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SHORT COMMUNICATION

Characterisation and Expression Analysis of Hydroxyphenylpyruvate Reductase Derived from *Orthosiphon aristatus*

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ABSTRACT

Herbal products are getting more popular as alternative medicines and food supplements. The therapeutic effects of herbal medicines are mainly attributed to their bioactive secondary metabolites. *Orthosiphon aristatus*, locally known as 'Misai Kucing', is known for its various health benefits. One of the main chemical constituent of *O. aristatus* is rosmarinic acid, a plant polyphenol that has been proven to have antioxidant, anti-inflammatory and antimicrobial activities. Hydroxyphenylpyruvate reductase (HPPR) is one of the enzymes involved in rosmarinic acid biosynthetic pathway. Here we report the effect of UV on HPPR expression and the isolation of a full-length *hppr* cDNA from *O. aristatus* via rapid amplification of cDNA ends polymerase chain reaction. An increase in the expression was detected when the plant was exposed to UV and detected via the expression of *hppr* transcript. A 1116 bp nucleotide putative cDNA was isolated corresponding to 307 predicted amino acid. We have also isolated the 5' and 3' untranslated regions with a length of 54 bp and 123 bp, respectively. Sequence similarity analysis was performed against NCBI genebank and the BLAST result showed that the putative *hppr* cDNA isolated from *O. aristatus* exhibited high similarities with other *hppr* cDNA of the members of the Lamiaceae family such as *Perilla frutescens*, *Salvia officinalis*, *Salvia miltiorrhiza* and *Solenostemon scutellarioides*.

Keywords: Phydroxyphenylpyruvate reductase (HPPR), *Orthosiphon aristatus*, rapid amplification of cDNA ends (RACE), rosmarinic acid

Orthosiphon aristatus [syn.: *O. grandiflorus*, *O. spicatus*, *O. stamineus*] is locally known as "Misai Kucing" belonging to the family Lamiaceae. It is a perennial herb that can grow to about 0.4 to 1.5 m high. The leaves are green and simple with a lanceolate leaf blade (Jaganath & Ng, 2000). The flowers have long protruding stamens, making it look like cat's whiskers (Figure 1(a)). It has been used in Southeast Asia countries such as Malaysia, Indonesia, Thailand and Vietnam in traditional medicine for treatment of fever, epilepsy, gallstones, hepatitis, rheumatism, hypertension, syphilis, gonorrhea, tonsilitis, hepatitis, gout and diabetes (Akowuah *et al*., 2005; Kiong *et al.*, 2008).

Various ranges of scientific studies support the traditional use of the plant. Studies have showed that the plant exhibited anti-pyretic activity (Yam *et al*., 2009), radical scavenging or antioxidant (Akowuah *et al.*, 2005; Khamsah *et al*., 2006), anti-apoptotic (Abdelwahab *et al*., 2011), anti-microbial (Tong *et al*., 2011), anti-bacterial activity, anti-fungal activity (Hossain *et al*., 2008) and chemo-preventive activity (Salleh *et al*., 2011). More than 20 compounds have been isolated and identified in *O. aristatus* (Tezuka *et al*., 2000) and rosmarinic acid is one of the major constituent (Chin *et al.*, 2009). Rosmarinic acid (RA) is commonly found within the families Boraginaceae and Lamiaceae (Li *et al*., 2005) and Figure 1(b) illustrates the pathway of rosmarinic acid biosynthesis in *Coleus blumei* now known as *Plectranthus scutellarioides* (Kim *et al*., 2004). RA has been proven to have anti-microbial, anti-viral, anti-pyretic and anti-oxidant effects (Petersen & Simmonds, 2003). UV is an abiotic stimulus that has the potential to induce accumulation of secondary metabolites which in turn affect numerous physiological functions (Matsuura *et al*., 2013; Paul & Gwynn-Jones, 2003). A study by Luis *et al*. (2007) showed significant increase of rosmarinic acid concentration in rosemary when exposed to

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UV-B. Meanwhile, hydroxyphenylpyruvate reductase (HPPR) is one of the enzymes that are involved in the biosynthetic pathway of RA production and involved in reducing hydroxyphenylpyruvates to hydroxylphenyl-lactates from dependence of NAD(P)H (Petersen *et al.*, 2009). Here we report the isolation of a full length *hppr* cDNA and preliminary correlation study of the expression of *hppr* gene and length of ultraviolet exposure on *O. aristatus.*

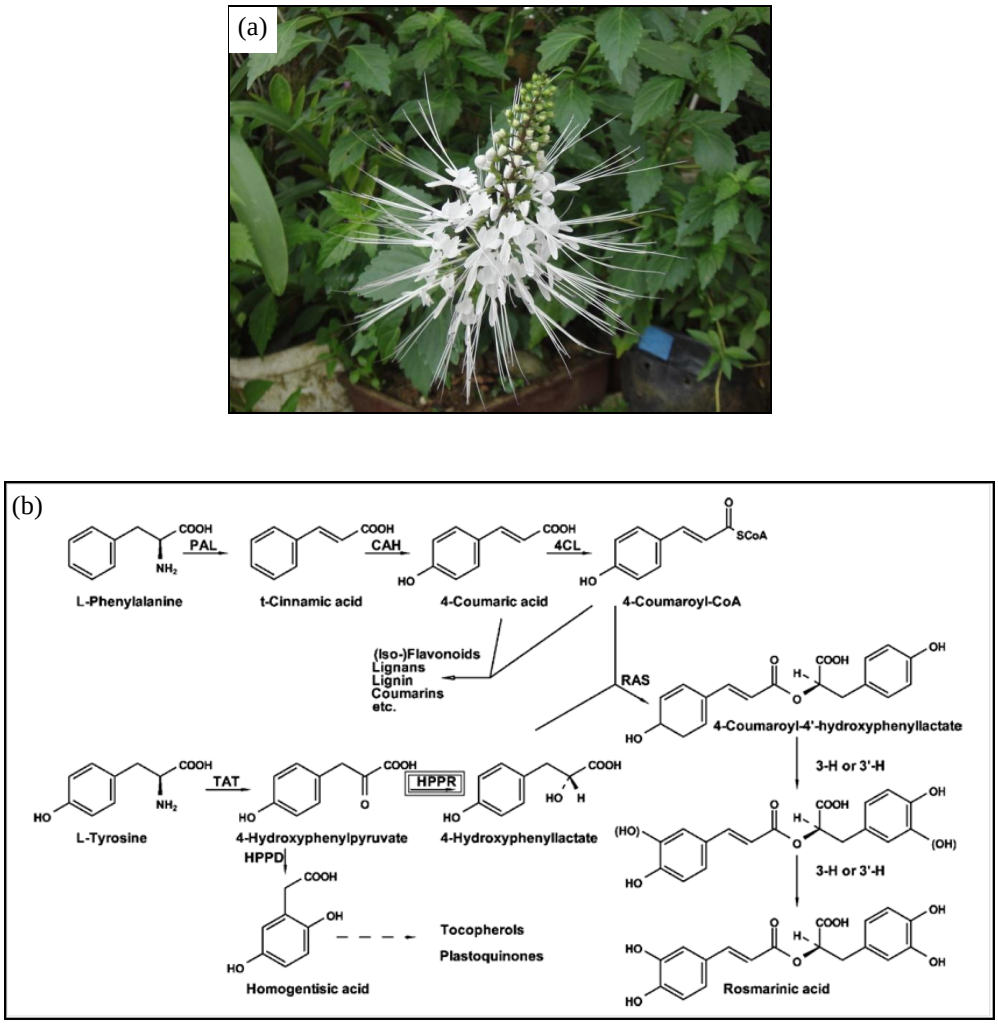

Figure 1. (a) Plant and flowers of *Orthosiphon aristatus* (Photo courtesy Ahmad, Z), and (b) the biosynthesis pathway for rosmarinic acid (adapted from Kim *et al*., 2004).

The *O. aristatus* plant samples were grown from stem cuttings in Universiti Malaysia Sarawak (UNIMAS). Prior to RNA extraction, all plant materials were rinsed with water, surface sterilized with 70% ethanol for 1 minute and final rinse with distilled water. The total RNA was isolated using the modified method of Gasic *et al*. (2004), quantified by spectrophotometer and analysed on ethidium bromide-stained 1% agarose gel. Total RNA was reverse transcribed using RevertAid Reverse Transcriptase (Fermentas). First strand cDNA were synthesized using primer oligo $(dT)_{15}$ACP as recommended by the manufacturer. Gene specific primers (GSP) were designed from conserved regions based on alignment with *hppr* from other plant species obtained from NCBI database. The gene specific forward primer haHppr-f (5'-AGAAGGGGGTTAGGGTTACCA-3') and reverse primer haHppr-r (5'-TCCCTACATGTGGCAACAGGA-3') were used to amplify the internal fragment of *hppr* transcript. PCR was performed in a total volume of 20 µl containing 2X GoTaq® Green Master Mix (Promega), forward and primer,

nuclease-free water and cDNA template. The PCR amplification was conducted in a thermal cycler (MyCycler, Biorad) with 35 cycles at annealing temperature of between 50-70°C. A positive control assay was done using the elongation factor-1 alpha (*ef-1α*) mRNA sequence (Nicot *et al*., 2005).

The 3' rapid amplification of cDNA ends (RACE) was amplified using modified touchdown PCR (Roslan, pers. comm.) utilizing oligo $(dT)_{15}$ACP and SolsHppr-f (5'-CTCTGCAGGCCGAGTCTATC-3'). The PCR product was then diluted and nested PCR was performed using primer oligo $(dT)_{15}$ACP and haHppr-f to increase specificity of the PCR amplification. Meanwhile, the 5'RACE, was done as described above and using the primer haHppr-r in combination with zaHppr01-f (5'-AGGAGCGGGGCGTGGAA ATT-3') primer.

The resulting PCR products were purified using GF-1 gel DNA Recovery Kit (Vivantis) and ligated into pGEM-T vector (Promega). The recombinant plasmids were sent for sequencing to First BASE Laboratories Sdn Bhd. (Malaysia). The software CLC Sequence Viewer was used to analyse the sequence. Semi-quantitative RT-PCR was conducted for samples treated with UV to investigate the expression of *O. aristatus hppr* under different UV exposure. The plants were exposed under UV for 15 minutes, 30 minutes, 45 minutes and 60 minutes. Total RNA was then extracted from leaves of *O. aristatus*, reversed transcribed into cDNA and amplified using hydroxyphenylpyruvate reductase internal primers. The RNA extraction from the leaves of *O. aristatus* yielded total RNA of approximately 200 µg of total RNA per g of fresh weight (Figure 2).

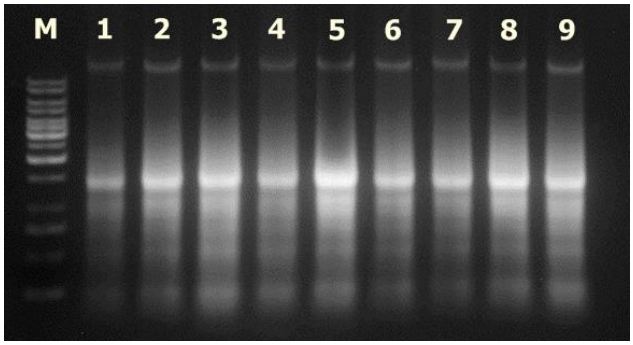

Figure 2. Agarose gel electrophoresis total RNA derived from *O. aristatus*. Lane M; 1 kb ladder (Fermentas), Lane 1-9; plant sample.

Several amplification using different combinations of primers were undertaken to construct the full-length *hppr* cDNA (Figure 3). Amplification using specific primer haHppr-f and haHppr-r produced a fragment of approximately 600 bp (Figure 3B). Sequence analysis on the fragment showed 91% similarities to *S. scutellarioides hppr* mRNA with a size of 1127 bp (Figure 3A). The 3'RACE-PCR produced a fragment of approximately 800 bp including a poly-A tail at the 3'-end (Figure 3C). Sequencing analysis showed 89 to 91% similarities with other plants *hppr*. Meanwhile, 5'RACE-PCR using zaHppr01-f and haHppr-r produced a fragment approximately 750 bp (Figure 3D). The nucleotide sequence analysis showed 86% similarities to other plant species. Merging of the 5'-end and 3'-end sequence produced a full-length *hppr* sequence of 1116 bp that includes the 5' (54 bp) and 3' (123 bp) untranslated regions (UTR), and encodes for 307 predicted amino acids (Figure 4). Meanwhile, Table 1 shows that the *hppr* of *O. aristatus* has sequence similarity of between 86% to 89% to other *hppr* genes.

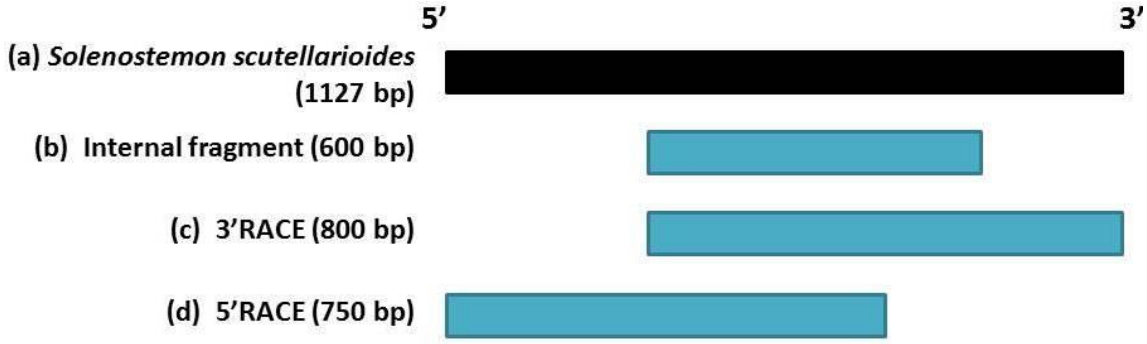

Figure 3. A schematic representation of the strategy used in amplifying the *hppr* cDNA from *O. aristatus*. (a) The full-length of *hppr* gene of *Solenostemon scutellarioides* used as a comparison to the amplification strategy; (b) The internal fragment of *hppr* gene in *O. aristatus*; (c) and (d) are the amplifications of the 3'-end and 5'-end, respectively.

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   1 accactcaaacacca tctccctcccccgcc accaacgccgccgct gcccttcaaATGgat
   1                                                          M  D

  61 gccatcagcgttctg atgatgtgccccatg agcgactacttggag caagagctcgacaag
  21  A  I  S  V  L   M  M  C  P  M   S  D  Y  L  E   Q  E  L  D  K

 121 cgcttcaagctcttc cgttactggactcag ccgcagtttccggcc gagtcaatccgcgca
  41  R  F  K  L  F   R  Y  W  T  Q   P  Q  F  P  A   E  S  I  R  A

 181 gtggtcggtaactcc accgccggcgccgac tccgacttgatcgat gaactgccgaaattg
  61  V  V  G  N  S   T  A  G  A  D   S  D  L  I  D   E  L  P  K  L

 241 gagattgtatcttgc tatagcgtgggtctc gataaagtcgacttg atcaagtgcaaggag
  81  E  I  V  S  C   Y  S  V  G  L   D  K  V  D  L   I  K  C  K  E

 301 aaggggggttagggtt accaacacgcccgat gtgctgacggatgac gtggcggatttggct
 101  K  G  V  R  V   T  N  T  P  D   V  L  T  D  D   V  A  D  L  A

 361 atcgggttgatgttg gctgttttgaggcgg atttgtgagtgtgat aagtatgtgaggagt
 121  I  G  L  M  L   A  V  L  R  R   I  C  E  C  D   K  Y  V  R  S

 421 ggggcgtggaaattt ggcgacttcaagttg acgactaagttcagc ggcaaaagagttggt
 141  G  A  W  K  F   G  D  F  K  L   T  T  K  F  S   G  K  R  V  G

 481 atcattggattgggc agaattggcttagca gttgctgagcgagca gatgtatttgattgt
 161  I  I  G  L  G   R  I  G  L  A   V  A  E  R  A   D  V  F  D  C

 541 cccataaattactac tcaagatccaagaaa cccaacacaaactac aagtactacaacagc
 181  P  I  N  Y  Y   S  R  S  K  K   P  N  T  N  Y   K  Y  Y  N  S

 601 gttgttgaattggcg aggaacagtgacatc ctagtcgtagcatgt gccctgactccagaa
 201  V  V  E  L  A   R  N  S  D  I   L  V  V  A  C   A  L  T  P  E

 661 acaactcacattgtg aatcgagaagtaatc gatgcattgggtcca aagggaattctcatc
 221  T  T  H  I  V   N  R  E  V  I   D  A  L  G  P   K  G  I  L  I

 721 aacatcggaagggga cctcatgtcgatgaa acagagttggtgtta gctcttgtggagggc
 241  N  I  G  R  G   P  H  V  D  E   T  E  L  V  L   A  L  V  E  G

 781 cgtctgggtagcgcg ggtcttgatgtcttc gaaagggaaccggag gtaccggagcagctc
 261  R  L  G  S  A   G  L  D  V  F   E  R  E  P  E   V  P  E  Q  L

 841 tttgggctcgagaat gttgtcctgttgcca catgtagggagtggc actgtggaaacgcgc
 281  F  G  L  E  N   V  V  L  L  P   H  V  G  S  G   T  V  E  T  R

 901 aaggttatggctgac cttgttctgggaaat ttggaagctcacttt tccagcaagcctctg
 301  K  V  M  A  D   L  V  L  G  N   L  E  A  H  F   S  S  K  P  L

 961 ttaactcctgtggtt TGAtactgaccgttc atcttatttatgcag aagcaataatgtttc
 321  L  T  P  V  V   *

1021 atacctcagatttta gtctttgatgaattc atattttggattgtt acattcaattaaaat
1081 aacgtcgatgattat ttcttaaaaaaaaaa aaaaaa
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Figure 4. The cDNA sequence and the deduced amino acid sequence of *O. aristatus hppr* gene. The start codon (ATG) is in italics and the stop codon (TGA) is in bold. The sequence contained the 5' and 3' untranslated regions (small caps). The putative poly (A) signal is boxed (ATTAAA) and the D-isomer specific 2-hydroxyacid dehydrogenases NAD-binding signature is shaded.

Table 1. BLAST search result for the putative *O. aristatus hppr* cDNA sequence.

Plant	Length (bp)	GenBank accession number	Homology (%)*
Perilla frutescens	942	HM587131.1	89 (826/927)
Perilla frutescens	426	HM152567.1	88 (377/426)
Salvia miltiorrhiza	3564	EF458148.1	88 (461/523)
Salvia officinalis	942	JX566894.1	87 (820/942)
Solenostemon scutellarioides	1127	AJ507733.2	87 (877/1008)
Salvia officinalis	890	EU924744.1	87 (772/892)
Salvia miltiorrhiza	1174	DQ266514.1	86 (861/998)
Salvia miltiorrhiza	1117	DQ099741.1	86 (861/998)

*The percentage is based on the BLAST nucleotide search. Numbers in bracket indicate the number of bases (query/subject) that was compared.

In silico analysis using InterProScan identified several domains including a putative catalytic domain for hydroxyphenylpyruvate reductase, D-isomer specific 2-hydroxyacid dehydrogenase, in amino acid residues 55 to 324, and NAD-binding domain in the amino acid residues 159 to 186 (Figure 5). These domains are usually found as a domain in the family of D-isomer-specific 2-hydroxyacid dehydrogenases (Barberini, 2013). Multiple alignment of the D-isomer–specific 2-hydroxyacid dehydrogenase using CLUSTAL Omega with several species were also conducted (Figure 6). The D-isomer specific 2-hydroxyacid dehydrogenase NAD-binding signature motif, -G-X-G-X-X-G-, is indicated at amino acid residues 163 to 168 (-G-L-G-R-I-G-).

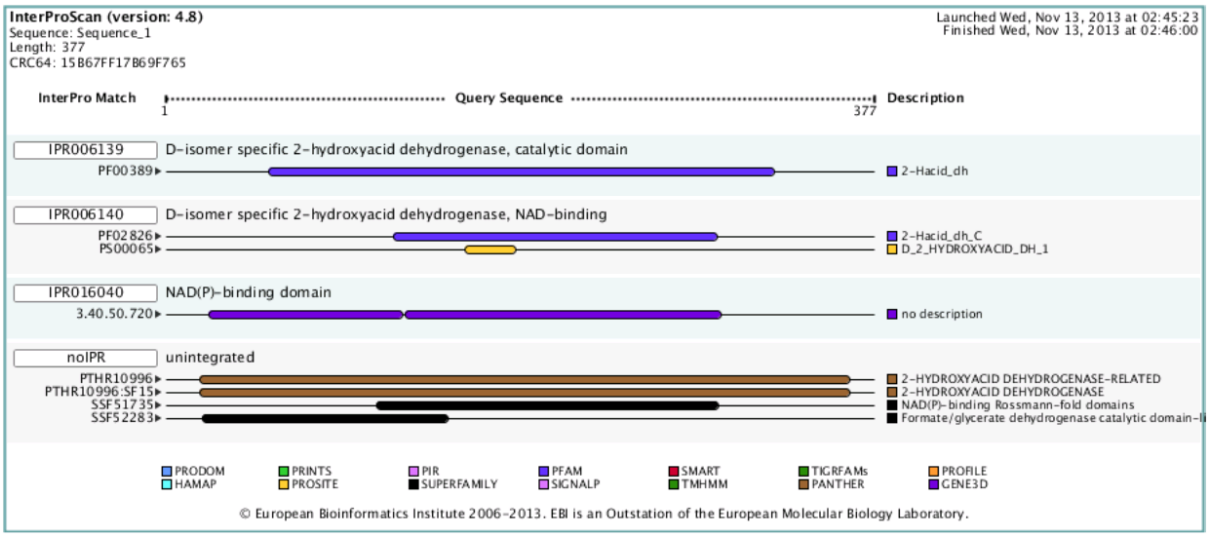

Figure 5. Schematic representation of *O. aristatus* protein domains analysed using InterProScan.

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CLUSTAL Omega(1.1.0) multiple sequence alignment
Orthosiphon         -----------------------------------MDAISVLMMCPMSDYLEQELDKRFK
Solenostemon        -----------------------------------MEAIGVLMMCPMSTYLEQELDKRFK
S.Miltiorrhiza      -----------------------------------MEAIGVLMMCPMNSYLEQELDKRFK
Perilla             -----------------------------------MEAIGVLMMCPMNNYLEQELDKRFK
S.officinalis       -----------------------------------MEAIGVLMMCPMNSYLEQELDKRFK
                                                        *:**.*******. ***********

Orthosiphon         LFRYWTQPQF------PAESIRAVVGNSTAGADSDLIDELPKLEIVSCYSVGLDKVDLIK
Solenostemon        LFRYWTQPAQRDFLALQAESIRAVVGNSNAGADAELIDALPKLEIVSSFSVGLDKVDLIK
S.Miltiorrhiza      LFRYWTQPKQREFLAQQAESIRAIVGNSTSGADADIIDSLPKLEIVSSFSVGLDRIDLLK
Perilla             LFRYWTQPRQREFLTQQAESIRAVVGNSTVGADAELIDALPKLEIVSCFSVGLDKVDLIK
S.officinalis       LLRYWTQPKQSEFLAQQADSIRAVVGNASAGADAALIDALPKLEIVSNFSVGLDKVDLVK
                    *:******         *:****:***:. ***: :** ******** :*****::**:*

Orthosiphon         CKEKGVRVTNTPDVLTDDVADLAIGLMLAVLRRICECDKYVRSGAWKFGDFKLTTKFSGK
Solenostemon        CEEKGVRVTNTPDVLTDDVADLAIGLILAVLRRICECDKYVRRGAWKFGDFKLTTKFSGK
S.Miltiorrhiza      CKEKGIRVTNTPDVLTEDVADLAIGLMLAVLRRICECDKYVRSGAWKLGDFKLTTKFSVK
Perilla             CKEKGIRVSNTPDVLTDDVADLAIGLMLAVLRRICECDKYVRRGAWKFGDFKLTTKFSGK
S.officinalis       CKEKGVRVTNTPDVLTDDVADLAIGLILAVLRRICECDKYVRRGAWKLGDFKLTTKFSGK
                    *:***:**:*******:*********:*************** ****:********** *

Orthosiphon         RVGII**GLGRIG**LAVAERADVFDCPINYYSRSKKPNTNYKYYNSVVELARNSDILVVACAL
Solenostemon        RVGIIGLGRIGLAVAERAEAFDCPISYFSRSKKPNTNYTYYGSVVELASNSDILVVACPL
S.Miltiorrhiza      RVGIIGLGRIGLAVAERAEAFDCPINYYSRSKKANTNYTYYGSVVELASNSDILVVACAL
Perilla             RIGIIGLGRIGLAVAERAEAFDCPINYYSRSKKPNTNYTYYSSVVELASNSDILVVACAL
S.officinalis       RVGIIGLGRIGLAVAERAEAFDCPINYYSRSKKANTNYTYYGSIVELASNSDILVVACAL
                    *:*****************:.*****.*:***** ****.** *:**** ********* *

Orthosiphon         TPETTHIVNREVIDALGPKGILINIGRGPHVDETELVLALVEGRLGSAGLDVFEREPEVP
Solenostemon        TPETTHIINREVIDALGPKGVLINIGRGPHVDEPELVSALVEGRLGGAGLDVFEREPEVP
S.Miltiorrhiza      TPETTHIVNREVMDALGPKGVLINIGRGPHVDEAELVSALVKGRLGGAGLDVFEKEPEVP
Perilla             TPETTHIVNREVIDALGPKGVLINIGRGPHVDEPELVSALVEGRLGGAGLDVFEREPEVP
S.officinalis       TPETTHIVNREVIDALGPKGVLINIGRGPHVDEAELVSALVEGRLGGAGLDVFEKEPEVP
                    *******:****:*******:************ *** ***:****.*******:*****

Orthosiphon         EQLFGLENVVLLPHVGSGTVETRKVMADLVLGNLEAHFSSKPLLTPVV*Y*---PFILFM
Solenostemon        EKLFGLENVVLLPHVGSGTVETRKVMADLVVGNLEAHFSGKPLLTPVV**CGSSVKILFM
S.Miltiorrhiza      EQLFGLENVVLLPHVGSGTVETRKVIADLVLGNLEAHFSSKPLLTPVV**LFESR*SHLL
Perilla             EQLFGLENVVLLPHVGSGTVETRKAMADLVLGNLEAHFSSKPLLTPVV*-----------
S.officinalis       EQLFGLENVVLLPHVGSGTVETRKAMADLVLGNLEAHFSSKPLLTPVV*-----------
                    *:*********************.:****:********.*********

Orthosiphon         QKQ*CFIPQILVFDEFIFWIVTFN*NNV-----DDYFLKK-KKKPPIEFAAFP
Solenostemon        LRQSCFIPTLEI-------------------LIM--------------------
S.Miltiorrhiza      RGTHVWYPQSVMNSYFGSC*L-FHSNKKTSIIILFFFFQKKTKKKKKK-----
Perilla             ------------------------------------------------------
S.officinalis       ------------------------------------------------------
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Figure 6. Multiple alignment of HPPR amino acid sequences of *O. aristatus* and from *S. scutellarioides*, *S. miltiorrhiza*, *P. frutescens*, and *S. officinalis*. Dashed lines (-) are gaps introduced to maximize identity. Asterisk (*) indicates positions which have a single, fully conserved residue. Colon (:) indicates conservation between groups of strongly similar properties. Period (.) indicates conservation between groups of weakly similar properties. The D-isomer specific 2-hydroxyacid NAD-binding signature is shaded in the *O. aristatus* sequence, meanwhile the NAD(P)H binding motif is indicated in bold and underlined.

From the cDNA sequence, a predicted structure of *O. aristatus* HPPR was generated via comparative modeling of three-dimensional structure using Expasy SWISS-MODEL and compared to the crystal structure of the HPPR enzyme of *P. scutellarioides* has been determined by Janiak *et al*. (2010) (Figure 7). Protein structure for *O. aristatus* HPPR was predicted based on template *3ba1*, structure of hydroxyphenylpyruvate reductase from *P. scutellarioides* (Figure 7). Similarity in the structure with the reference protein indicated that the *O. aristatus* HPPR share the same biological activity. Using the software Raptor and a Molecular Graphic Visualization Tool, RasMol, the predicted *O. aristatus* HPPR structure was determined to contains 18 helices, 27 beta sheet strands with 25 turns.

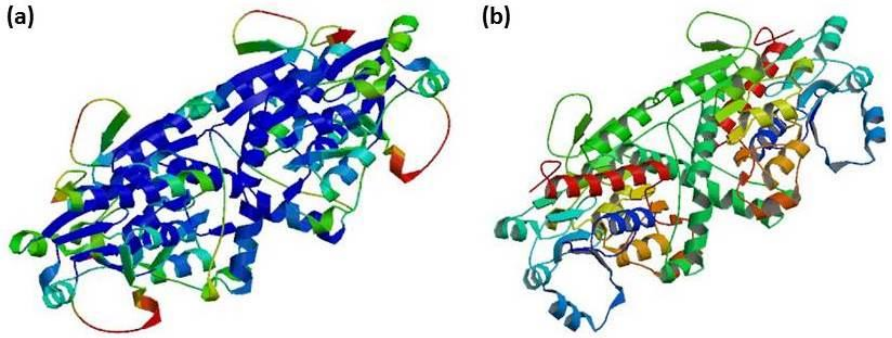

Figure 7. Structures of HPPR predicted protein of *O. aristatus* (a) and *P. scutellarioides* (b).

A preliminary work to investigate the correlation between HPPR expression and exposure to ultraviolet (UV) was done via RT-PCR analysis. Total RNA was extracted at exposure times of 15 minutes, 30 minutes, 45 minutes and 60 minutes. The RT-PCR was conducted using specific internal primers (zaHppr02 forward and reverse primers with an expected size of approximately 400 bp) and results showed increase in the *O. aristatus hppr* cDNA detected upon exposure to UV. The amplification intensities increased with the time of UV exposure (Figure 8a). HPPR is an enzyme that is involved in the tyrosine-derived pathway branch of the biosynthetic pathway of rosmarinic acid. Luis *et al*. (2007) found that exposure to UV approximate doubled the phenolics content including rosmarinic acid, naringin, cirsimaritin and carnosic acid. Another study by Iwai *et al*. (2010) on *Perilla frutescens* showed that artificial illumination is effective for inducing the accumulation of rosmarinic acid and luteolin. In this study the *hppr* transcripts were shown to increase with exposure to UV to which we predict may affect the level of rosmarinic acid biosynthesis in *O. aristatus*.

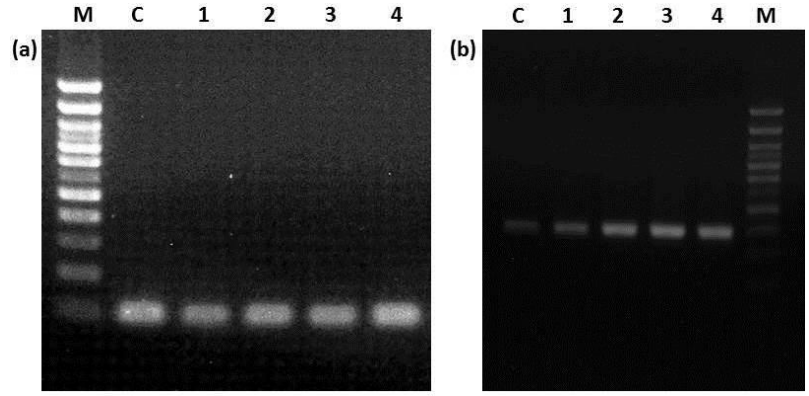

Figure 8. RT-PCR amplification of *ef-1α* and *hppr* at different UV exposure time. M denotes for 100 bp ladder marker (Fermentas) and C is the control (no exposure to UV at 0 minute). (a) Amplification of *ef-1α*. 1; 15 minutes UV exposure, 2; 30 minutes UV exposure; 3; 45 minutes UV exposure; 4; 60 minutes UV exposure. (b) Amplification of *hppr*. 1; 15 minutes UV exposure, 2; 30 minutes UV exposure, 3; 45 minutes UV exposure, 4; 60 minutes UV exposure.

We have thus far shown that *hppr* is present in *O. aristatus* with the isolation of the full-length cDNA sequence. From the cDNA sequence and predicted protein structure, it was determined to be HPPR. Nevertheless, further characterization is needed which should consists of characterizing fully the effect and range of UVs towards the expression of *hppr* transcript and at the same time, quantitate the level of rosmarinic acid produced.

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