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RESEARCH ARTICLE

Extraction and Isolation of Tyrosine Ammonia Lyase (TAL) and Diosgenin from *Persea americana* as Potent Inhibitors of Melanin Synthesis Involving *In Vivo* Study on Zebrafish Embryos

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Abstract

Tyrosine ammonia lyase is an enzyme in the natural phenol biosynthesis pathway. L-tyrosine is deaminated to p-Coumaric acid with the help of the enzyme tyrosine ammonia lyase. Tyrosine Ammonia Lyase (TAL) is extracted from the leaves of *Persea americana.* Diosgenin involved in the inhibition of intracellular tyrosinase is extracted along with the enzyme Tyrosine Ammonia Lyase from leaves of *Persea americana,* significantly increasing the activity of Tyrosine Ammonia Lyase. Diosgenin in the current study on analysis is highly increasing the activity of enzymes. The ability of the enzyme along with diosgenin to increase the activity of the enzyme is analysed in the current study. The enzyme along with diosgenin is used for depigmentation of skin. The methodology of protein separation on treatment with acetone was utilized for the extraction of the enzyme. p-Coumaric acid formation detected using a UV-Visible spectrophotometer at 380nm confirmed the presence of the enzyme. Defatting followed by microwave-assisted extraction and ultrasound-assisted extraction methodology were used for the extraction of diosgenin from leaves of *Persea americana.* The role of diosgenin in increasing the activity of the enzyme was carried out by analysing the enzyme activity at 410nm. The enzyme-activating ability of diosgenin was compared with that of magnesium sulphate-purified Tyrosine Ammonia Lyase enzyme and sodium sulphate-purified Tyrosine Ammonia Lyase enzyme. Zebrafish embryos were used as model organisms for the conduction of *in vivo* studies. kojic acid was used as a control in the *in vivo* studies to compare the effect of the enzyme extracted along with the diosgenin with that of kojic acid.

Keywords: Diosgenin, P-Coumaric Acid, *Persea americana,* Tyrosine Ammonia Lyase, Zebrafish

1. Introduction

The mechanism of inhibition of melanin pigment has been widely studied in the current period[1]. Many pharmaceutical companies are largely involved in the discovery of elements which are involved in the inhibition of melanin synthesis that can be used as a source to the treatment of hyperpigmentation. Hyperpigmentation is a condition where an excess amount of melanin pigment is released because of the conversion of a large amount of tyrosine to melanin leading to darker skin colour[2]. The objective of the current study is to inhibit the conversion of L-tyrosine to further products in the melanin synthesis pathway leading to depigmentation utilizing the enzyme Tyrosine ammonia lyase, an enzyme involved in the deamination of L-tyrosine to p-Coumaric acid[3]. *Persea americana* containing a huge amount of nutrition, antioxidant and biochemical profile is marketed as a superfood[4]. In the current study, leaves of *Persea americana* have been used in the extraction of Tyrosine Ammonia Lyase for depigmentation. The human skin colour based on the melanin pigment is divided into two categories: Constitutive or intrinsic skin colour and

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Facultative or inducible skin colour[5]. The genetically determined cutaneous melanin pigmentation is designated by constitutive skin colour, facultative level is characterised as the increase in the level of melanin pigmentation above the constitutive level that is obtained from a complex interplay of solar radiations and hormones[5,6]. Solar radiation leading to facultative skin colour changes is known as suntan[7]. In the current study, the darkening of the skin colour due to the hormonal and suntanning effects is treated through the process of depigmentation of the particular area where excess melanin pigment is expressed[8]. There have been numerous beauty products manufacturing companies that have been involved in manufacturing many products leading to depigmentation; all these beauty products contain many synthetic chemical agents that act as major components in depigmentation, leading to effects that are temporary and also give rise to many side effects like effecting the inner membrane linings that will slowly show the effect, as a result of this an attempt has been made to use natural elements extracted from natural resource to obtain depigmentation in the above study[9]. *Persea americana,* commonly known as avocado, is a species of Mexico Central America[10]. Oil of *Persea americana* has been widely used for many Dermatological applications - the use of *Persea americana* in the treatment of many Health-related conditions has been reported by ethnopharmacological information. Research work has also proved that *Persea Americana* may improve hypercholesteremia and has shown many positive effects in the treatment of hypertension, inflammatory conditions, and diabetes[11-13]. The leaves of *Persea americana* are involved in the treatment of many disorders due to the presence of many important vitamins and minerals in them. Diosgenin, a biologically active phytochemical has significantly been utilized for the treatment of various types of diseased conditions like inflammation, hypercholesteremia and cancer (Figure 1)[14]. This steroid has been proved to be the subject of interest worldwide by many researchers due to its huge industrial importance, especially in the field of cosmetics manufacturing industries[15]. The tremendous anti-inflammatory properties of diosgenin make it suitable for its use in dermatological Products. Diosgenin extract has been proven to have

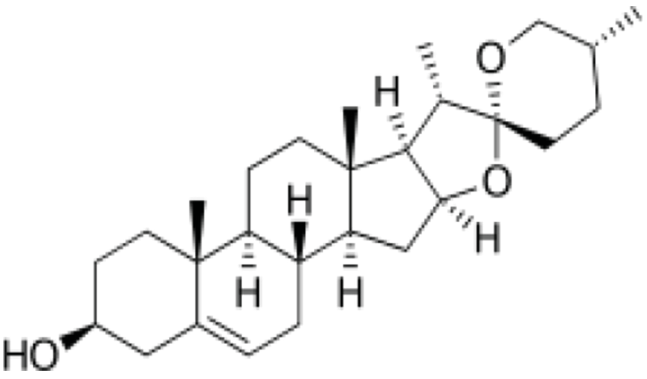

Figure 1. Structure of diosgenin.

a great amount of anti-collagenase effect proving to be suitable for its use in anti-ageing products, hence justifying itself to be a rich element in fighting many skin degenerative syndromes[16]. Considering all its applications, in the above study, diosgenin has been targeted to be used as an element increasing the activity of Tyrosine Ammonia lyase to increase the activity of the enzyme in comparison with that of kojic acid that is been used as control.

Zebrafish Danio is a freshwater species belonging to the minnow family of Cypriniforms order[17]. Zebrafish is a significantly used vertebrate model organism in research by scientists due to its fully sequenced genome[18]. The embryonic development stages of Zebrafish embryos are well studied. Zebrafish is an organism that was cloned first in the research area[19]. The embryos of Zebrafish Danios are transparent, with natural pigments[20].

2. Materials

Reagents: Chilled acetone, chilled distilled water, embryonic water, Tris HCl, HCl 1(N), L-tyrosine, methanol, $MnSO_4$, NH_4SO_4, kojic acid, hexane solvent, ethanol, diosgenin, methanol, p-anisaldehyde, ethyl acetate, H_2SO_4, L-ascorbic acid.

Instruments: Chilled grinder, cooling centrifuge, UV-spectrophotometer, incubator, breeding tank, ph-meter, soxhlet apparatus, reflux unit, ultrasound bath, microwave, magnetic stirrer.

Other Requirements: Mortar and pestle, glass wares, centrifuge tubes, micropipettes, fresh leaves of *Persea americana.*

3. Methods

3.1 Extraction and Isolation of Tyrosine Ammonia Lyase

Persea americana leaves were collected from northern areas of Bangalore. The water content of the leaves was removed completely by drying them at room temperature. 26.6 g of leaves were weighed and ground with 100 ml of distilled water using a mixer grinder. The muslin cloth was used and the prepared mixture was filtered. The obtained filtrate was measured using a measuring cylinder. The volume of the measured cylinder was found to be 110 ml, it was then refrigerated in the freezer for 24 hrs[21]. An equal amount of chilled acetone was added to the filtrate and kept undisturbed at -2°C for 24 hrs in a deep freezer. After precipitation two layers were formed, the upper layer was discarded and the precipitate containing protein was collected. The precipitate obtained was then centrifuged at 5000 rpm for 5 minutes at low temperature in a cooling centrifuge. The pallet obtained after the process of centrifugation was collected, it was washed using acetone and the obtained mixture was then poured into Petri plates to dry the acetone present in it. The acetone powder obtained was weighed. 1.0 g of the powder was homogenized with 20 ml of 1 mM Tris HCl (pH 8.2) in a pestle and mortar. The mixture obtained was centrifuged at 5000 rpm for 5 mins. The crude enzyme extract was obtained in a supernatant form, which was used for further analysis[22].

3.2 Assay of Tyrosine Ammonia Lyase

Reaction mixture tubes T_1, T_2, and T_3 were prepared, T_1 tube was prepared using 0.5 ml of enzyme source, 0.2 ml of 1mM L-Tyrosine (substrate) and 1.3 ml of 0.1M Tris HCl buffer (pH 8.9). T_2 was prepared using 1 ml of enzyme source, 0.2 ml of 1mM L-Tyrosine(substrate) and 0.8 ml of 0.1M Tris HCl buffer (pH 8.9). T_3 was prepared using 1.5 ml of enzyme source, 0.2 ml of 1mM L-Tyrosine (substrate) and 0.6 ml of 0.1M Tris HCl buffer (pH 8.9). A control tube was also prepared using 1 ml of distilled water instead of enzyme extract, 0.2 ml of 1mM L-Tyrosine (substrate) and 0.8 ml of 1mM Tris HCl buffer (pH 8.9). All the tubes were incubated in an incubator at 37°C for 30 mins. After incubation reaction is terminated by the addition of 0.5 ml of 1N HCl. Tyrosine Ammonium Lyase enzyme deaminates L-Tyrosine and produces p-coumaric acid with the release of ammonia. Then it was quantitatively measured by using a UV-Visible spectrophotometer at 380nm[23,24].

4. Enzyme Activity Analysis using Diosgenin Extracted from *Persea americana*

Diosgenin, a steroid an element that inhibits melanin production, is extracted from leaves of *Persea americana* to analyse its ability to increase the activity of Tyrosine Ammonia Lyase by defatting followed by Microwave-assisted extraction and ultrasound-assisted extraction[25,26].

4.1 Defatting

About l00 g of fresh leaves of *Persea americana* were weighed. The leaves were washed thoroughly and dried completely. The dried leaves of *Persea americana w*ere powdered finely using a mixer grinder. 20 g of fine powder of *Persea americana* leaves were taken and defatted using a Soxhlet apparatus. 10 g was accurately taken, and appropriately sized thimbles were made. 100 ml of hexane solvent was used, and the sample was defatted. About four cycles were carried out that lasted around for 4 hrs[27].

4.2 Extraction by Microwave-Assisted Extraction (MAE)

Microwave-assisted extraction of leaves of *Persea americana* was performed[28]. 2 g of defatted sample was weighed and taken in a conical flask, and 10 ml of 80% of ethanol 1:5 w/v was added to the sample. Extraction of the sample was carried out in a microwave (IFB, Microwave oven) at 200W for 50s and 80s, respectively. The extracted sample was centrifuged at 200 rpm for 10 mins. The obtained supernatant was filtered using a Whatman filter paper No. 1. The obtained filtrate was evaporated for a period of two days. The obtained product was then dissolved in ethanol to give a 5% concentration of extract. The sample obtained was used for analysis of enzyme activity.

4.3 Extraction by Ultrasound-Assisted Extraction (UAE)

Ultrasound-assisted extraction of defatted leaves of *Persea americana* was carried out[29]. An ultrasound

bath was used for extracting the sample. 2 g of defatted sample was weighed in a 50 ml conical flask 10 ml of 80% ethanol was added to the sample 1:5 w/v solid to sample ratio was maintained similar to that of microwave-assisted Extraction. The extraction was carried out for 5-6 mins[29]. The obtained extract was filtered using a Whatman filter paper No. 1. The obtained filtrate was evaporated for two days. The sample obtained was used for analysis of enzyme activity.

4.4 Analysis

The obtained extracts were used and made up to a concentration of 10 µg/ml. Four test tubes marked as Control, T_1, T_2 and T_3 were taken. 1 ml of distilled water followed by 0.8 ml of Tris HCl buffer and 0.2 ml of Tyrosine were taken in the test tube marked as control. The T_1 test tube contained 1 ml of the untreated enzyme (without the diosgenin-containing extract), 0.8 ml of Tris HCI buffer 0.2 ml of Tyrosine.1 ml of enzyme extract, 0.8ml of Tris HCl buffer, 0.2 ml of tyrosine and 0.2 ml of Microwave-assisted extract was added in T_2 test tube, whereas T_3 was added with 1 ml of 1 ml of enzyme extract, 0.2 ml of tyrosine, 0.8 ml of Tris HCl buffer followed by 0.2 ml of ultrasound-assisted extract. Incubation of all the test tubes was carried out at 37˚C for 30 mins. 0.5 ml of 1N HCl was used to terminate the reaction after incubation. The UV-visible spectrophotometer was used to measure the samples quantitatively at 410 nm^2.

5. Enzyme Activity Analysis using Partially Purified Enzyme from NH_4SO_4 and $MnSO_4$

5.1 Purification of Enzyme using NH4SO4

The NH_4SO_4 Purification method is used to purify proteins by altering their solubility. The solubility of proteins decreases at low salt concentrations. Salting in and salting out are the two commonly used methods. The salting out method was used in the purification of Tyrosine Ammonia Lyase. About 0.286 g of NH_4SO_4 salt was weighed and added to 2 ml of diluted extract of the enzyme (50% dilution), and it was kept in ice bath followed by constant stirring. The solution was centrifuged for about 8 mins at 5000 rpm. The obtained pellet was dissolved in 2 ml of Tris HCl buffer of pH 8.9. Test tubes marked as Control, T_1 and T_2 were taken for further estimations. The control tube was added with 1 ml of distilled water, 0.2 ml of Tyrosine and 0.8 ml of Tris HCl buffer (pH 8.2). 1 ml of untreated enzyme extract followed by 0.8 ml of Tris HCl buffer (pH 8.2) and 0.2 ml of Tyrosine was added in T_2, whereas T_3 was added with 1 ml of NH_4SO_4 purified enzyme, 0.2 ml of Tyrosine and 0.8 ml of Tris HCl buffer (pH-8.2). All the tubes were incubated for about 30 minutes at room temperature. 0.5 ml of 1N HCl was used to terminate the reaction after incubation. UV-visible spectrophotometer was used to measure it quantitatively at 410 nm^2.

5.2 Purification of Enzyme using MnSO4

Enzymes were purified using the $MnSO_4$ purification method where all unwanted biomolecules and nucleic acids were removed to prevent their interference with the substrate. About 1.69 g of $MnSO_4$ was utilized and added drop by drop gradually to 2 ml of the diluted enzyme extract that was placed on the ice bath followed by continuous stirring. The solution was centrifuged for about 8 mins at 5000 rpm. The obtained pellet was dissolved in 2 ml of Tris HCl buffer of pH 8.9. Test tubes marked as Control, T_1 and T_2 were taken for further estimations appropriately. The control tube was added with 1 ml of distilled water, 0.2 ml of Tyrosine and 0.8 ml of Tris HCl buffer (pH 8.2). 1 ml of untreated enzyme extract followed by 0.8 ml of Tris HCl buffer (pH 8.2) and 0.2 ml of Tyrosine was added in T_2, whereas T_3 was added with 1 ml of NH_4SO_4 purified enzyme, 0.2 ml of Tyrosine and 0.8 ml of Tris HCl buffer (pH 8.2). All the tubes were incubated for about 30 minutes at room temperature. 0.5 ml of 1N HCl was used to terminate the reaction after incubation. UV-visible spectrophotometer was used to measure it quantitatively at 410 nm^2.

6. *In Vivo* Experimental Study on Zebrafish Danios

Zebrafish were maintained in lab conditions and allowed to mate in a breeding tank. Embryos of Zebrafish were taken after 20 hrs of egg fertilization before the pigmentation was produced and transferred in a Petri plate containing embryo water. Embryos were maintained under proper lab condition and their developmental stages were observed under microscope[21,23].

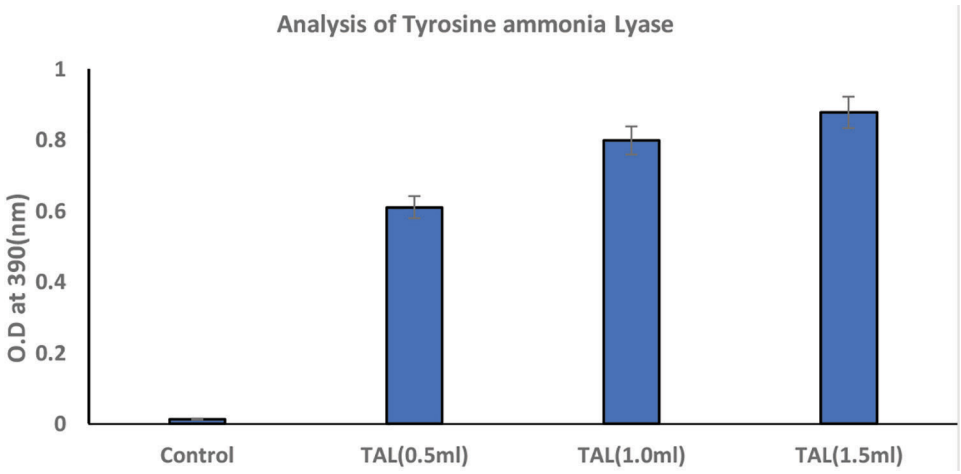

Figure 2. Quantitative analysis of p-coumaric acid.

6.1 Experiment on Zebrafish (*In Vivo*): to Obtain Depigmentation

Embryos of Zebrafish were taken after 20 hrs of fertilization before the pigmentation[23] was produced and kept in Petri plates containing embryo water. Six small petri plates were taken and marked as untreated, control, T_1, T_2, T_3 and T_4. In each Petri plate, three embryos and 2 ml of embryo water were taken. Then 0.1 ml of kojic acid was added to the plate marked as control. 0.1 and 0.3 ml of enzyme extract were added to T_1, T_2 samples. 0.1 ml of the enzyme along with MAE diosgenin and UAE diosgenin was added to plates marked as T_3 and T_4, and the untreated plate was left as such without enzyme. All the plates were kept at optimum temperature and observed under the microscope after 24 hrs. After observation, the embryo water was cleaned and the plates were again added with samples. Then, it was kept at optimal temperature and observed after 24 hrs for better results[2,16].

7. Results

The presence of the enzyme tyrosine ammonia lyase was confirmed in the leaves of *Persea americana*. The standard conditions were followed and the enzyme assay was performed. The UV-visible spectrophotometer was utilized to find the activity of the enzyme at 380 nm[2]. Diosgenin was extracted from the leaves of *Persea americana*[23], where in microwave-assisted extraction method and ultrasound-assisted extraction method were utilized to extract the diosgenin. The extracted diosgenin significantly increased the activity of the enzyme Tyrosine ammonia lyase on analysing the activity of the enzyme using a UV-visible spectrophotometer at 410 nm, and hence, was proved to be an activator of the enzyme Tyrosine Ammonia Lyase. NH_4SO_4 and $MnSO_4$ salts were utilized in partially purifying the enzyme to analyse the activity of the partially purified enzyme with that of the crude enzyme extract. *In vivo* studies performed utilizing Zebrafish embryos showed a high depigmenting effect in the samples in which enzyme extract was utilized. The effect of depigmentation of enzyme samples on Zebrafish embryos was compared with that of samples where kojic acid was used as a control[2]. The enzyme Tyrosine Ammonia Lyase along with microwave-assisted extract of diosgenin showed a high level of similarity with kojic acid on comparison.

7.1 Enzyme Assay of Tyrosine Ammonia Lyase extracted from Persea americana

Each experiment was performed in triplicate, and the results are presented as Mean±S.D. Figure 2 shows that the sample marked as T_3 showed the formation of a high amount of P-Coumaric acid as a result of deamination of L-Tyrosine that was assayed quantitatively at 380 nm, where 1.5 ml of enzyme source was taken when compared to that of T_1 and T_2 in which 0.5 ml and 1 ml of enzyme sample was added, indicating that a gradual rise was seen in the formation of the product P-Coumaric acid on deamination of L-Tyrosine by the

enzyme Tyrosine Ammonia Lyase with the increasing volume of enzyme sample utilized for assay that was quantitatively measured at 380nm. The control indicated that there was no formation of P-Coumaric acid observed, as the enzyme sample extract was not added in the test tube marked as control.

7.2 Enzyme Activity Analysis using Diosgenin Extracted from Persea americana

Each experiment was performed in triplicates, and the results are presented as Mean±S.D.

Quantification of P-Coumaric acid formed was used to analyse the activity of the TAL enzyme. Diosgenin obtained from the leaves *of Persea americana* increased the activity of the enzyme Tyrosine Ammonia Lyase when added to the test samples containing crude enzyme extract. It was extracted by Microwave-assisted extraction method and ultrasound-assisted extraction method. Enzyme in the test sample containing microwave assisted diosgenin extract showed a high increase in the activity of the enzyme when compared to that of diosgenin extracted from ultrasound-assisted extraction method. The enzyme extracts along with diosgenin extracted from the microwave-assisted extraction method showed an increase in the ability of the enzyme to form P-Coumaric acid as a result of deamination of L-Tyrosine by the enzyme in comparison with that of the enzyme-containing ultrasound-assisted extract (Figure 3).

7.3 Enzyme Activity Analysis using Partially Purified Enzyme from NH4SO4 and MnSO4

Each experiment was performed in triplicates, and the results are presented as Mean±S.D. The activity of the TAL enzyme was analysed by quantifying the amount of P-Coumaric acid formed as a result of the reaction of the enzyme with that of substrate Tyrosine, where the capability of Tyrosine Ammonia lyase to deaminate Tyrosine to p-coumaric acid was examined. The amount of P-Coumaric acid formed by the enzyme extract was examined, and the enzyme partially purified with Ammonium sulphate, and enzyme partially purified with magnesium sulphate were assessed. The enzyme purified with ammonium sulphate and magnesium sulphate showed a decrease in the activity of the enzyme when compared to that of the unpurified crude enzyme (Figure 4).

7.4 In Vivo Experimental Study on Zebrafish Danios

The obtained eggs of Zebrafish danios were collected very carefully from the breeding tank. The eggs were placed in different Petri plates containing embryo watermarked as test samples and control. The eggs in the petri plate were placed in a sterilized incubator. The eggs in each Petri plate were visualized under the microscope to examine their viability and to analyse

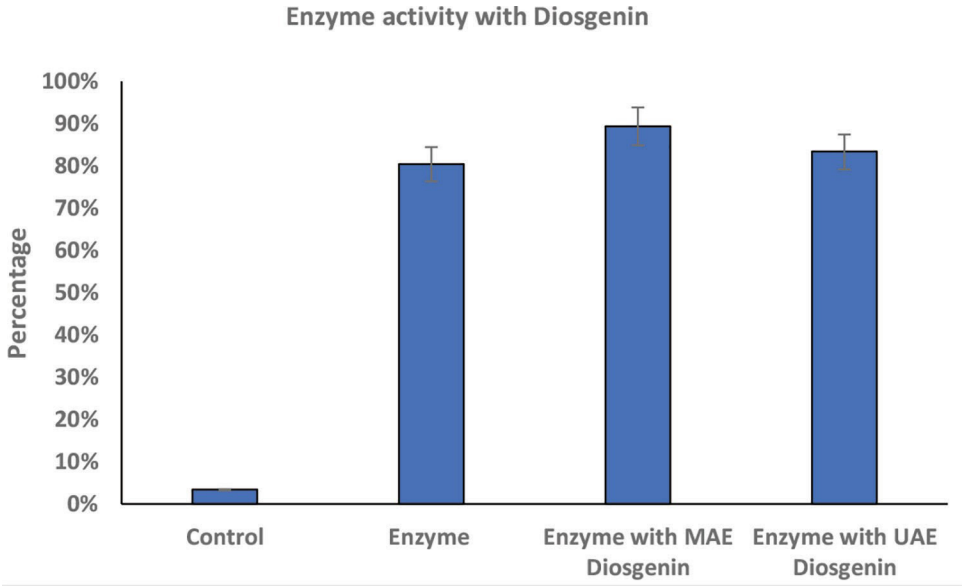

Figure 3. Activity of Tyrosine Ammonia Lyase (TAL).

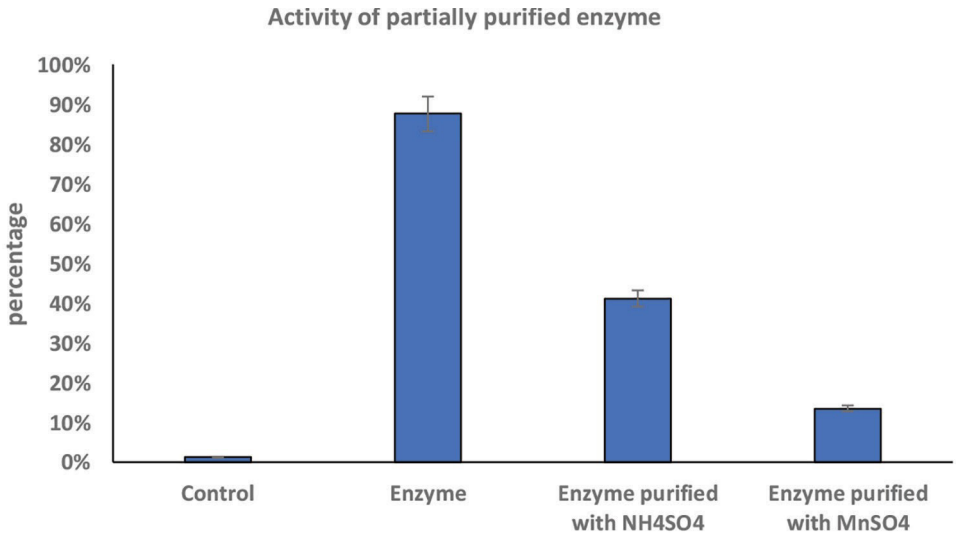

Figure 4. Activity assay of partially purified Tyrosine Ammonia Lyase (TAL).

each development stage of the embryos inside the eggs. The selected eggs with well-developing embryos are chosen to carry out further tests (Figures 5-8).

7.5 Experiment on Zebrafish (In Vivo) to Achieve Depigmentation

Figures 9-14 represent that the development of pigment was marked in embryos that were not treated with the enzyme sample. A slight pigment development was still observed in embryos that were treated with 0.1 ml of enzyme extract, whereas higher depigmentation similar to embryos which were treated with control containing 2% kojic acid was marked in embryos that were treated with 0.3 ml of enzyme extract. Clear Depigmentation was marked in embryos that were treated with a mixture of TAL enzyme and Microwave assisted extracted diosgenin and enzyme with ultrasound-assisted extracted diosgenin proving that diosgenin acted as a potent activator of TAL enzyme.

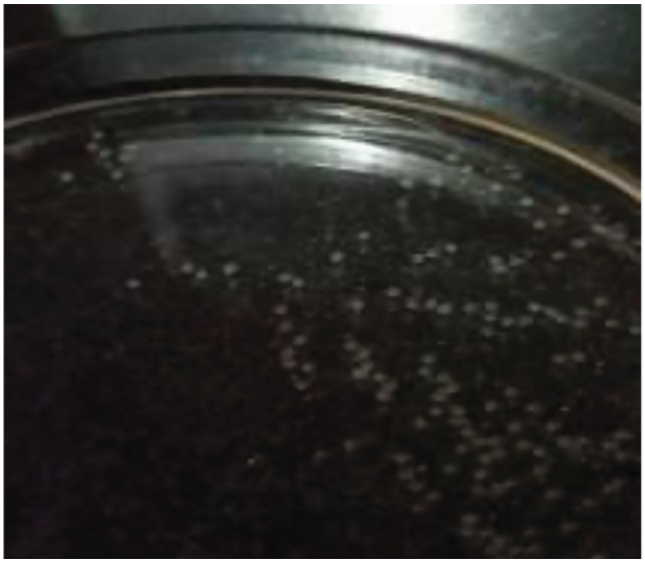

Figure 5. Zebrafish eggs in embryo water.

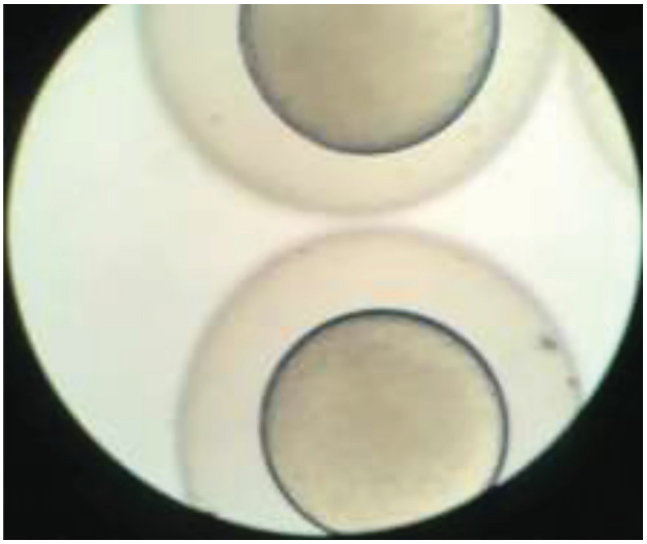

Figure 6. Zebrafish eggs under 40x. magnification.

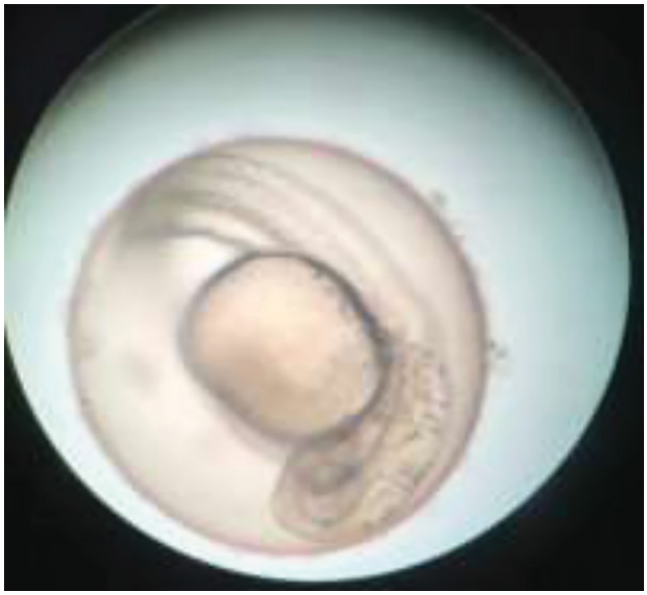

Figure 7. Embryo development inside an egg.

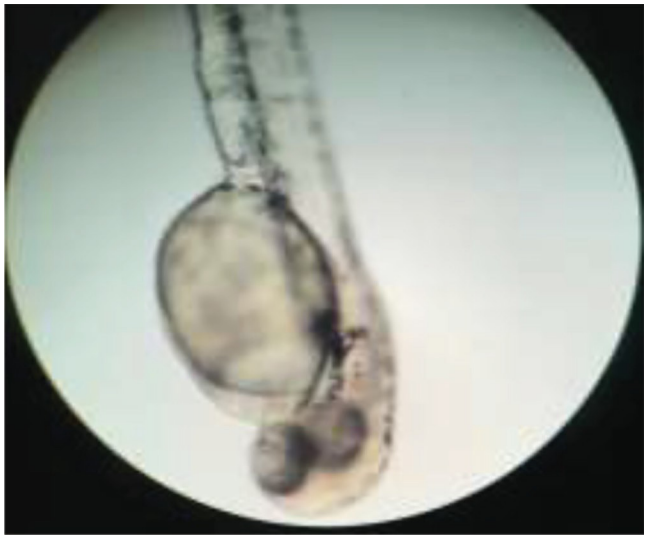

Figure 8. Developed embryo.

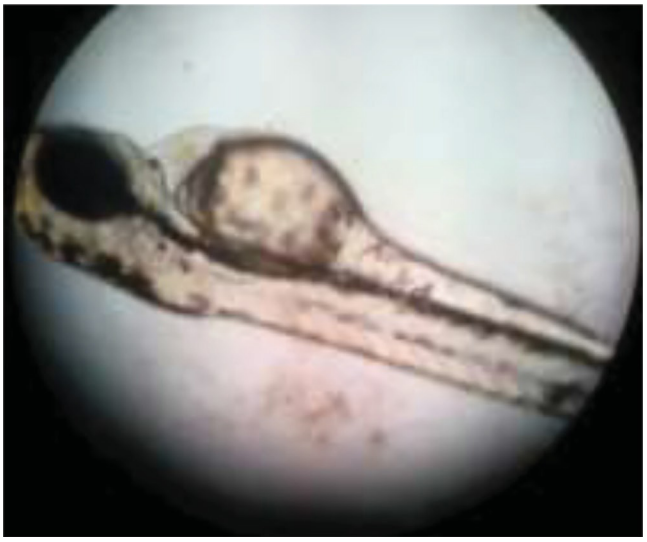

Figure 9. Untreated without TAL enzyme.

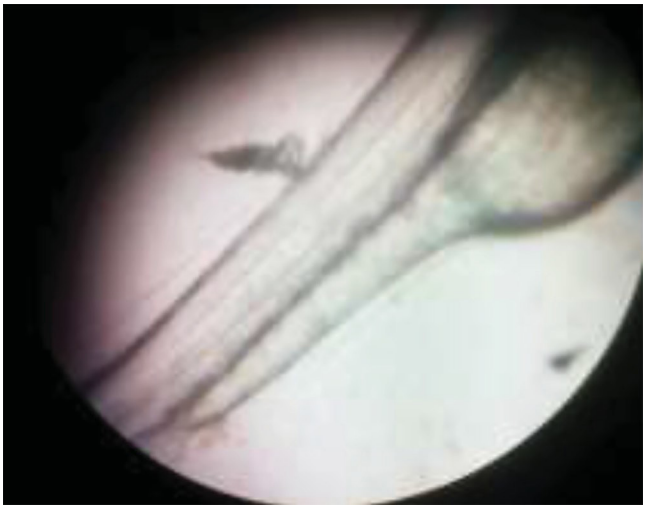

Figure 10. Control 2% kojic acid.

8. Conclusion

The main objective of our study was to identify a natural compound and utilize it for depigmenting the area of the skin, where an excess amount of melanin pigment is released from the melanin pigment production pathway and darkens the area of the skin to a greater extent, the enzyme Tyrosine ammonia lyase that has been extracted from the leaves of *Persea americana* is utilized in the treatment of such hyperpigmentation conditions and Suntans that has been proved significantly from the *in vivo* studies that has been carried out on the Zebrafish embryos, where in kojic acid a skin whitening chemical element is used as control to compare the effect of Tyrosine ammonia lyase extracted with that of kojic acid. The enzyme Tyrosine ammonia lyase extracted from leaves of *Persea americana* showed a similar effect as that of kojic acid used as control. Diosgenin, a melanin-inhibiting element extracted from the leaves of the same plant acted as a potent activator and increased the activity of the enzyme showing a markable significant effect of depigmentation. This particular study carried out indicates that the use of natural enzymes extracted from plants along with their natural activating elements

Figure 11. 0.1 ml of TAL enzyme.

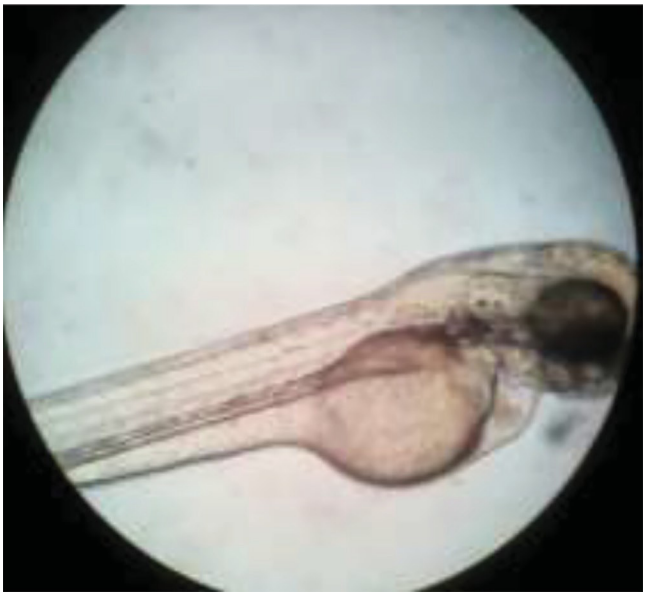

Figure 13. 0.1 ml of TAL with MAE of diosgenin.

Figure 12. 0.3 ml of TAL enzyme.

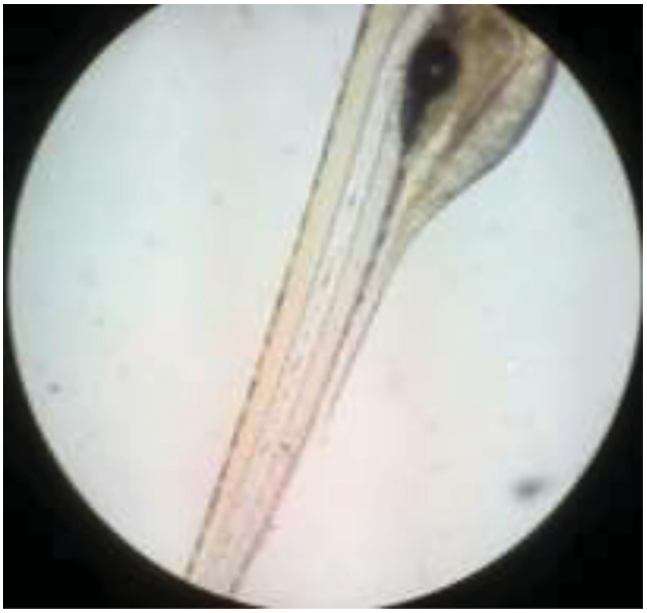

Figure 14. 0.1 ml of TAL with UAE of diosgenin.

can be utilized in the treatment of Hyperpigmentation as well as lightening the skin without any harmful effect.

9. Acknowledgements

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10. References

1. Jimbow K, Obata H, Pathak MA, Fitzpatrick TB. Mechanism of depigmentation by hydroquinone. Journal of Investigative Dermatology, 1974; 62(4):436-49. https://doi.org/10.1111/1523-1747.ep12701679
2. Difran AJ, Abhinaya HV, Verma S, Sampath S, Umaima U, Swetha BN, *et al*. Extraction of Tyrosine Ammonia Lyase (TAL) from *Murraya Koenig*ii plant for the treatment of

hyperpigmentation-involves in-vivo analysis on zebrafish embroys and molecular docking. World Journal of Pharmaceutical Sciences. 2016; 5(17):69-80.
3. Singh KG, Umaima SU, Jangid K. *In vivo* study of depigmentation using Tyrosine Ammonia Lyase from *Trigonella foenum-graecum* on zebra fish embryos. International Journal of Current and Research and Review. 2019; 11(8):13-16. https://doi.org/10.31782/IJCRR.2019.1316
4. Bhuyan DJ, Alsherbiny MA, Perera S, Low M, Basu A, Devi OA, *et al*. The odyssey of bioactive compounds in avocado (*Persea americana*) and their health benefits. Antioxidants. 2019; 8(10): 426-69. https://doi.org/10.3390/antiox8100426
5. Fitzpatrick TB, Miyamoto M, Ishikawa K. The evolution of concepts of melanin biology. Archives of Dermatology. 1967; 96(3):305-23. https://doi.org/10.1001/archderm.1967.01610030083015
6. Fitzpatrick TB. The epidermal melanin unit system. Dermatologische Wochenschrift. 1961; 147:481-9.
7. Naughton GK, Reggiardo D, Bystryn J-C. Correlation between vitiligo antibodies and extent of depigmentation in vitiligo. Journal of the American Academy of Dermatology. 1986; 15(5):978-81. https://doi.org/10.1016/S0190-9622(86)70260-0
8. Daniels F, Brophy D, Lobitz WC. Histochemical responses of human skin following ultraviolet irradiation. Journal of Investigative Dermatology. 1961; 37(5):351-7. https://doi.org/10.1038/jid.1961.131
9. Dooley TP. Topical skin depigmenting agents. Journal of Dermatological Treatments. 1997; 8(4):275-83. https://doi.org/10.3109/09546639709160535
10. Dabas D, Shegog RM, Ziegler GR, Lambert JD. Avacado (*Persea americana*) Seed as a source of Bioactive Phytochemical. Journal of Current Pharmaceutical Design. 2013; 19(34):6133-40. https://doi.org/10.2174/1381612811319340007
11. Anaka ON, Ozolua RI, Okpo SO. Effect of the aqueous seed extract of *Persea americana* mill (Lauraceae) on the blood pressure of Sprague Dawley rats. African Journal of Pharmacy and Pharmacolog. 2009; 3(10):485-90.
12. Patel K, Gadewar M, Tahilyani V, Patel DK. A review on pharmacological and analytical aspects of diosgenin: A concise report. Natural Products and Bioprospecting. 2012; 2(2):46-52. https://doi.org/10.1007/s13659-012-0014-3
13. Jesus M, Martins APJ, Gallardo E, Silvestre S. Diosgenin: Recent highlights on pharmacology and analytical methodology. Journal of Analytical Methods in Chemistry. 2016; 16(8):243-52. https://doi.org/10.1155/2016/4156293
14. Binic I, Lazarevic V, Ljubenovic M, Mojsa J, Sokolovic D. Skin ageing: Natural weapons and strategies. Evidence-Based Complementary and Alternative Medicines. 2013; 13(14):1-10. https://doi.org/10.1155/2013/827248
15. Raines F, Pauli D, Rereio D. Fish Base Faroe Islands. 2007; 19(5):34-40.
16. Howe K, Clark MD, Taroja CF. The zebrafish reference genome sequence and its relationship to the human genome. The Zebra Fish Genome. 2013; 16(8):498-503. https://doi.org/10.1038/nature12111
17. Goldshmit Y, Sztal TE, Jusuf PR, Hall TE, Nguyen-Chi M, Currie PD. Fgf-dependent glial cell bridges facilitate spinal cord regeneration in zebrafish. The Journal of Neuroscience. 2012; 32(22): 7477-92. https://doi.org/10.1523/JNEUROSCI.0758-12.2012
18. Dham R. Zebra fish exposed. American Scientist. 2016; 94(5):446-53. https://doi.org/10.1511/2006.61.1006
19. Harwood LM, Moody CJ. Experimental organic chemistry: Principles and Practice (Illustrated ed). International Journal of Pharma and Bioscience. 1989; 5(1):76-82.
20. Watts KT, Mijts B, Lee P, Manning AJ, Schmidt-Dannert C. Discovery of a substrate selectivity switch in Tyrosine Ammonia-Lyase, a member of the aromatic amino acid lyase family. Journal of Chemical Biology. 2006; 13(5):1317-26. https://doi.org/10.1016/j.chembiol.2006.10.008
21. Rosler J, Kerekal F, Amrehein N, Schmid J. Maize phenlalanine ammonia-lyase has Tyrosine Ammonia-Lyase activity. Journal of Plant Physiology. 1997; 113(1):175-9. https://doi.org/10.1104/pp.113.1.175
22. Louie GV, Bowman ME, Moffitt MC, Baiga TJ, Moore BS, Noel JP. Strutural determinants and modulation of substrate specificity in Phenylalanine Tyrosine Ammonia-Lyase. Chemistry and Biology. 2006; 13(12):1327-38. https://doi.org/10.1016/j.chembiol.2006.11.011
23. Arya P, Kumar P. Comparison of ultrasound and Microwave-assisted extraction of diosgenin from *Trigonella Foenum Graecum* seeds. Ultrasonic Sonochemistry. 2021; 74(72): 274-85. https://doi.org/10.1016/j.ultsonch.2021.105572
24. Liu B, Lockwood GB, Gifford LA. Supercritical fluid extraction of diosgenin from tubers of *Dioscorea nipponica*. Journal of Chromatography A. 1995; 690(2):250-3. https://doi.org/10.1016/0021-9673(94)01117-W
25. Wani SA, Bishnoi S, Kumar P. Ultrasound and Microwave-assisted extraction of diosgenin from fenugreek seeds and fenugreek supplemented cookies. Journal of Food Measurements and Characterization. 2016; 10(16):527-32. https://doi.org/10.1007/s11694-016-9331-2
26. Sharma RD, Raghram TC, Roa NS. Effect of fenugreek on blood glucose and serum lipids in Type 1 Diabetes mellitus. Search Life Science Literature. 1990; 44(4):301-6.
27. Rothrock JW, Hammes PA, McAleer WJ. Isolation of diosgenin by acid hydrolysis of saponin. Journal of Biochemistry. 1957; 49(2):186-8. https://doi.org/10.1021/ie50566a025

28. Zang Y, Thang L, An X, Fu E, Ma C. Modification of cellulase and its application to extraction of diosgenin from *Dioscorea zingiberensis* C.H. Wright. Biochemical Engineering Journal. 2009; 47(3):80-6. https://doi.org/10.1016/j.bej.2009.07.006

29. Dsouza M, Rufina K, Hana D. Extraction of diosgenin from fenugreek and evaluation of its Pharmacological role in alleviating metabolic syndrome *in vitro*. Research Journal of Biotechnology. 2018; 13(12):10-17. https://doi.org/10.1016/j.bej.2009.07.006