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DETECTION OF PHYTOCONSTITUENTS OF CALLUS AND LEAVES OF *SCHINUS TEREBINTHIFOLIUS* RADDI

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ABSTRACT

The present study focuses on the development of callus from *Schinus terebinthifolius* Raddi and the detection of its phytoconstituents. The first step of research work focuses on the development of callus by using plant growth regulators- auxin (2,4-Dichlorophenoxyacetic acid ie 2,4-D) and cytokinin (Kinetin). 2,4-D and Kinetin both induced callus germination individually and concentration of plant growth regulators had positive effect on callus growth. 2,4- D showed the better callus indexes compare to the kinetin. The callus with best callus index and explant used (leaf) was used for GC-MS analysis for identification of phytoconstituents. Phytochemical analysis showed more concentration of most of the phytoconstituents in callus as compared to leaf, serving it as a possible tool for extracting phytoconstituents.

Keywords*: -* *Schinus terebinthifolius*, Callus, phytoconstituents, micropropagation

INTRODUCTION

The plant *Schinus terebinthifolius* Raddi; also known as Brazilian pepper tree, belongs to the Anacardiaceae family. It is an evergreen shrub that grows in central and south America (Morton,1978; Leite *et al* 2011). Since 1999 a gel-based aqueous bark extract of *S. terebinthifolius*is being marketed for the treatment of vaginitis and cervical vaginitis. The decoction of *S. terebinthifolius* is recommended by Brazilian pharmacopeia as a natural anti-inflammatory agent (Carvalho *et al* 2013). The leaf extract has been found to exhibit anti-oxidant anti-allergic, anti-inflammatory, anti-cancer, antimicrobial, antiulcer, and anti-adherent properties as well as wound-healing properties (Gomes *et al*,2010; Barbieri *et al, 2014;* Silva *et al*, 2017; Farag, 2008; El-Massry *et al 2009*). Chemical studies of extract of *S. terebinthifolius* leaves showed polyphenolic and flavonoid are major constituents (Santana *et al 2012;* Moustafa, 2007). The leaves of this plant are popularly used for healing ulcers and wounds, combating oral candidiasis in children (Bendaoud *et al,* 2010)

Callus tissue is a group of parenchyma cells that are irregularly shaped and formed on the wound or cutting areas of the plant. For the growth of callus, a different parts of the plant like leaf, stem, knot, and tuber can be used on artificial media such as Murashige and Skoog (MS) medium, White's medium, and the woody plant medium, etc (Lieber,2013; Perianez-Rodriguez *et al*,2014; Schell *et al, 1993*), equipped with at least one of plant growth regulators. During the callus development, the cells undergo multiple changes, like change in the size, shape, composition, and the significant increase in some important structural processes such as protein binding and nucleic acids (Finer *et al,1989*). The secondary metabolite or drug can be directly extracted from callus tissue without sacrificing the whole plant making this technique as a possible alternative for the conservation of medicinal plants (Schauer *et al,* 2005). There is no data available for callus culture from *S. terebinthifolia* leaf and its phytoconstituents till now. The present study focuses on the establishment of callus culture and phytochemical investigation of the callus that will pave the way for further research.

MATERIALS AND METHODS

Development of callus culture:

The leaves of plant cultivated in the Sangli district (MS); India were procured in the month of August for research work. The leaves of were rinsed in sterile distilled deionized water for 15 minutes. Followed by rinsing in a 1% sodium hypochlorite with two drops of tween 20 for

ten minutes. The tresses of chemicals were removed by rinsing leaves with sterile distilled deionized water three times for five minutes each. The Murashige and Skoog agar media with different concentrations of2,4, -Dichlorophenoxy acetic acid and kinetin were used. 1 gm of 2,4, -Dichlorophenoxy acetic acid and Kinetin were dissolved separetaly in 1 L of distilled water to make the stock solution. The solubility of kinetin was achieved by first dissolving it in 1% NaOH. The pH of the stock solution was then adjusted with hydrochloric acid. The leaf disc was placed adaxially side down into the test tube containing 50 ml of Murashige and Skoog media and kept in the incubator at 25± 2° C under 16 hr photoperiod of 2000 lux with white light. The relative humidity was maintained 60-70% during incubation. The subculturing was done after 4 weeks.

Callus index calculation –

The formula for the calculation of the callus index

$$\text{Callus index} = \left(n \times \frac{G}{N}\right) 100$$

n = Total number of callused explants; G = Average weight of callus rating on explants; N = Total number of cultured explants; Statically analysis complete randomized design

HUMAN

STATASTICAL ANALYSIS

The statistical analysis and error graph plotting was done by using Microsoft excel software. Anova: Two-Factor with Replication was applied to check the P -value.

Extraction of callus (Figueiredo *et al, 1996*)

Prepared callus was dried in the oven at 70^{0}C for 48 hrs. 0.5 gm of dry callus powder was added to the 25 ml of 50% ethanol and ultrasonicated for 30 minutes at 30^{0}C. The resultant homogenate was centrifuged at 3000 rpm for 15 minutes. After centrifuged, superannuant was retrieved and used to determine the phytoconstituents.

Plant extraction ((Figueiredo *et al, 1996*)

0.5 gm of dry leaves powder was added to the 25 ml of 50% ethanol and ultrasonicated for 30 minutes at 30^{0}C. The resultant homogenate was centrifuged at 3000 rpm for 15 minutes. After centrifuged, supernatant was retrieved and used to determine the phytoconstituents.

Gass chromatography mass spectrometry analysis (GC-MS)

GC-MS (Shimadzu GC-2010 plus, Tokyo Japan) analysis was done at Shivajiuniversity, Kolhapur, Maharshtra, India. GC-MS Set up was with a VF5ms model; fused silica capillary column of 30 m length, 025mm internal diameter, and 0.25 µm film thickness. A Quadrupole system was used for GC-MS detection. Sample size 1 µl was manually injected in splitless mode with scan ranges up to 650 amu for twenty minutes in the total running time of GC-MS analysis.

RESULTS AND DISCUSSION:

Fig no 1: *Schinus terebinthifolius* Raddi

Fig no 2: Developed callus

Table no1: Final weight of callus by using 2,4- D (Wet callus weight)

Concentration of 2,4-D (mg L^{-1})	Weight of wet callus (media 1) (gm)	Weight of wet callus (media 2) (gm)	Weight of wet callus (media 3) (gm)	Weight of wet callus (media 4) (gm)	Average weight of callus ±S.D (gm)	Callus index
0.02	0.047	0.047	0.048	0.0485	0.0476 ±0.00075	4.27
0.03	0.053	0.0491	0.054	0.055	0.0527 ±0.0025	5
0.04	0.059	0.065	0.068	0.064	0.064 ±0.0037	6.01
0.06	0.079	0.078	0.0784	0.0793	0.0786 ±0.00058	6.88

Table 2: Final weight of a callus by using kinetin (wet callus weight)

Concentration of kinetin (mgL^{-1})	Weight of a wet callus (Media 1) (gm)	Weight of a wet callus (media2) (gm)	Weight of a wet callus (media3) (gm)	Weight of a wet callus (media4) (gm)	Total weight of a callus ±S.D (gm)	Callus index
0.02	0.029	0.030	0.028	0.0292	0.029 ±0.00082	2.65
0.03	0.031	0.0321	0.0326	0.0298	0.0313±0.00124	2.85
0.04	0.0334	0.0378	0.0391	0.0365	0.0371±0.00244	3.45
0.06	0.0489	0.0492	0.0486	0.0496	0.0490±0.00042	4.66

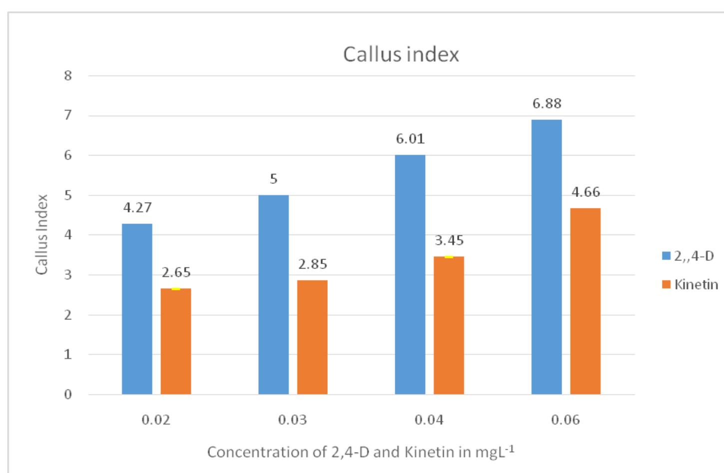

Fig no 3: Graphical representation of Callus development

Table 3: Statistical analysis of data (Anova: Two-Factor With Replication)

SUMMARY	Gr 0.02	Gr 0.03	Gr 0.04	Gr 0.06	Total	
2,4-D						
Count	4	4	4	4	16	
Sum	190.5	211.1	256	314.7	972.3	
Average	47.625	52.775	64	78.675	60.76875	
Variance	0.5625	6.669167	14	0.3425	155.7103	
kinetin						
Count	4	4	4	4	16	
Sum	116.2	125.5	146.8	196.3	584.8	
Average	29.05	31.375	36.7	49.075	36.55	
Variance	0.676667	1.549167	5.966667	0.1825	65.656	
Total						
Count	8	8	8	8		
Sum	306.7	336.6	402.8	511		
Average	38.3375	42.075	50.35	63.875		
Variance	99.11125	134.3679	221.4971	250.5564		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	4692.383	1	4692.383	1253.426	3.14E-22	4.259677
Columns	3074.148	3	1024.716	273.7214	1.09E-18	3.008787
Interaction	156.4984	3	52.16615	13.93458	1.82E-05	3.008787
Within	89.8475	24	3.743646			

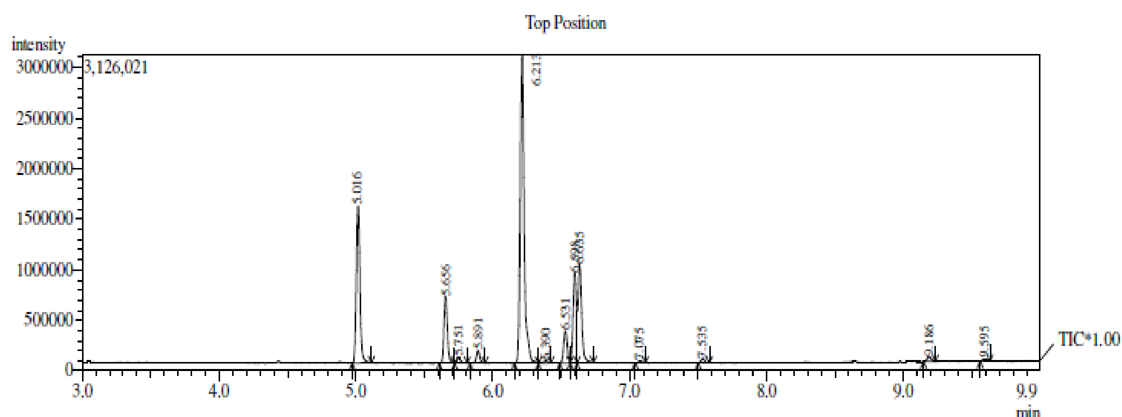

Fig no 4: GC-MS analysis of ethanolic callus extract of *Schinus terebinthifolius* Raddi

Table 4: Phytoconstituent identified in ethanolic callus extract of *Schinus terebinthifolius* Raddi

Sr. no.	Compound name	Molecular weight g/mol	Molecular formula	Area %	Retention time	Invert time	Flat time
1.	α- pinene	136	C10H10	42.64	5.016	4.975	5.115
2.	Bicyclo (3.1.0) hexane, 4-methylene-1-(1- methylethyl)-	136.23	C10H16	18.90	5.656	5.61	5.72
3.	Bicyclo (3.1.1)heptane,6,6-dimethyl,2-methylene (1s)-	136.23	C10H16	1.765	5.751	5.72	5.815
4.	o- Cymene	134	C10H14	3.360 9	6.531	6.49	6.565
5.	β- phellandrene	136	C10H10	29.66 2	6.635	6.615	6.765
6.	Cyclohexane, 3-methylene-6-(1-methylethylidene)-	136.23	C10H16	1.29 8	7.535	7.5	7.58
7.	Terpinene-4-ol	154.25	C10H18O	1.62 6	9.186	9.145	9.235
8.	Bicyclo(3.1.0)hexane-3- ol,4-methylene-1-(1- methylethyl)-,(1alpha,3alpha,5alpha)	154.24	C10H18O	0.74 4	9.595	9.56	9.63

Fig no 5: GC-MS analysis of ethanolic leaf extract of *Schinus terebinthifolius* Raddi

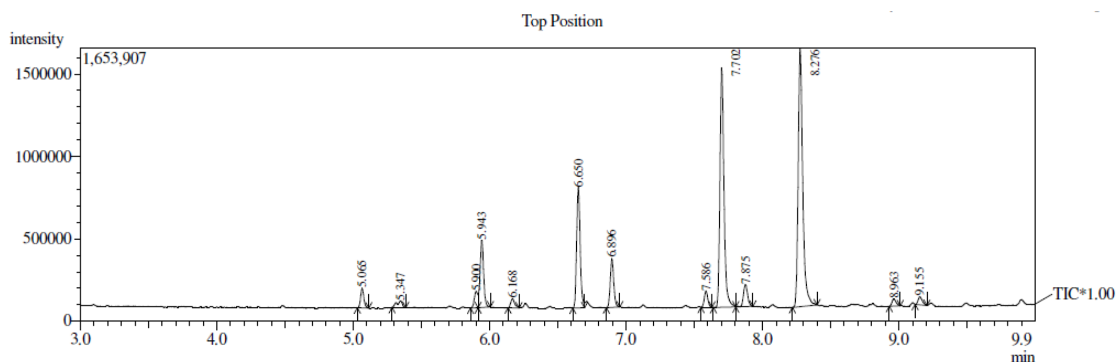

Fig no 5: GC-MS analysis of ethanolic leaf extract of *Schinus terebinthifolius* Raddi

Table 4: Phytoconstituent identified in ethanolic extract of leaf of*Schinus terebinthifolius* Raddi

Sr. No.	Compound name	Molecular weight	Molecular formula	Area%	Retention time	Invert time	Flat time
1	α- pinene	136	C10H10	20.6808	5.016	4.975	5.115
2	Bicyclo (3.1.0) hexane, 4-methylene-1-(1- methylethyl)-	136.23	C10H16	9.1666	5.656	5.61	5.72
3	Bicyclo (3.1.1)heptane,6,6-dimethyl,2-methylene (1s)-	136.23	C10H16	0.8564	5.751	5.72	5.815
4	β-myrcene	136.23	C10H16	1.681	5.891	5.835	5.945
5	o-cymene	134	C10H14	4.531	6.531	6.49	6.565
6	β- phellandrene	136	C10H10	14.3866	6.635	6.615	6.765
7	Terpinene-4-ol	154.25	C10H18O	0.78888	9.186	9.145	9.235
8	Cyclohexane,3-methyl- 6-(1-methylethylidene)	136.23	C10H16	0.6299	7.535	7.5	7.58
9	α- phellandrene	136.23	C10H16	46.9163	6.21	6.16	6.335
10	Bicyclo(3.1.0)hexane - 3-ol,4-methylene-1-(1- methylethyl)-,(1alpha,3alpha,5alpha)	154.24	C10H18O	0.3612	9.595	9.56	9.63

DISCUSSION:

The P-values ensured that the data obtained was of high significance. As compared to the kinetin, 2,4-D showed promising callus growth. The callus growth was supported by the use of only auxin or cytokinin. All concentration of plant growth regulators showed growth of callus, indicating the plant growth regulators support the callus growth independantly.

2,4-D have more positive effect on the callus index. Upon comparison of the GC-MS analysis, the callus extract shows more percentage of many of the phytoconstituents like α-pinene , Bicyclo (3.1.0) hexane, 4-methylene-1-(1-methylethyl)-, β- phellandrene, Cyclohexane, 3-methylene-6-(1-methylethylidene)-, Terpinene-4-ol, Bicyclo(3.1.0) hexane-3-ol,4-methylene-1-(1-methylethyl)-(1alpha,3alpha,5alpha). While β-myrcene and α- phellandrene were found to be missing in callus extract. Only o- Cymene has more area percent in leaf extract as compare to callus extract.

CONCLUSION:

Schinus terebinthifolius Raddi is a tree with many secondary compounds that have a variety of applications. The biosynthesis pathway of secondary metabolite and primary metabolite is the same, but their function is quite different. As compared to primary metabolites, secondary metabolites are not essential for growth and development and are not equally distributed among the taxonomic groups. The secondary metabolite has different applications as natural products, which include dyes, flavoring agents, oils, waxes, perfumes, and drugs. Secondary metabolites are also used as an aid in plant survival by protecting against herbivory and microbial infection by acting as attractancy in the case of pollinating and seed-dispersing animals and as an allopathic agent.

This study focused on the development of callus from *Schinus terebinthifolius raddi* and the detection of its secondary metabolite. The concentration of plant growth regulator affects the callus growth. 2,4- D showed better result as compare to the kinetin. The GC-MS analysis revealed the presence of more number of phytocnstitutents in leaf exctract as compared to callus but the the overall percentage of all the phytoconstituents present in callus extract was much more than that in leaf extract. This can pave a way for further research for establishing relationship between the plant growth and phytoconstituents as well as a sustainable alternative for extraction of phytoconstituents.

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