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STUDY OF ANTIBACTERIAL PHENOLIC COMPOUNDS OF VITEX GENUS LEAVES EXTRACTIONS BY HPLC AND SNPS OF THE FIRST STRUCTURAL BIOSYNTHESIS PATHWAY GENE (*PAL*)

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Abstract

Phenylalanine ammonia-lyase (*PAL*; EC 4.3.1.24) is an important plant enzyme in pathogen defense. The purpose of this work was to analyze the phylogenetic relationships of a partial *PAL* gene sequence approximately 370 bp from *Vitex nrgundo L.*(VN) , *Vitex agnus castus L.*(VAC) and *Vitex pseudo negundo L.*(VPN) Considering the importance of *PAL* in plant responses to biotic stresses, so a partial-lenth genomic DNA sequence of a *PAL* gene from Vitex species were obtained , to characterize the single nucleotide polymorphism (SNPs) within this three species ,and find the relationship between this SNPs, concentrations of the antibacterial phenolic compounds and antimicrobial activity of the leaves of Vitex species against one Gram-positive bacteria *Staphylococcus aureus* and two Gram-negative bacteria *Escherchia coli* and *Pseudomonas aeruginosa*. ethanolic extracts was prepared and studied for antibacterial activity using disc diffusion method, Results showed promising antibacterial activity with two extractions VAC and VPN. a property that supports traditional use of the plant in the treatment of some diseases as broad spectrum antibacterial agents.

***Keywords*:** *PAL*, Vitex, Antibacterial, SNPs, Phenols, Medicinal plants

Introduction

The use of herbal drugs, forming a major part of complementary and alternative medicine or traditional medicine, is on the rise world-wide (Feudis and Drieu, 2004). The increased interest in plant derived drugs is mainly because of the wide spread belief that herbal medicine is safer than costly synthetic drugs which possesses side effects. Hence, there is need to screen medicinal plants for promising biological activity. Further, there is a continuous development of resistant strains which pose the need for search and development of new drug to cure diseases (Silver, 1993).

Phenolic compounds encompass a considerable range of substances such as simple phenols, phenolic acids, acetophenols, phenylacetic ,hydroxycinnamic acids, phenylpropanes, (iso) coumarins, chromones, xanthones, stilbenes, flavonoids, lignans, neolignans, lignins, catechol, melanins and others. All these classes of compounds are derived from phenylalanine and, to a limited extent, from tyrosine, or directly from shikimate pathway intermediates (Harborne, 1980; Hahlbrock and Scheel, 1989). In this study, we used candidate gene (*PAL*) from known biosynthetic pathway (Fig. 2 B) to identify SNPs that explain variation in the concentrations of PSMs that defend vitex leaves against both negative and positive pathogenic bacteria (Carsten *et al*., 2001).

Studies have indicated that the phytochemicals as flavonoids and other phenolic compounds provide significant antibacterial activity and health benefit (Puupponen–Pimia *et al.,* 2001; Sarıbaz *et al.*, 2007), *PAL* enzyme catalyzes the non-oxidative elimination of ammonia from L-phenylalanine to give trans-cinnamic acid a substrate common to the biosynthesis of different phenylpropanoid products (Xu *et al*., 2008), and *PAL* is the first enzyme in the phenylpropanoid pathway, it responsible for coordinating this pathway. *PAL* activity could still be regulating the total flow of carbon into phenolics, therefore, the expression of this gene and the enzyme activity could be considered as markers of this defense response (Distéfano *et al*., 2008; Shoresh *et al*., 2004), So that *PAL* has received most of the attention in studies of the regulation of phenolic biosynthesis. In general the activity of *PAL* increases dramatically when plants are subjected to conditions resulting in the stimulation of phenolic synthesis (Camm, 1973a), it is regulated at the transcriptional level in response to different stresses, such as pathogenic attack, UV radiation, low supply of nitrogen, phosphate, or iron (Olsen *et al.* 2008, Pina and Errea 2008, Sullivan *et al.* 2009). Phenolic Secondary metabolites p-hydroxybenzoic acid, Casticin and Rutin is biosynthesis by this pathway, so that the *PAL* gene had been detected and sequencing to determine SNPs within this three species of medicinal plant vitex and its relationships with its phenolic compounds concentrations.

PAL is an important enzyme not only for plant development but also for biotic and abiotic stress response. In some plants, *PAL* gene expression is believed to be activated by the jasmonic acid/ethylene signal pathway during induced disease resistance (Diallinas and Kanellis, 1994; Mitchell and Walters, 1995; Kato *et al*., 2000; Distefano *et al*., 2008). The researches on the characteristics and roles of *PAL* genes in Vitex genus's are scarce specifically in VN, VAC and VPN, there is no available information about the phylogeny of this a *PAL* gene sequence from *Arachis hypogeae L.* (peanut) has been recently published in GenBank (accession No.GU123139). This knowledge is, however, essential for a better understanding of primers design to detecting conserved regain gene in vitex genus.

Table 1: Taxonomical Classification. Venkateswarlu., 2012

Kingdom	**Plants**
Sub kingdom	Vascular plants
Super Division	Seed plant
Division	Flowering plant
Class	Dicotyledons
Sub Class	Asteridea
Order	Lamiales
Family	Verbenaceae
Genus	Vitex
Species	*V.negundo* *V. agnus castus* *V.pseudo negundo*

Materials and Methods

Chemicals

All chemicals and solvents used in this study were of analytical grade and supplied from Sigma-Aldrich (Steinheim, Germany).

Plant Extracts

Vitex genus leaves collected ,cleaned ,dried and powdered by grinding and extracted with ethanol 70% for 72 hour using magnetic stirrer in dark without heat, The mixture was filtered through a filter paper (Whatman No.1), Resulting solution was evaporated under Rotary Evaporate to 10 ml, then freeze-dried at -50 with a lyophilizer for 24 hour, Extract was kept in a freezer for further experiments such antibiotic activity and HPLC (Gulcin., 2005).

Microorganisms

The microorganisms employed in the current study were procured from microbiology laboratory of science in Tikrit University . which includes clinical isolates of three genus belong to positive and negative gram: *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli.*

Disc Diffusion Assay

The agar cup method was followed to doubly ensure the antibacterial activity of the extract (Barry, 1980). Overnight Nutrient Agar Broth culture of the test organisms were firmly seeded over the Muller Hinton Agar (MHA) plates. Using sterile borer Wells of 8 mm diameter were made. the bottoms of the wells were sealed by pouring 25-50 µl of molten MHA into the scooped out wells. 100-200µl of 200 mg/ml extract were poured into the wells. The water was allowed to evaporate and the plates were incubated at 37°C for 18-24 h. After the incubation period the plates were observed for a clearance zone around the discs which indicates a positive antibacterial activity of the respective extracts.

High Performance Liquid Chromatography (HPLC)

Qualitative identification of phenols in extracts obtained from extraction methods above was authenticated by HPLC, this identification was made by comparison of the retention time obtained at identical chromatographic conditions. The analyzed sample and the authentic standard conditions were:

- Mobile phase: liner gradient of solvent A 0.05% trifluoro acetic acid TFA in deionized water ; solvent B 0.05% trifluoro acetic acid in methanol , pH 2.5
- Column: C18 2.0 mm x 50 mm
- Flow rate: 1.1 ml /min
- Detection: UV. Detector at λ 280 nm.
- Injection volume: 20 µL
- Injection concentration: 200ug /ml

DNA EXTRATION

In order to obtain a *PAL* gene sequence from vitex species, genomic DNA from young leaves was isolated using CTAP extraction, PCR primers forward (5'AAGCACCACCCTGGTCAAATTGAG-3') and reverse (5'-GACAAGCTCGGAGAATTGAGCAAAC-3') were designed based on conserved sequences of *PAL* genes from *A. hypogaea*. PCR was performed with 20 reaction volume: 10 ul of master mix, 1 ul of each primer, 3ul of template DNA solution and complete the reaction to 20 ul with distal water. temperature profile was as follows: initial denaturation at 95°C for 3 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1 min, and a final extension step at 72°C for 10 min.

Results

The Hplc results for three vitex species shows that leaves of vitex species have that three phenolic secondary metabolites (SM), *P*-Hydroxybenzoic acid whit retention time (2.0) ,Casticin with retention time (3.19) and Rutin with retention time (5.75) (figure.1) and the concentrations of this compounds are calculated by the formula

Concentration mg/ml = sample area / standard area *dilution factor *concentration

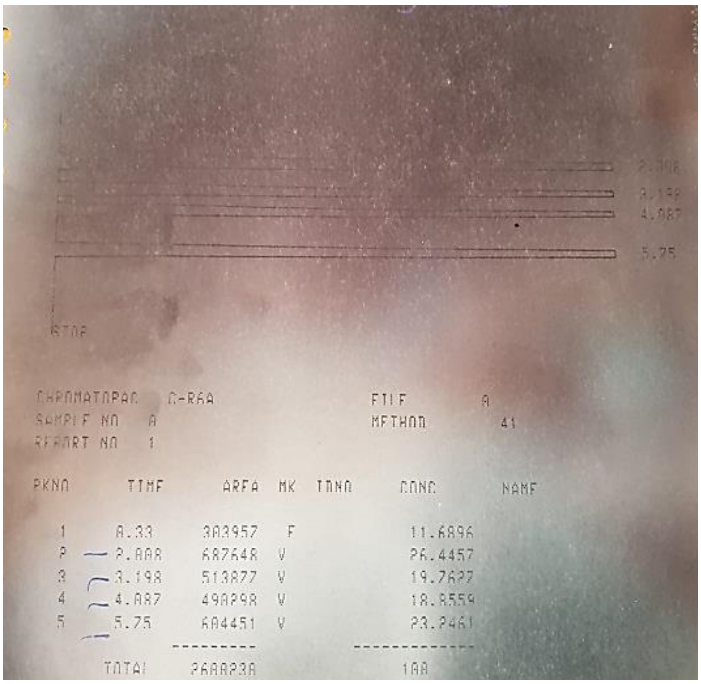
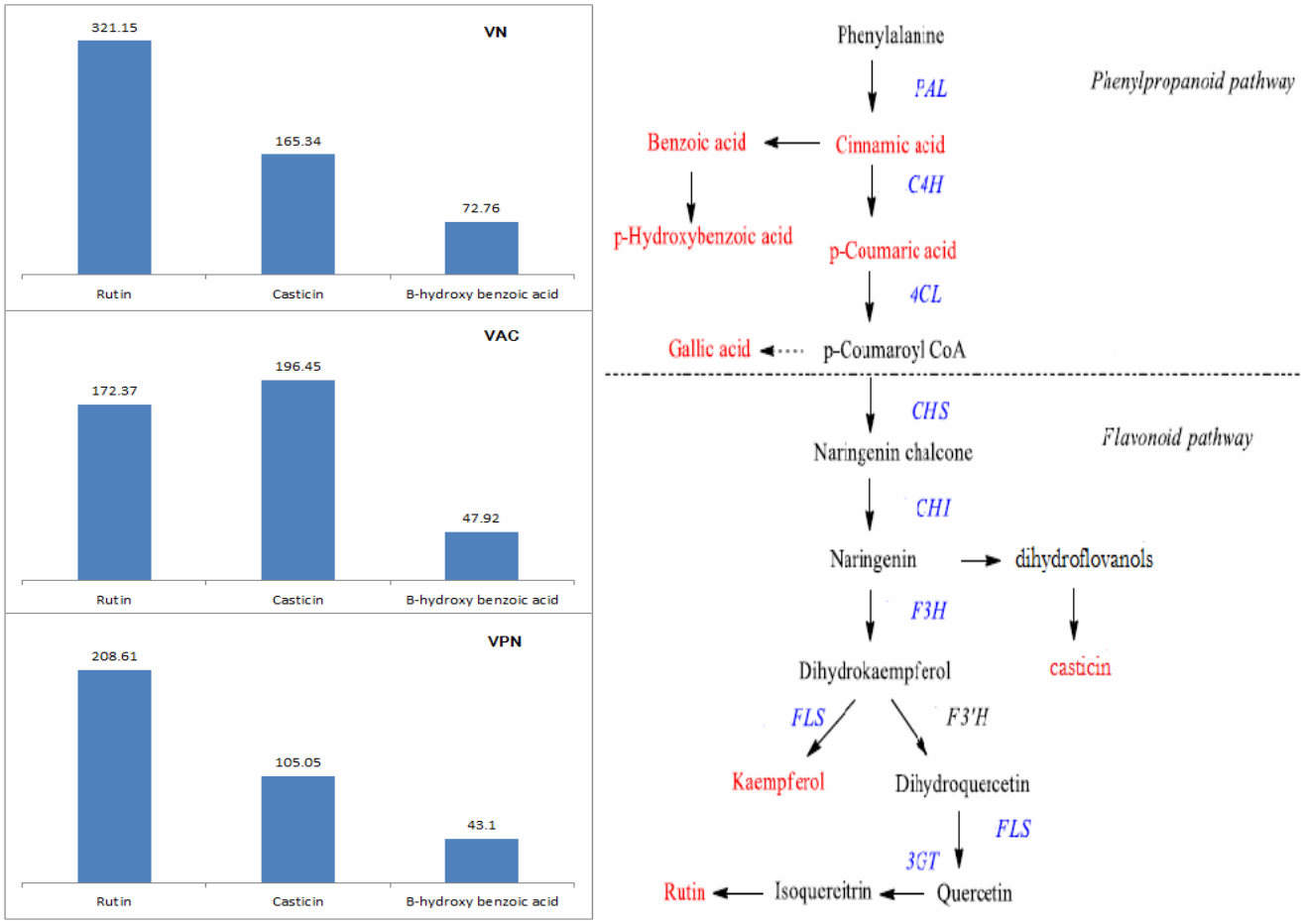


Fig. 1 : HPLC peaks of phenolic standard

Table 2 : HPLC results for Rutin ,Casticin and PHPA standards

Peak	Retention time (min)	Area	Identified compounds
1	0.33	303959	Unknown
2	2.0	687648	p-hydroxy benzoic acid
3	3.19	513877	Casticin
4	4.08	490298	Unknown
5	5.75	604451	Rutin

The specie VN shows the highest concentration of PHBA and Rutin were are VAC shows the highest concentration of Casticin and the lowest concentration of Rutin (figure 2a), all this three phenolic secondary metabolites are biosynthesis by phenylpropanoid pathway and the *PAL* gene which encoded the enzyme phenyl alanine amonialyase is the first structural gene in this pathway. so that changing in concentrations of SM is related with the *PAL* gene variation which change in enzyme structures and activity. sequencing results for *PAL* gene shows that the specie VN have lowest SNPs and in the other hand more Rutin and PHPA, were as the specie VPN shows the highest SNPs numbers and in the other hand lowest concentrations of PHBA and Casticin. figure. 2 (a,b) shows that the highest concentration of casticin in the specie VAN is accomplished with the lowest concentration of Rutin and vise versa.



a b

Fig. 2: (A) concentrations and (B) phenylpropanoid pathway for PHBA, Rutin and Casticin biosinthesis (genes are in blue) .

This results was correlated with biological activity against bacterial species. the disk diffusion assay shows antibacterial activity of the specie VN against two types of bacteria (*E.coli* and *Staphylococcus aureus*), the specie VPN show an antibacterial activity against *Staphylococcus aureus*, were are the specie VAC don't show any activity against all this three types of bacteria (table.3)

Table 3: Antibacterial activity of ethanolic leaves extract of vitex species

Sample	*Escherichia coli*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
V. negundo	+	-	+
V. agnus castus	-	-	-
V. pseudo negundo	-	-	+

electrophoresis showed a fragment of approximately 370 bp was amplified, custom sequenced for forward strand. Vitex species were sequenced in (Macrogen, Korea) and analyzed using MEGA 6 SOFTWARE (Tamura *et al*., 2007) (Figure 3).

A high level of similarity was found within *PAL* gene in *Vitex* species in addition to some SNPs between them (Figure.4), the sequencing results with MEGA 6 showed that the three species has 10 SNPs within the coding regain of *PAL* gene, all the SNPs have identical nucleotides in two species and the third one is changed, the first specie VN showed the lowest polymorphisms by one mutant base every 300 nucleotide, VAC showed 3 mutant whereas VPN showed the height polymorphisms by 6 mutant base (Table 4).

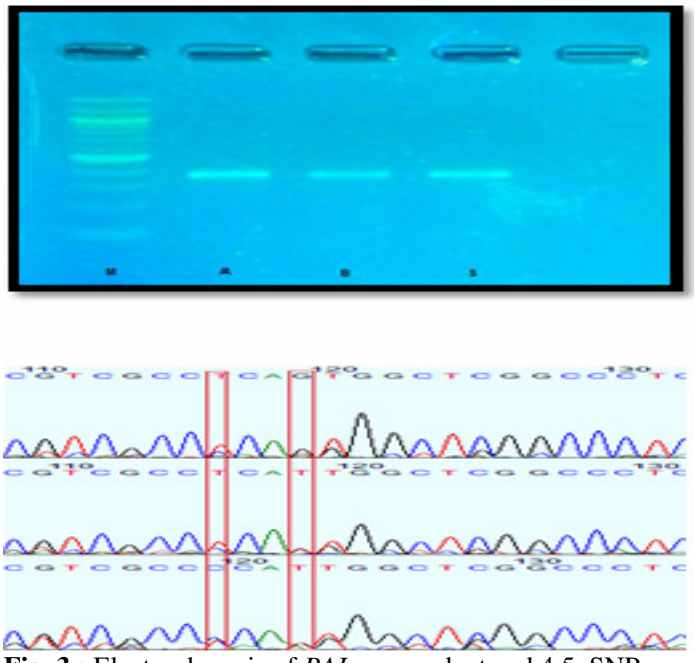


Fig. 3 : Electrophoresis of *PAL* pcr product and 4,5 SNPs between the three vitex species using MEGA6 SOFTWARE

Table 4: Type and number of mutant bases

A	T	G	C	Mutant bases	Samples
0	0	1	0	1	*V.negundo*
1	1	1	0	3	*V.agnus castus*
4	1	0	1	6	*V.pseudo negundo*
5	**2**	**2**	**1**	**10**	Total

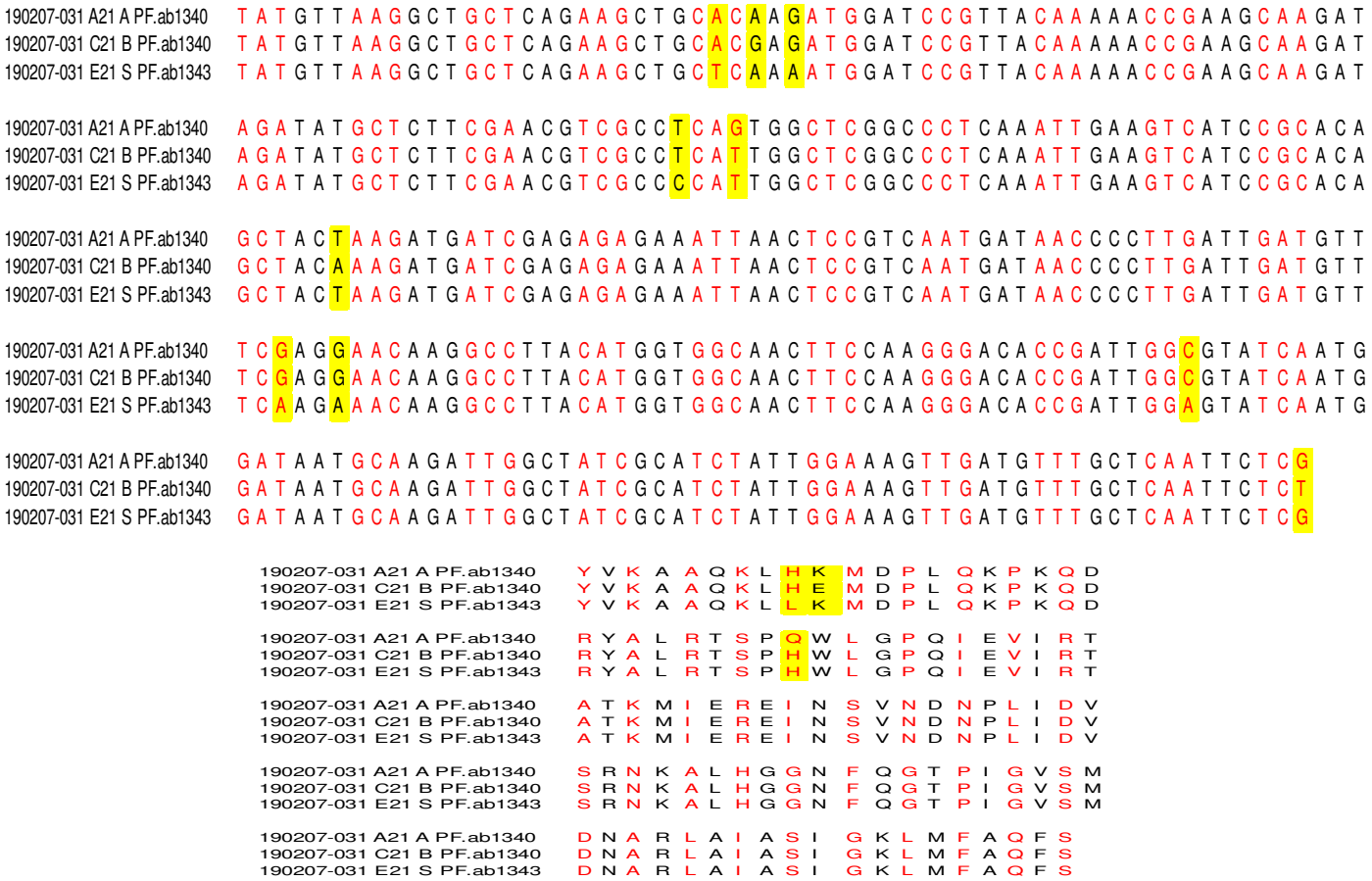


Fig. 4 : Scheme for identical and mutant nucleotides and amino acids of *PAL* gene sequence in VN,VAC and VPN

the previous sequencing (Figure.4) shows the changes in amino acids types resulting by SNPs of *PAL* gene in the three vitex species, this changes reflected on increasing in concentrations of Glu, His, Leu and Gln and decreasing in concentration of Lys (Table 8), and this variations reflected on *PAL* enzyme structure and activity.

Table 5: *PAL* gene SNPs in the three vitex species

V.negundo	*V.agnus castus*	*V.pseudo negundo*	Base sequence
A	**A**	T	26
A	G	A	28
G	G	A	30
T	T	C	84
G	T	T	87
T	A	T	126
G	G	A	183
G	G	A	186
C	C	A	231
G	T	G	297

Table 6: Silent mutations

2nd base of aminoacides codon	*V.pseudo negundo*	*V.agnus castus*	*V.negundo*	Amino sequence	Base sequence
Cytosine	Pro (P)	Pro (P)	Pro (P)	28	84
Cytosine	Thr (T)	Thr (T)	Thr (T)	42	126
Guanine	Ser (S)	Ser (S)	Ser (S)	61	183
Guanine	Arg (R)	Arg (R)	Arg (R)	62	186
Guanine	Gly (G)	Gly (G)	Gly (G)	77	231
Guanine	Ser (S)	Ser (S)	Ser (S)	99	297

Silent mutations is a different codons encode to same amino acids within the bases of the *PAL* coding regain gene. Thus, there was no change in the form and activity of this enzyme in the three plant species. the previous table (6) shows that the second base of amino acids codons is one of the tow hydrogen bases guanine or cytosine, show the figure (5).

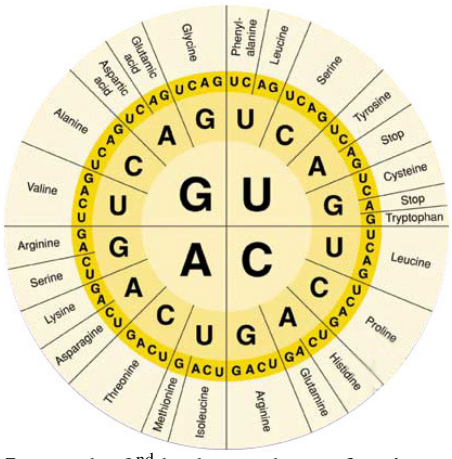


Fig. 5 : see the 2nd hydrogen base of amino acides

Table 7: Missense mutations

2nd base of aminoacides codon	*V.pseudo negundo*	*V.agnus castus*	*V.negundo*	Amino sequence	Base sequence
Adenine/Thymine	(L) Leu	(H) His	(H) His	9	26
Adenine	(K) Lys	(E) Glu	(K) Lys	10	30
Adenine	(H) His	(H) His	Gln)Q(	29	87

Missense mutations is a change in the codons leads to a different amino acid and these mutations are affecting the structure and activity of this enzyme. the previous table (7) shows that the second base of amino acids codons is one of the tow hydrogen bases adenine or thymine, show the figure (5).

In addition to that the *Pal* gene of VAC specie which shows lowest ratio of Rutin and in the other hand highest ratio of Casticin (figure 2a) have tow missense mutations for histidine amino acide and one amino acid of another type, whereas the other tow Vitex species VN and VPN shows one missense mutant of Histidine amino acid in addition to tow deferent amino acides, so that three deferent types of amino acid may be the cause of that secondary metabolites ratio changes.

Table 8 : Amino acids ratios in vitex species

Amino sequence	*V. negundo*	*V. agnus castus*	*V. pseudo negundo*
Ala(A)	9.090909091	9.090909091	9.090909091
Sys(C)	0	0	0
Asp(D)	5.050505051	5.050505051	5.050505051
Glu(E)	4.04040404	3.03030303	3.03030303
Phe(F)	3.03030303	3.03030303	3.03030303
Gly(G)	6.060606061	6.060606061	6.060606061
His(H)	3.03030303	2.02020202	2.02020202
Ile(I)	8.080808081	8.080808081	8.080808081
Lys(K)	7.07070707	8.080808081	8.080808081
Leu(L)	8.080808081	8.080808081	9.090909091
Met(M)	4.04040404	4.04040404	4.04040404
Asn(N)	6.060606061	6.060606061	6.060606061
Pro(P)	6.060606061	6.060606061	6.060606061
Gln(Q)	6.060606061	7.07070707	6.060606061
Arg(R)	6.060606061	6.060606061	6.060606061
Ser(S)	6.060606061	6.060606061	6.060606061
Thr(T)	4.04040404	4.04040404	4.04040404
Val(V)	5.050505051	5.050505051	5.050505051
Trp(W)	1.01010101	1.01010101	1.01010101
Tyr(Y)	2.02020202	2.02020202	2.02020202

Homologous genes were identified using NCBI blast and the best identic result Among *PAL* sequences from vitex species and other plants species showed that *PAL* gene fragment had a nucleotide sequence identity of 98% with *sesamum* (99% query coverage) and 79% (99% query coverage) with *Arachis hypogaea L* . confirming that even among evolutionary distant taxa, *PAL* sequences are highly conserved (Butland *et al*., 1998; Kumar and Ellis, 2001). This character were employed in previous primers design.
Alignment whiten NCBI also was done between every two of this three species to determine the SNPs among *PAL* gene sequences from this three vitex species, the results shows that the vitex *PAL* gene fragment had a nucleotide sequence highest identity by 98.51% between the two species *V. agnus castus-V.negundo* and 5 SNPs within this species, and med identity of 96.06 % within *V.negundo-V.pseudo negundo* and 13 SNPs, where are alignment of the two species *V.agnus castus-V.pseudo negundo* showed lowest identical with 95.45% and 15 SNPs, In addition to 1,2,3 GAPs in this three alignment (figure 6).

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AGCATTCTTGATGGTAGC-GCATATGTTAAG
......A....C..C...T............
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Fig. 6 : Part of SNPs and GAPs between VN and VPN *PAL* gene by NCBI alignment.

Table 9 : Alignment characterizations of *PAL* gene between vitex species

Idint	transversion	transition	gaps	SNPs	Sample
98.51	3	1	1	5	*Agnus castus-negundo*
96.06	4	7	2	13	*negundo-pseudo negundo*
95.45	4	8	3	15	*Agnus castus-pseudo negundo*

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