October, then it showedup-regulation in November and expectedly while coming to December, January, February PALGene expression profile showed many folds increase in expression level certainly due to stress condition which arises due to winter season. The analysis shows PAL gene expression to increase in percentage of 60,122,194.340,224 in months November, December, January, February, and March respectively in comparison to base line of October month which was chosen as October month is transition stage from Summer to winter season.

In GC MS graph which has shown the graphical representation of essential oil. The graph which was derived from plotting the area to months shows various result which implies that the concentration of essential oil and other component varies with change in season.

The graphs showed that eugenol concentration remains low in October month but with the coming of November, it increases, but again in December it decreases drastically after which it increases slightly again and reached to high fold in month of February.

In contrast to eugenol, isoeugenol shows a great increase in October month then it slightly increases and then decrease. It repeats this slight increase decrease concentration.

Methyl eugenol showed the zig -zag pattern of increase decrease in concentration. It previously increased in October, decreased from November to January then high concentration in

February and again decreased in March.

Chavicol the odorant compound, in same respect showed similarity in concentration to

methyl eugenol. It increased in October, then started to decrease from November to January,

then again increased in February, and then decreased.

Chalcone is the compound, which showed minimal variation with respect to season. It onlyshowed increase in months of November and then decrease until March, after then aslight increase in concentration.

These all result shows that except isoeugenol all theessential oil components as eugenol, chavicol, meth eugenol, chalcone all shows agreater concentration in the winter season.

Our finding is in line which the theory that when stress occurs plant develop a kind of safety mechanism to cope with the stressful condition and in various kind of defense mechanism elevation of flavonoids secretion and essential oil secretion is common among various processes.

V. CONCLUSION:

The transcriptional analysis observed through real time PCR analysis reflected that the transcription of flavonoids biosynthesis genes depended on the stress condition, which prevails with variation in seasons. It can be exclaimed that the transcription of PAL gene is high in the month of winter season i.e. from November to March. Similarly,the result which derived from GC-MS chromatograph also shows that theessential oil secretion also increases to greater folds in winter season,with the exception to isoeugenol.

O.tenuiflorum which has bundles of therapeutic activities can be widely used to extract

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the bioactive components which are responsible for therapeutic components. Our study throw light on the duration in which the secretion of essential oil and flavonoids is maximum so that if the extraction process of bioactive components is to be done then these months can be considered for better extraction process.

Drawbacks:

Though an elaborate study needs to be done on patient in case control manner to know in which season desire bioactive components when given is specific dose give better result. Apart from this detailed analysis of subdivision of PAL gene expression pathways need to be done to know more associated factor which can impact the secretion pathway of states gene.

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Disclosure:
None.

REFERENCE:

(1) Eckert AJ, Hall BD: Phylogeny, historical biogeography, and patterns of diversification for Pinus (Pinaceae): phylogenetic tests of fossil-based hypotheses. Mol PhylogenetEvol 2006, 40:166-182.
(2) de Laubenfels DJ: The status of "conifers" in vegetation classifications.Ann Assoc Am Geogr 1957, 47:145-149.
(3) Adomas A, Heller G, Li G, Olson A, Chu T, Osborne J, Craig D, Zyl LV, Wolfinger R, Sederoff R, Dean RA, Stenlid J, Finlay R, Asiegbu FO: Transcript profiling of a conifer pathosystem: response of Pinus sylvestris root tissues to pathogen (Heterobasidionannosum) invasion. Tree Physiol 2007, 27:1441-1458.
(4) Dixon RA, Paiva NL: Stress-induced Phenylpropanoid metabolism. Plant Cell 1995, 7:1085-1097.
(5) Koukal J, Conn EE: The metabolism of aromatic compounds in higher plants. IV. Purification and properties of the phenylalanine deaminase of Hordeum vulgare. J Biol Chem 1961, 236:2692-2698.
(6) Anbarasu K, Vijayalakshmi G. Improved shelf life of protein-rich tofu using Ocimum sanctum (Ocimum tenuiflorum L) extracts to benefit Indian rural population. J Food Sci. 2007;72:M300–05. [PubMed] [Google Scholar]
(7) Kelm MA, Nair MG, Strasburg GM, DeWitt DL. Antioxidant and cyclooxygenase inhibitory phenolic compounds from Ocimum sanctum Linn. Phytomedicine. 2000;7:7–13. [PubMed] [Google Scholar]
(8) Shishodia S, Majumdar S, Banerjee S, Aggarwal BB. Urosolicacidinhibits nuclear factor-kappaB activation induced by carcinogenic agents through suppression of IkappaBalpha kinase and p65 phosphorylation: Correlation with down-regulation of cyclooxygenase 2, matrix metalloproteinase 9, and cyclin D1. Cancer Res. 2003; 63:4375–83. [PubMed] [Google Scholar]
(9) Johnson M, WeselyEG, Hussain MIZ and N Selvan, In vivo and in vitro phytochemical and antibacterial efficacy of Baliospermummontanum(Willd.) Muell. Arg, Asian Pac J Trop Med, 2011, 3 (11),894–897
(10) Vrinda B and Uma Devi P. Radiation protection of human lymphocyte chromosomes in vitro by orientin and vicenin. Mutat Res. 2001 Nov 15;498(1-2):39-46.
(11) Singh.E, Sharma.S, Dwivedi.J and Sharma.S, Diversified potentials of Ocimum sanctum Linn (Ocimum tenuiflorum L): An exhaustive survey, J. Nat. Prod. Plant Resour., 2012, 2 (1):39-48
(12) Renu IK, Kumar M, Rai A,HaqueI,Poddar R, Mukhopadhyay K. (2014),Characterization and functional analysis of eugenol O-methyltransferase gene reveal metabolite shifts, chemotype specific differential expression and developmental regulation in Ocimum tenuiflorum L, Molecular Biology Reports 41:7-10.