Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Disease

journal homepage:www.elsevier.com/locate/apjtd

Document heading

Phytochemical evaluation and molecular characterization of some important medicinal plants

Varahalarao Vadlapudi [1*] and D.S.V.G.K.Kaladhar [2]

[1]*Department of Biochemistry, Dr Lankapalli Bullayya P G College, Visakhapatnam-530013, Andhra Pradesh, India*
[2]*Department of Bioinformatics, GIS, GITAM University, Visakhapatnam-530045, Andhra Pradesh, India*

ARTICLE INFO

Article history:
Received 15 June 2012
Received in revised form 27 June 2012
Accepted 18 October 2012
Available online 28 October 2012

Keywords:
Pimenta officinalis
Piper betel
agar well diffusion method
Artemia salina
lethal dosage (LD50)
BLASTN
matK gene

ABSTRACT

Objective: Phytochemical evaluation and molecular characterization of plants is an important task in medicinal botany and drug discovery. In the current study, Ocimum species, *Pimenta officinalis* and *Piper betel* were considered as medicinal plants by evaluation of phytochemical composition like phenol content, Flavonoid content, antioxidant content and other activities like antibacterial, antifungal, lethal dosage (LD 50) of the plant extracts. Among the selected plants *P. officinalis* shown higher medicinal properties and is selected for molecular characterization. **Methods:** **A**ntimicrobial activity by agar well diffusion method and also estimated Total phenols, flavonoids content, Total Antioxidants, Cytotoxic assay on *Artemia salina* for determining lethal dosage (LD50), matK gene was sequenced by using ABI Prism 3700. Leaf extract of *P. officinalis* plant is further selected for GC-chromatographic analysis to know its chemical composition. DNA was isolated by different protocols, optimized, and is used for the PCR amplification of trnL-gene which is a universal marker among plants in molecular taxonomy. The trnL-gene is amplified by using PCR. The product obtained from PCR is purified and the sample is used for sequencing so that it can be used for comparative studies. **Results:** P.offcinalis has shown good antimicrobial activity against all organisms . A. flavus is resistant against *O. sanctum* (B). Phenolic content (26.5 μg/g) is found to be rich in *P. betel* where as flavonoid and Antioxidant content are significant in *P. betel*. The chromatogram revealed the presence of high concentration of Eugenol in the leaf sample. On submitting to BLASTN, the genetic sequence has found similarity with Pimenta dioica plastid partial matK gene and Ugni molinae trnK gene. MatK did not shown any interactions with trnK or trnL genes. MatK has shown interactions with various genes like ycf5, pclpp, psbh, atph, NDVI, rpoc1, ndha, ndhd, psai. **Conclusions:** we can conclude for this investigation of chemical composition and antioxidative properties of the essential leaf oil of *P. officinalis* that contain eugenol, methyl eugenol and β-caryophyllene were found as main components with this capacity. P.offcinalis can be used as alternative source to synthetic drugs against Klebsiella, Pseudomonas, A. niger, A. flavus *T. versicolor* infections. *In silico* analysis did not predicted any interactions between matK and trnL or trnK genes.

1. Introduction

The discovery and developments of antibiotics have led to a dramatic improvement in the potential treatment of infectious diseases and are among the key advances of the 20th century. The development of competent antibacterial agents has been accompanied by the emergence of drug-resistant organisms because of failure to complete a course of treatment, the irrational and overuse of antibiotics, genetic versatility of microbes and horizontal transfer of resistant genes among bacterial species. All the proposed factors reduce the clinical effectiveness of antibiotics [1]. Medicinal plants represent a rich source of antimicrobial agents [2-6]. Based on World Health Organization (WHO) reports, more than 80% of the World population relies on traditional medicine for their primary healthcare needs [7, 8]. These living factories are able to generate endless biochemical compounds [9]. *Pimenta officinalis* Lindl. syn. Pimenta dioica (L.) Merr. (Family -Myrtaceae) commonly known as Clove pepper, Jamaica pepper, Pimento, Allspice. Pimenta offcinalis is native to the West Indies, Southern Mexico, and Central America and is widely cultivated in warm regions of the world [10]. *P. officinalis* is considered an anodyne against rheumatism and neuralgic, powdered

*Corresponding author: Dr. Varahalarao Vadlapudi.Department of Biochemistry, Dr Lankapalli Bullayya P G College, Visakhapatnam-530013, Andhra Pradesh, India.
Phone: 9985670299
email: drvadlapudi@yahoo.in

fruits are used against flatulence, dyspepsia and diarrhea. It is also used in the preparation of tonics and purgatives. Pimento berry oil is used as a carminative and stimulant. It shows bactericidal, fungicidal and antioxidant properties. All spice is used as a paste to soothe and relieve toothache and as a mouth wash to freshen the breath. *Piper betel* (Local name 'Pan') belongs to Piperaceae and is a dioecious, perennial creeper, climbing by many short adventitious rootlets. Ocimum bacillicum is a member of Lamiaceae (mint family) has medicinal properties.Some basil varities are annuals while others are perennials. Ocimum canum is populaely known as holy basil. And belongs to the family Lamiaceae. It is an erect herb found through out india. *O. gratismum* is originating in the Orient and is widespread throughout tropical countries including Brazil. The Ocimum species is used in traditional medicines in South America and Africa for a variety of therapeutic purposes which includes its use in treating bacterial infections, diabetes, diarrhea [11], respiratory-tract infections, fever, pneumonia and coughs [12]. Ocimum santum belongs to the family Lamiaceae. It is commonly called as Vishnu priya, tulasi, Bharati, krishnamul, holi basil, Basil saint, Kala tulasi, Humpina, Shiva tulasi, Karitulasi, Madura tulla and others. It is a small herb found through out india. It is cultivated near hindu houses and temples and the leaves,seeds and roots of this plant are very useful.

2. Materials and methods

Pimenta offcinalis, *Piper betel*, Ocimum basilicum, Ocimum canum, Ocimum gratissimum, Ocimum sanctum (brown) and Ocimum sanctum (green) plant samples were collected from Vijayawada of Krishna district. They were stored in sterile polythene bags and transferred to the laboratory and stored at 4 ℃temperatures till the time of use. All the chemicals used for estimations and media preparation were procured from Himedia Laboratories Pvt. Ltd, Mumbai and Merk India Pvt. Ltd, Mumbai, Fisher Inorganic and Aromatics Limited, Chennai.

2.1 Tested organisms

All the organisms used were procured from Microbial Type Culture Collection and Gene Bank (MTCC), IMTECH, Chandigarh. The lyophilized form of pure strain was reconstituted in sterile water and produced a suspension of the microbial cells. Inoculation was done with sterile inoculating loop to liquid broth medium. Liquid cultures are then incubated to allow cell replication and adequate growth of the culture, for use in bioassays. Following incubation, liquid cultures are refrigerated to store for further use. Typically, 24 hours will provide sufficient growth to allow visibly thick spread of the microbes as required for bioassay. The bacterial strains are maintained and tested on Nutrient Agar (NA) and Potato Dextrose Agar (PDA) for fungi.

2.2 Estimation of Total phenols

The total phenolic content of the purified fractionswas determined using the Folin Ciocalteau method reported by [13]. Briefly, to 0.1mL of the extract, 0.5mL of Folin Ciocalteau reagent and 5.0mL of sodium carbonate were added. There action mixture was allowed to stand for 30min and the absorbance was measured at 640 nm. Gallic acid was used as the standard.

2.4 Estimation of flavonoids content

To estimate the amount of flavonoids content in the plant extract, Quercitin is used as standard. Quercitin of known concentration is mixed with Methanol, Alluminium chloride and Potassium acetate solution followed by incubation at room temperature for 30 minutes. The color developed is measured at 420 nm by [14].

2.5 Estimation of Total Antioxidants

To estimate the reducing strength (anti-oxidant activity) of the plant extract, Ascorbic acid is used as standard. Ascorbic acid of known concentration is mixed with Phosphate buffer, Potassium ferry cyanide, followed by incubation at 50℃ for 20 minutes. Then TCA is added and the contents are centrifuged at 10,000 rpm for 2 min to obtain supernatant. Volume of the supernatant is doubled with distilled water and Ferric chloride is added. The color developed is measured at 680nm by using [15].

2.6 Preparation of plant extracts

The collected plant materials were chopped into small pieces and coarsely powdered in Willy mill. The coarsely powdered material weighed and extracted with methanol using a soxhlet extractor for five to six hours at temperature not exceeding the boiling point of the solvent. For each gram of dry material 2 ml of solvent was used. The extracted solvents were filtered through Whatman no-1 filter paper and subsequently concentrated under reduced pressure (in vacuo at 40℃) using a rotary evaporator. The residue obtained were designated as crude extracts and stored in a freezer at -20℃ until bioassayed.

The dried plant extract residues obtained were redissolved in 0.1% Dimethyl Sulfoxide (DMSO) to get different concentrations (100 mg/ml, 200 mg/ml, 300 mg/ml, 400 mg/ml, and 500 mg/ml) of crude extracts and filtration through a 0.45 μm membrane filter and stored in sterile brown bottles in a freezer at 20℃ until bioassayed.

The prepared hexane, chloroform and methanol extract samples were tested for antibacterial and antifungal activity against the test organism's using the agar cup plate / well diffusion method.

2.7 Determination of antimicrobial activity

The crude extracts of the different plant parts of different species were subjected to antimicrobial assay using the agar well diffusion method of [16] modified by [17]. 20 ml of nutrient agar was dispensed into sterile universal bottles these were then inoculated with 0.2 ml of cultures mixed gently and poured into sterile petri dishes. After setting a number 3-cup borer (6mm) diameter was properly sterilized by flaming and used to make three to five uniform cups/wells in each petri dish. A drop of molten nutrient agar was used to seal the base of each cup. The cups/wells were filled with 50 μl of the extract concentration of 100mg/ml, 300mg/ml and 500 mg/ml and allow diffusing for 45 minutes. The solvents used for reconstituting the extracts were similarly analyzed. The plates were incubated at 37℃ for 24 hours for bacteria. The above procedure is allowed for fungal assays but except the media potato dextrose agar instead of nutrient agar and incubates at 25℃ for 48 hours. The zones of inhibition were measured with antibiotic zone scale in mm

2.8 Preparation of plant Extract for cytotoxic assay

Plant leaf sample was air dried and one gram of sample is taken. This sample is ground with gradual addition of 20% Methanol. After grinding filter the solution and allow to air dry. Then 1ml of DMSO (Dimethyl sulfoxide) is added and mixed well. The solution can be stored till time of use in a refrigerator.

Cytotoxic assay of plant extract by using *Artemia salina* (Brine shrimp) for determining lethal dosage (LD_{50}) using [18]. Plant extract prepared in above manner is used for the cytotoxic assay of Plant extract by using *Artemia salina* (Brine shrimp).The plant extract was diluted to 1 ppm concentration from these different dilutions were prepared for analyzing the lethal dosage against *Artemia salina* on watch glasses. In this analysis three watch glasses were taken and 10 Artemia were added to each watch glass. Then, 1ml of different dilutions of concentration 5, 50 and 500 ppm were added to three watch glasses and incubated at room temperature for 24 hr.The lethal dosage is calculated by comparing the live ones against dead ones and percentage of live Artemia were calculated.

2.9 Extraction of plant material

Pimento leaf sample was air dried and 50g of sample was taken.The leaf sample was powdered and extracted with Petroleum ether (60-80 ℃).The extract is concentrated by distilling the solvent and dried in an oven at 50 ℃ by heating. The Marc was dried in an air oven below 50 ℃. Then, the marc is extracted with Toluene, Chloroform, Acetone and Methanol respectively as in case of Petroleum ether. Finally, the marc was extracted with water by macerating it with for 24hr.Thus the ingredients of leaf extract were extracted with polar and non-polar solvents so that the sample is completely extracted.

2.10 Development of chromatogram

For analysis DB-Wax capillary column of dimensions 30mx0.32mm, 0.5 μ is chosen. Injection volume is 0.1 μl (with 10 μl Hamilton syringe) and injection temperature is 240 0C.Helium gas is used as carrier and the oven conditions at 40℃ (3 min) to 220℃ for 8 min. FID (Flame ionization detector) is used as detector unit and detector temperature is 240 ℃ with split ratio of 1:20. The analyzer unit used is a computer (Shimadzu 17 A Japan, GC equipped with FID detector) and the software employed is Spinchrom CFR software. By using above conditions, the chromatogram is obtained in the form of a graph.

2.11 Isolation of plant DNA and its optimization

Isolation of DNA by using SN-Buffer by [19]. 0.3gm of plant sample was weighed chopped in to pieces and it was grinded completely with 200 μl of TE-buffer and to this 300 μl of SN-buffer was added and incubated for 10minutes at 65℃ and it was centrifuge for 2minutes at 10,000rpm. The supernatant was collected and to it 100 μl of sodium acetate (3M) was added, mixed well by many inversions and again centrifuged for 2minuts at 10,000rpm. Again the supernatant was collected and to that 600 μl of ice cold isopropanol was added and centrifuged at 10,000rpm for 2min.after centrifuged the supernatant was discarded and the pellet was washed with 90% ethanol, the supernatant was discarded and the pellet was again washed with 70% ethanol for removing co contaminants and salts. The pellet was completely dried until the ethanol is evaporated. Finally the pellet was stored at -20℃ by dissolving in TE buffer.

2.12 Isolation of DNA by using ST buffer

About 0.3gm of plant sample was weighed chopped in to pieces and it was grinded completely with 200 μl of TE-buffer and to this 300 μl of ST-buffer was added and incubated for 10 minutes at 65℃ and it was centrifuge for 2 minutes at 10,000 rpm. The supernatant was collected and to it 100 μl of sodium acetate (3M) was added, mixed well by many inversions and again centrifuged for 2minuts at 10,000rpm. Again the supernatant was collected and to that 600 μl of ice cold isopropanol was added and centrifuged at 10,000 rpm for 2 min.after centrifuged the supernatant was discarded and the pellet was washed with 90% ethanol, the supernatant was discarded and the pellet was again washed with 70% ethanol for removing co contaminants and salts. The pellet was completely dried until the ethanol is evaporated. Finally the pellet was stored at -20℃ by dissolving in TE buffer [20].

2.13 matK-gene sequencing

The amplified and purified matK sequence sample is sequenced by using ABI Prism 3700 carried out at MWG Eurofins India Pvt Ltd, Bangalore

3. Results

The amount of phenol content, Flavonoid content and antioxidant content has been estimated in various selected medicinal plants. Phenolic content (26.5 μg/g) is found to be rich in *P. betel* whereas flavonoids and Antioxidant content are significant in *P. betel* (Table 1).

All the extracts tested against the growth of microorganisms are shown good inhibition zones which are varied and effective. *P.offcinalis* has shown good antimicrobial activity against all organisms (Table 2). A. flavus is resistant against *O. sanctum* (B).

With the increasing the concentrations of *P. offcinalis* extract from 5, 50, 500 μg worked affectively No surviving cells of A. salina (Brine shrimp) were decreased from 9, 3, 0 consequently and found the lethal dosage (LD50) at 500 μg is more effective among all concentrations.

G C analysis *P. offcinalis* plant leaf sample is extracted with different solvents and subjected to Gas chromatography. The chromatogram revealed the presence of high concentration of Eugenol in the leaf sample. The chromatogram and details of analysis is presented in Figure 1

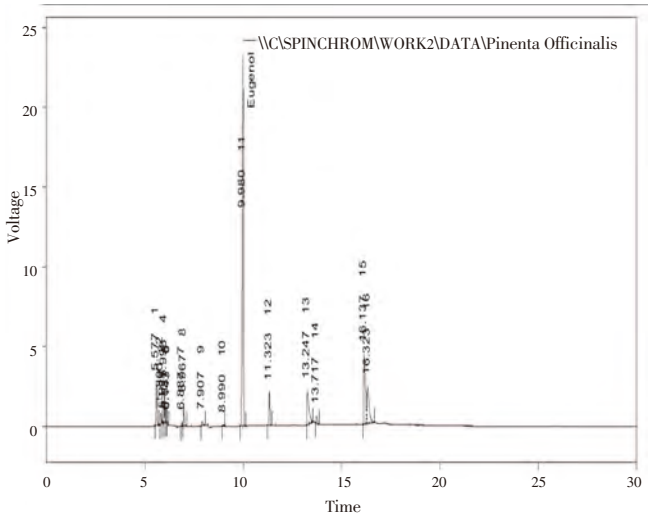

Figure 1: GC- chromatogram of *P.offcinalis*

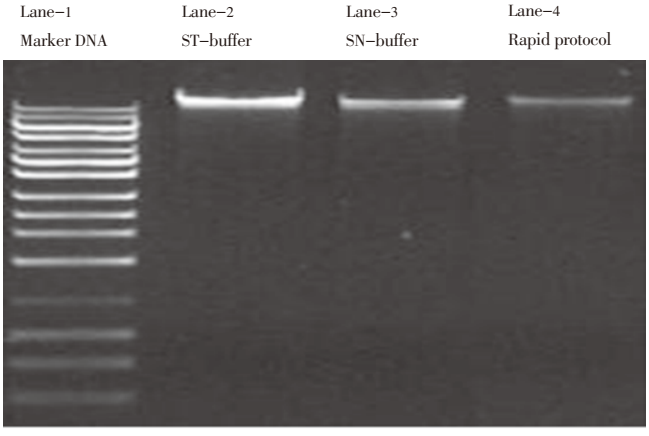

Figure 2: Plant DNA isolation

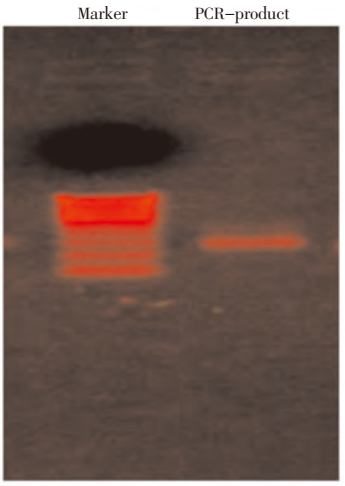

Figure 3- PCR amplification of plant chloroplast trnL-gene and its purification

The PCR product is purified by treating with various reagents to get rid of contaminants like enzymes and other reaction ingredients like dNTPs (Figure 3 and 4). Then, the purified product is subjected to Agarose gel Electrophoresis to evaluate its purity. The pattern of gel running is as follows.

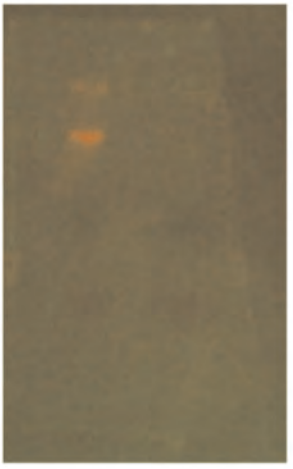

Fig 4- PCR product purification

matK sequence: 845bps

AATTTCACATTTAGATTATGTGTTGGATGTACTAATGCCCTA
TCACATCCATCTAGAAATCTTGGTTCAAACCCTTCGCTACTG
GGTGAAAGATGCCTCTTCTTGCATTTATTACGTTTCTTTCTCC
ACGAGTATTGGAATAGTCTTATTACTCCAAAGAAACATATT
CCTATTTTTTCAAAAGGGAATCCAAGATTATTCTTGTTCCTA
TATAATTCTCATGTGTGTGAATACGAATCTATCTTTCTTTTTC
TCCGTAATCAATCTTTCATTTACGGTCAACATCTTCTGGAAT
CTTTTTTGAGCGAATATATTTCTATGTAAAAATAGAACATTT
TGCCAAAGTCTTCTTTGATAATGATTTTCAGTGCATCCTATG
GTTCTTCAAAGATCCTTTCATGCATTATGTTAAATATCAAGG
AAAATCAATTCTGGCTTCAAAAGATACGCCTCTTTTCATGAA
TAAATGGAAATATTACCTTGTTAATTTATGGCAATATCATTT
TTACGCGTGGTTTCAACCAGTAAGGATCGATATAAACCAAT

TATGCAATTATTCTATTGACTTTTTAGGCTATCGTTCAAGCG
TGCGACTAAATTCTTCAGTGGTACGAAGTCAAATGCTAGAA
AATGCATTTATAATAAATAATGCTATGAAGAAGTTCGAGAC
AATAGTTCCGATTATTCCTCTGATTGGATCATTGTCTAAAGC
GAATTTTTGTAACACATTAGGGCATCCCATTAGTAAACCGA
CCCGGGCTGATTCATCAGATTCCGATATTATCGACCGTTTTT
TGCGTATATGCAGAAATCTTTCTCATTATCCACCAGGGATCC
TCAAAAAAA

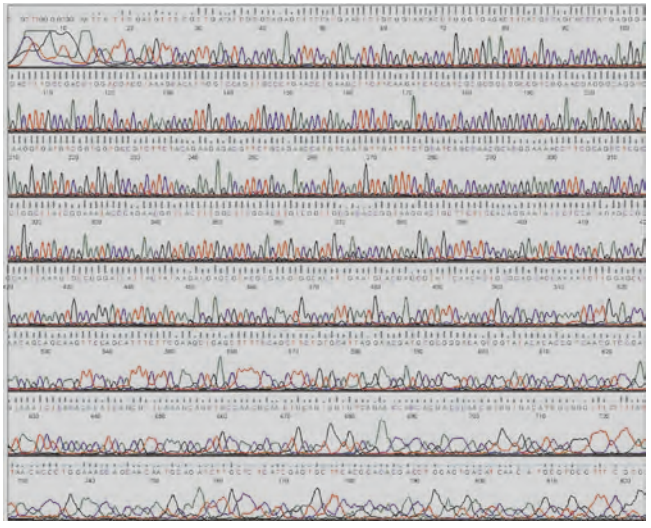

Fig 5: trnl- gene sequencing

Figure 5 has shown the sequence of isolated trnL gene with 545bps length.

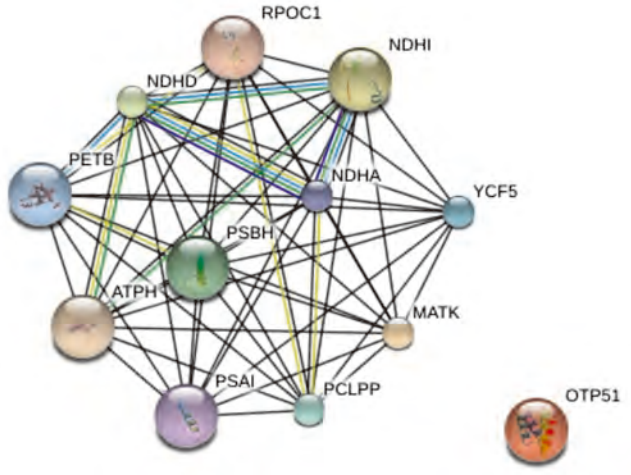

Figure 6: protein-protein interaction of Matk with other genes

4. Discussion

The main characteristic of an antioxidant is its ability to trap free radicals and also reduce the risk of chronic diseases including cancer, central nervous system injury, arthritis and heart diseases. However, the strength of the existing data is not enough to suggest a reasonable mode of action for antioxidant effects. The data of this study may just enrich the existing comprehensive data of antioxidant activity of plant materials. Though several plants are

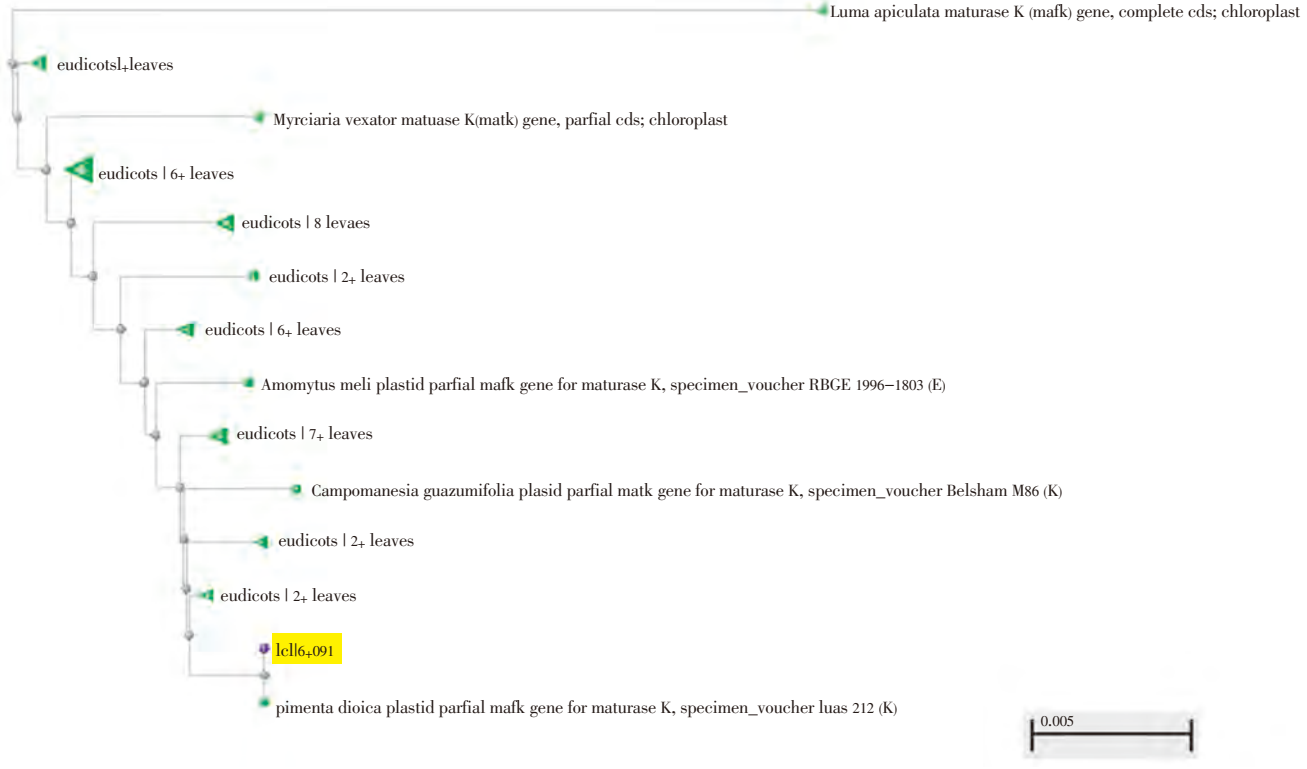

Figure 7 has shown the phylogenetic relationship of matK gene of Campomanesia guazumifolia and Pimento dioica.

Figure 7: Phylogenic tree of matK gene sequence

Table 1

Phytochemical estimation

S.No	Plant name	Phenol content in μg/g	Flavonoid content in μg/g	Antioxidant content (μg/g)
1	O. basilicum	17.5	55	4.6
2	O. canum	18	48.75	6
3	O. gratissimum	16	40	4.6
4	*O. sanctum*(brown)	7.2	93.75	3.33
5	*O. sanctum*(green)	11	25	2.66
6	*P. offcinalis*	7.7	116.25	4.3
7	*P. betel*	26.5	123.5	5.6

Table 2

Assay of antimicrobial activities

S.no	Zone of inhibition in cm				
	Plant name	Klebsiella	Pseudomonas	*A. niger*	*A. flavus*	*T. versicolor*
1	O.basilicum	0.85	0.77	0.5	0.5	0.65
2	O.canum	0.8	0.57	0.55	0.37	0.57
3	O.gratissmum	0.6	0.6	0.47	0.27	0.65
4	O.sanctum(B)	0.82	0.77	0.47	0.21	0.65
5	O.sanctum(G)	0.85	0.72	0.45	0.45	0.55
6	P.betel	0.9	0.55	0.44	0.35	0.75
7	*P.offcinalis*	1.1	0.85	0.57	0.47	0.95

extensively used in traditional medicine. Some authors found correlation between the total phenolic content and the antioxidant activity, others found no such relationship. *P.offcinalis* is good plant against majority of pathogens. Antimicrobial activity may be due to active components which are present in plant extracts. The differences in antimicrobial activity is due to the potential difference in the susceptibility of conidia, germinated conidia and hyphae to antimicrobial compound and the time duration for the exposure of the compound. It is not surprising that there are differences in the antimicrobial activities of plant groups, due to the phytochemical differences between species. Number of surviving cells of *Artemia salina* decreased by increased concentration theof *P. offcinalis* extract in μg. On submitting to BLASTN, the genetic sequence has found similarity with Pimenta dioica plastid partial matK gene and Ugni molinae trnK gene. To study the interaction of matk and trnk gene, we has conducted an *in silico* experimentation by submitting the genes to the string v9 server. The interactions has been presented in Figure 6. MatK did not show any interactions with trnK or trnL genes. MatK has shown interactions with various genes like ycf5, pclpp, psbh, atph, NDVI, rpoc1, ndha, ndhd, psai.

5. Conclusion

Therefore, we can conclude for this investigation of chemical composition and antioxidative properties of the essential leaf oil of *P. officinalis* that contain eugenol, methyl eugenol and β-caryophyllene were found as main components with this capacity. Pimento oil manifested greater antiradical activity with respect to OH• in comparison with DPPH radicals, attested by the respective LC50 values. We found *P. offcinalis* extract lethal dosage (LD50) at 500 μg is more effective among all concentrations on A. salina (Brine shrimp). The inhibitory capacity of pimento oil on OH• exceeded that of quercetin. The oil was a Fe^{3+}-chelator, too, thus preventing the initiation of hydroxyl radicals. Pimento oil inhibited xanthine oxidase activity, which caused a decrease of the generation of superoxide radicals; moreover, there was a mechanism involved – the scavenging of superoxide radicals. Pimento oil was capable of an effective inhibition of both the conjugated diene formation and the generation of secondary products from lipid peroxidation. The use of some methods to determine the antioxidative properties of medicinal plant essential oils confirms the findings that antioxidative capacity detected by only a single method should be interpreted with some caution . *P.offcinalis* can be used as alternative source to synthetic drugs against Klebsiella, Pseudomonas, *A. niger, A. flavus T. versicolor* infections. According to the literature, it was possible to conclude that depending on the chemical compositon of the Pimenta oils, they can act as anti-inflammatory affecting the arachidonic metabolism or the cytokines production, or on the modulation of pro-inflammatory gene expression. The antioxidant and anti-inflammatory activities of the essential oils are well documented; nevertheless their uses can be hampered due to the chemical variability of the oils. Several factors including harvesting time of the aromatic plant, climatic and agronomic conditions, vegetative development of the plant, the plant part used, type of extraction used can be considered as responsible for fluctuations in their chemical

compositions. The established flavnoids and antioxidant properties of pimento oil thus broaden the scope for its implementation in food industry and medicine. The product obtained from PCR is purified and the sample is used for sequencing so that it can be used for comparative studies. *In silico* analysis did not predicted any interactions between matK and trnL or trnK genes. We believe that information provided can be useful to many research groups considering the huge interest in the search for new natural sources of functional ingredients.

Acknowledgements

I am thankful to Department of Biochemistry, Dr L B PG College, Visakhapatnam, India for constant Financial support from department internal source.

Referances

[1] Amit R, Shailendra S. Ethnomedicinal approach in biological and chemical investigation of phytochemicals as antimicrobials. Available at http://www.phamainfo.net. Accessed April- 28, 2007.

[2] Suresh KP. Anti-fungal activity of Leptadenia reticulata in rat animal model in vivo. *J Basic & Appl Biol* 2008; **2**(1): 9-13.

[3] Emamuzo ED, Miniakiri SI, Tedwin EJO, Ufouma O, Lucky M. A*nalgesic and anti-inflammatory activities of the ethanoTrop Med* 2010; **3**(5): 341-347.

[4] Haripriya D, Selvan N, Jeyakumar N, Periasamy RS, Johnson M, Irudayaraj V. The effect of extracts of Selaginella involvens and Selaginella inaequalifolia leaves on poultry pathogens.*Asian Pac J Trop Med* 2010; **3**(9): 678-681.

[5] Selvamaleeswaran P, Wesely EG, Johnson M, Velusamy S,Jeyakumar N. The effect of leaves extracts of Clitoria ternatea Linn against the fish pathogens. *Asian Pac J Trop Med* 2010; **3**(9):723-726.

[6] Hasan MF, Khan A, Rahman M, Sikdar B. Determination of antibacterial and antifungal activities of Polygonum hydropepper L. root extract. *J Basic Appl Biol* 2009; **3**(1&2): 6-10

[7] Duraipandiyan V, Ayyanar M, Ignacimuthu S. Antimicrobial activity of some ethnomedicinal plants used by paliyar tribe from Tamil Nadu, India. *BMC Complement Altern Med* 2006; **6**:35.

[8] Duraipandiyan V, Ignacimuthu S. Antibacterial and antifungal activity of cassia fistula l.: An ethnomedicinal plant. *J Ethnopharmacol* 2007;**112**: 590-594.

[9] Abdallah EM. Plants: An alternative source for antimicrobials. *J. Applied Pharmaceutical Sci* 2011; **1**: 16-20.

[10]Cowan MM. Plant products as antimicrobial agents. Clin. Mic.*Rev* 1999; **12**(4): 564-586.

[11]Dorman HJD, Deans SG. Antimicrobial agents from plants: Anti-bacterial activity of plant volatile oils. *J. App. Microbiol* 2000; **88**: 308-316.

[12]Riffle, R.L..The Tropical Look. Timber Press, Portland, OR. 1998.

[13]Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phospho tungstic acid reagents. *American Journal of Enology and Viticulture* 1965; **16**(3): 144-158,

[14]Kumar S. Antioxidant and free radical scavenging potential of Citrullus colocynthis (L.) Schrad. Methanolic fruit extract, *Acta Pharm* 2008; **58**: 215-220.

[15]Shon, MY, Kim TH, Sung NJ. Antioxidants and free radical scavenging activity of Phellinus baumii (Phellinus of Hymenochaetaceae) extract. *Food Chem* 2003; **82**: 593-597.

[16] urray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken HR. Manual of clinical microbiology, 6th Edition, ASM Press,*Washington DC,* 1995, 15-18.

[17]Olurinola PF. A laboratory manual of pharmaceutical microbiology. Printed by National Institute for Pharmaceutical Researchand Development, Idu, Abuja, Nigeria, 1996, 69-105.

[18]McLaughlin J. L. In Methods in Plant Biochemistry, Hostettmann, K., Ed.; Academic Press Limited: London; Assays for Bioactivity 1991; 6: p 1-30.

[19]Khanuja SPS, Shasany AK, Darokar MP, Kumar S. Rapid isolation of DNA from dry and fresh samples of plants producing large amounts of secondary metabolites and essential oils. Plant Mol. Biol.Rep 1999; 17: 1-7.

[20]Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 1989.