In Vivo Therapeutic Study Of Repigmentation Of Depigmented Patches In Vitiligo Disorder Isolated Tyrosinase Of Moringa Oleifera And Other Extracts In Zebra Fish Embryo

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Abstract
Zebra fish embryos obtained by mating the Zebra fish Danios were used as a model for preclinical *in vivo* therapeutic studies. Tyrosinase enzyme isolated from the leaves of *Moringa oleifera* along with the crude extracts rich in polysterols, flavonoids, phenols and carotenoids analysed qualitatively and quantitatively were utilized in inducing pigmentation in the depigmented patches in the above diseased conditions. The depigmented developing embryos were treated with the Tyrosinase enzyme with a 1ml of concentration, showed a partial repigmentation in them. It was also found that the crude enzyme extract along with the enzyme Tyrosinase obtained also induced repigmentation in the depigmented embryos by involving in development of melanin cells and protecting the cells by enhancing the activity of enzyme Tyrosinase in inducing melanin pigments. It functions by inhibiting the conversion of L-tyrosine of melanin, as a result of this the normal Zebra stripped pattern on the skin was not formed, resulting in the formation of depigmented skin in the developing embryos. Firstly, Vitiligo or Leucoderma like condition is induced in Zebra fish embryos by carrying out depigmentation using kojic acid. The outcome of the above study indicated that the repigmentation induced in the depigmented embryos can be correlated to treatment of Vitiligo and Leucoderma by inducing repigmentation of depigmented patches in the above conditions.

Keywords: *Moringa oleifera,* Tyrosinase, Kojic acid, Zebra fish, Vitiligo, Leucoderma Melanin

Introduction

Vitiligo is a skin disorder which majorly effects skin among the people worldwide. The melanocyte and the melanin of the epidermis become inactive and loses it functioning activity leading to Vitiligo disorder. Leucoderma is also a similar condition as Vitiligo except that the difference between Vitiligo and Leucoderma is that, Vitiligo is found to be a disorder that is self-generated usually found out to be an autoimmune disorder, whereas Leucoderma occurs due to allergies from some harmful chemicals, infections of skin and also may be autoimmune disorder condition. The white patches are common case appears due to melanocytes degradation in the skin (Aggarwal and Shishodia, 2006, Ahmadiani et al., 2001, Alkhateeb et al., 2003). Melanin pigment gives skin its natural colour and protect it from U-V rays. When these cells get affected by environmental, genetic and immunological factors, it loses their functions and is inactivated leading to discoloration of skin in form of patches affecting various parts of the body (Alzoreky and Nakahara, 2003, Anwar et al., 2007, Aswar et al., 2010). Approximately around 2% of the world

population has been found affected by Vitiligo and 5% of it by Leucoderma (Baltas et al 2001). The colour loss rate in Vitiligo disorder is unpredictable (Baquer et al., 2011). The loss of colour of skin in Leucoderma is limited to a particular area and does not spread to the other portions as seen in cases of Vitiligo (Best et al., 2008, Banger et al., 2008, Bin-Hafeez et al., 2003). There are different methodologies that are being adopted in the current period which are being practised and are still under progress to be practised to regain the skin colour in the white patches that are formed. The current treatment procedures like skin tissue replacement surgeries, radiation therapies and numerous drugs would take long period of time to show its effect which may or may not show the result that has been usually observed from a long period of time, more over these following methods used may result in numerous types of side effects leading to other disorders et (Boersma et al., 1995). It is also found out that due to use of radiation therapies the inner layer of the skin is affected by destroying the collagen protein weaking the binding of tissues and cells to each other (Boulton and Marshall, 1985). The objective of the above study is to utilise important compounds and enzyme from the natural source from *Moringa oleifera* that has a got high ability to cause the repigmentation of the white patches and prevent the spread of the white patchy appearance by protecting the melanin cells in the above conditions (Brysk et al., et al., 1989, Cui et al., 1991). An effort is made to rule out all the ill effects and side effects that are caused from the current methodologies used in the treatment process by using the natural source having excellent nutritive values with highly efficient qualities to regain the lost pigment as well as significant capacity to protect the destruction of melanin cells.

Moringa oleifera known as Petrygosperma gaertn, belongs to the family of Moringaceae a Perennial angiosperm plant (Verma et al., 2009). Leaves of *M. oleifera* have been found to be highly rich in phytochemicals. It is found that the amount of Vitamin C present in the plant is more than that found in oranges, vitamin A more than carrots, calcium more than milk, proteins more than yogurt and iron more than spinach (Islam et al., 2021). The use of leaves of *M. oleifera* has been recommended in the utilization of the treatment of thyroid disorder, diabetic disorder and also in the treatment of many cardiovascular disorders from the ancient time periods itself without knowing the specific target components present, that are involved in the treatment (Mbikay, 2012). Hence due to its ability in the treatment some of the above diseased conditions and the presence of all vitamins, minerals and proteins, the leaves of the plants is used in the above research carried out.

The enzyme Tyrosinase is an enzyme enclosing copper within it. Tyrosinase enzyme (EC. 1.14.18.1) belongs to oxidoreductase category in the general classification of enzyme, the mechanism of oxidation and reduction in the epidermis is carried out by this enzyme (Laksmiani et al., 2022). This particular chemical reaction catalysed by this enzyme is of great importance in the melanin synthesis process, it is one of the most important key enzyme in mammalian melanin synthesis (Pillaiysr et al., 2017). The Tyrosinase enzyme is involved in production of pigment in two different cell types: the epithelial cells of retina and the melanocytes (Tief et al., 1996). Melanin is utmost spread in the plant and animal kingdom. It is a pigment that is widespread on the vertebrate surface area. The granules of melanin are shifted from melanocytes to epithelial cells and then involve in the formation of pigments of epidermis and hair (Moreiras et al., 2021). Hence current investigation aimed to identify the new biomolecule for the pigmentation issue using various process and extract of *M. olifera* leaves and their in vivo studies on Albino Zebra fish embryos.

Material and methods

Preparation of leaves extract of *Moringa oleifera*

Green matured leaves of *M. oleifera* were collected from north areas of Bengaluru. Two separate 10% raw and boiled extracts of fresh

leaves and completely dried (37˚C), powered leaves of *M. oleifera* and 10% methanolic extract of fresh leaves was prepared. 10% of all these extracts were used to quantitatively analyse the bioactive molecules, present in the leaves.10% raw extract of fresh leaves were used as crude sample in the in vivo studies. Fresh matured green leaves were homogenised using pestle and mortar, filtered and centrifuged at high speed. Similarly fresh matured green leaves were completely dried at room temperature for 2-3 days and finely powdered, the fine powder of the dried leaves were centrifuged at high speed. The supernatant obtained was used to prepare 10% raw extract of both fresh and dried leaves. Fresh leaves and dried leaf powder of *M. oleifera* was firstly homogenised and boiled in water to prepare 10% boiled extracts. The boiling was carried out for 10 minutes and cooled, later the 10% boiled extracts were centrifuged at high speed and both the raw and boiled leaf extracts along with 10% methanolic extracts were stored and used to quantitatively analyse the presence of Phytosterols, Flavonoids, Phenols and Carotenoids.

Quantitative analysis of bioactive molecules

Estimation of Phytosterol

Phytosterols are an important class of secondary plant metabolites. It has a chemical structure similar to that of cholesterol. Therefore, cholesterol can be used as a standard for estimation of phytosterols (Salehi *et al*., 2020). 0.005g of the cholesterol was diluted using 50ml of glacial acetic acid and was used as the stock solution, around 1mL of the solution from stock was diluted using 25mL of glacial acetic acid and used as working standards. Different aliquots from 00 to 5mL was added to different test tubes , 0.1 ml of the different sample extract were added in different test tubes and volumes in each was made up to 6mL using glacial acetic acid .Then 4ml of colouring Agent was added and the reaction mixture was incubated for approximately 30 minutes. The colour obtained was observed colorimetrically at 560nm. Standard curve of Cholesterol was made using the absorbance. Phytosterol estimation was done by using Zack's Method.

Estimation of total Flavonoids

The content of Flavanoids was estimated by using the most common method described by Kolgi et al., 2021). 5mg of Quercitin was added to 50mL of the methanol and the solution was used as stock solution. Working solution was prepared by taking 1mL from stock solution and then diluting with 25mL of the Methanol. The standard solution was taken in the aliquotes of 0.0 to 1 ml in different test tubes and 0.5mL of the sample extracts were taken as unknown solution. The volume was made up to 2mL by adding methanol. 0.1mL of 10 % Aluminum Chloride was added to each test tube followed by the addition of 0.1mL of 1M Potassium acetate and 2.8mL of the distilled water. Incubation of the reaction mixture was done at room temperature for 30 minutes; the absorbance of the reaction mixture was measured using UV/VIS spectrophotometer at 415 nm.

Estimation of total Phenols

To estimate the amount of total Phenols in the *Moringa olefeira* leaves Folin-Ciocalteau method with slight modification was used to determine the total phenolics (Ainsworth and Gillespie, 2007). This method is one of the most commonly used colorimetric assay. This is a very sensitive assay which requires no digestion. The FC method is based upon the transfer of the reducing equivalents (e^-) in alkaline medium from phenolic compounds forming a phosphomolybdic complex (mixture of sodium tungstate, sodium molybdate and sodium phosphate) along with copper sulphate resulting in a blue coloured complex which can be measured at 660nm.

Catechol was used as standard reference for plotting the standard curve. The mixture contained 0.5mL of Folin-Ciocalteau reagent which was mixed with around 0.1mL of aqueous methanolic extract. The test tubes containing the reaction mixturewere incubated for 3 minutes, 2mL of Sodium Carbonate solution was added. The test tubes were then incubated for the second time for approximately 10 minutes. This result in the formation of a blue coloured complex, absorbance was read using colorimeter at

660nm. The total phenolics were determined from the standard graph of Catechol. The amount of total phenols was expressed in mg/mL.

Estimation of Carotenoids

To estimate the amount of carotenoids in the leaves of *Moringa olefiera*.5mg of β-carotene was weighed, added to 50 ml of the ethanol in the standard flask. This forms the stock solution. 1mL of the solution from the stock was taken and made upto 25mL with the help of ethanol, which was then used as working standard. Standard β-carotene solution was taken in different aliquots from 0-5mL in different test tubes. The volume was made upto 5mL using ethanol. The absorbance was taken using the spectrophotometer at 450nm (Saini et al., 2014).

Preparation of Tyrosinase

Moringa oleifera leaves were collected from north areas Bangalore. Extraction of *M. oleifera* Tyrosinase was performed by the method of Velichkova with few modifications. The 26.6g of leaves were homogenized with 100ml of 50mM Tris HCl buffer (pH=5.8) in a blender. The homogenate was filtered using a muslin cloth. The filtrate was centrifuged at 10000rpm for 10 minutes and supernatant was collected. The supernatant obtained was used as a source of enzyme Tyrosinase (Zaidi et al., 2014).

Purification of the enzyme from crude extract by ammonium salt precipitation

Ammonium sulfate salt precipitation was done in an ice bath using the finally grounded Ammonium sulfate. 10ml of crude enzyme extract was subjected to 40% of salt precipitation i.e. 22.6g of salt was weighed for 40% salt precipitation process. The salt powder was weighed and added slowly to the extract by constant stirring to ensure complete solubility. Once complete salt get soluble in the crude extract it was then centrifuged at 10000 rpm for 10 min at 4°C. The obtained supernatant was then collected for dialysis process (Zaidi et al., 2014).

Dialysis

We know that dialysis is a protein purification process that separate protein from other small molecules. Activation of dialysis membrane was done before dialysis by processing the dialysis membrane in boiling water bath before and after treating with EDTA. Precipitate was then filled in dialysis bag tied tightly at both the ends without the introduction of any air bubble and leakage and made sure that the pressure exerted by the sample is high enough for efficient dialysis. Dialyzed bag was then placed in a 1000ml beaker containing 1000ml of 50mM Tris HCl buffer. Precipitate was then dialyzed against 50mM Tris HCl buffer (pH=5.8) on a magnetic stirrer for 2 hours by changing the buffer twice. The dialyzed fraction was used for Tyrosinase activity and Protein content (Zaidi et al., 2014).

Assay of Tyrosinase activity

The Tyrosinase activity assay was performed as reported by sung and cho spectrophotometrically, measuring conversion of L-DOPA to red colored oxidation product dopachrome. The initial rate of reaction is proportional to concentration of enzyme. An aliquot containing 5ml of Tyrosinase was incubated for 5 minute at 35°C/time initial level, 1ml of L-Dopa solution (4mg/mL) for measured at 475nm. After incubation of additional 5 minutes, the mixture was shaken again and a second reading was determined and was measured for 3 minutes. The change in absorbance was proportional to enzyme concentration. One unit of enzyme corresponded to the amount which catalyzed the transformation of 1µmol of substrate to product per minute under above condition (Zaidi et al., 2014).

In vivo study on Zebra fish of Tyrosinase enzyme of *M. olifera* leaves extract

Albino Zebra fish were maintained in lab condition and allowed to mate in a breeding tank. Embryos of Albino Zebra fish was taken after 20 hours of fertilization before the pigmentation produced and transferred in a petri plate containing embryo water. Two petri plates containing Zebra fish embryos were taken and marked as controlled and test.

Controlled plate with the Zebra fish embryos was left as such without the enzyme Tyrosine ammonia lyase, whereas test petri plate with Zebra fish embryos were kept as it is as control. Both the plates containing Albino Zebra fish embryos were kept at optimum temperature and observed under microscope after 24 hours. After observation the embryo water in the petri plate was cleaned and the marked as test were added with enzyme Tyrosinase enzyme extracted from *M. olifera* and the other marked as test plate 2 was added with Tyrosinase extract from *M. olifera* and crude extract. Then the petri plates containing Zebra fish embryos was kept at optimal temperature and observed after 24 hours for better result (Umme Umaima *et al.,* 2016).

Results and Discussion

The present study reveals that fresh raw leaves extract of *M. olifera* was the richest source of secondary metabolites among the other varieties of sample extracts. Highest phytosterol content was estimated in fresh raw leaves extract of *M. olifera* extract around 0.137 mg/mL. The results obtained were similar to those reported by Narkhede et al, (2015). The total Phenols of different varieties ranged from 0.28 to 0.623 mg/ml. The reports obtained were similar to the study conducted by Kchaou *et al*. (2013). Amount of carotenoids in the sample extracts were obtained with the help of standard curve of β-carotene. The methanolic extracts of leaves of *M. olefiera* samples were found to contain higher amount of Carotenoids. Dried boiled leaves of *M. olefiera* are found to be moderate source of Carotenoids (0.045mg/ml) as compared to other samples (Martín-Sánchez *et al*., 2014).

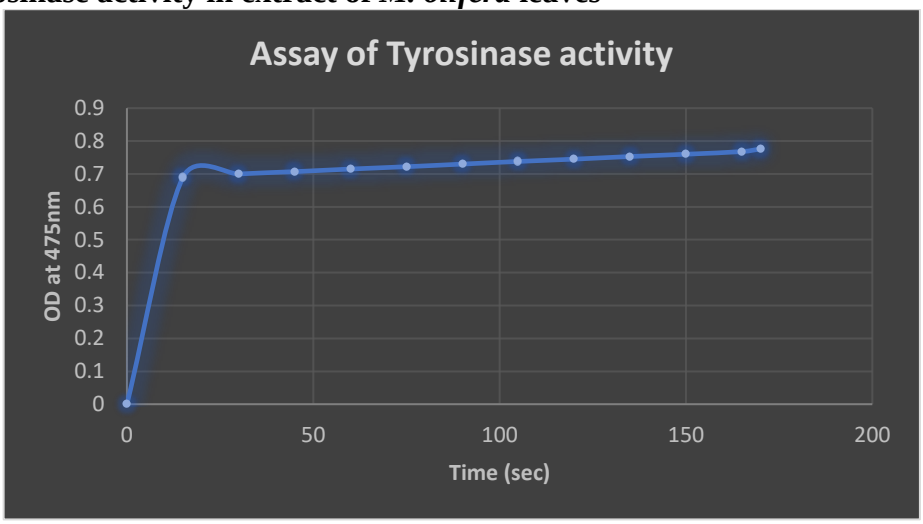
Table 1: Quantitative analysis of phytochemicals of various processed extracts of *M. olifera* leaves

Plants extracts	Total Flavanoids (Mg/Ml)	Total Phenols (Mg/Ml)	Phytosterols (Mg/Ml)	Carotenoids (Mg/Ml)
F.R	0.27±0.079	0.623±0.0404	0.047±0.0152	0.0242±0.0025
F.B	0.125±0.012	0.28±0.02	0.047±0.0058	0.0189±0.00134
D.R	0.0134±0.036	0.28±0.104	0.04±0.026	0.0456±0.0234
D.B	0.127±0.010	0.353±0.0305	0.067±0.0305	0.018±0.0043
M.E	0.173±0.0064	0.55±0.226	0.137±0.023	0.026±0.0073

Triplicates are performed and results are presented as mean±SD
F.R.: Fresh raw leaves extract of *M. olifera:* **F.B**: Fresh boiled leaves extract of *M. olifera*
D.R.: Dry raw leaves extract of *M. olifera;* **D.B**.: Dry boiled leaves extract of *M. olifera*.
M.E.: Methanolic extract of *M. olifera*

The Total Phenol content was found to be higher in the Fresh F.R – Fresh raw leaves extract of *M. olifera.* The total Flavinoid content was found to be higher in the Fresh F.R – Fresh raw leaves extract of *M. olifera*. The total Polysterol content was found to be higher in the Fresh F.R – Fresh raw leaves extract of *M. olifera*. The Total Carotenoid content was found to be higher in the Methanolic leaves extract of *M. olifera*. *M. oleifera* confirmed the presence of Tyrosinase. Enzyme source used is the buffer extract of *M. oleifera*. Estimation was carried out to detect the presence of protein using Lowry's method calorimetrically at 660nm. From the above estimation carried out, the amount of protein was found to be 2mg/ml in the given source *M. oleifera*. Enzyme assay was carried out as reported by Sung and Cho spectrophotometrically, by measuring conversion of L-Dopa to red colored oxidation product Dopachrome at various time intervals at 475nm.

Assay of Tyrosinase activity in extract of *M. olifera* leaves



Graph 1: Assay of Tyrosinase activity in extract of *M. olifera* leaves

Figure A & B: Control of Albino Zebra Fish Embryo without any Pigmentation

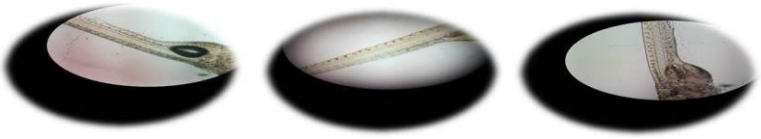


Figure C, D & E: Treated with Tyrosinase Enzyme of *Moringa olifera* Leaves Extract to Albino Zebra Fish Embryo and showing Repigmentation

Figure 1: Showing A and B control Albino Zebra embryo fish without any pigmentation and C, D & E treated with Tyrosinase enzyme extracted from *Moringa olefera* leaves along with raw leaf extract of *M. olifera* leaves and exhibited repigmentation in the embryonic development of Zebra fish

In the above figure it represents the increased activity of Tyrosinase at different interval of time. The depigmented developing embryos were treated with the Tyrosinase enzyme with a 1ml of concentration, showed a partial repigmentation in them. It was also found that the crude enzyme extract along with the enzyme Tyrosinase obtained also induced repigmentation in the depigmented embryos by involving in development of melanin cells and protecting the cells by enhancing the activity of enzyme Tyrosinase in inducing melanin pigments. The outcome of the above study indicated that the repigmentation induced in the depigmented embryos can be correlated to treatment of Vitiligo and Leucoderma by inducing repigmentation of depigmented patches in the above conditions.

The melanin is also involved in various biological functions. Phytosterols are plant derived alcoholic steroids, they are extensively found many medicinally useful plants (Salehi et al., 2020). They are found to be useful in the treatment of heart and cholesterol related disorders. Flavonoids are a category of naturally occurring antioxidants find abundantly in many plants. They are the phytochemicals that cannot be synthesized by humans (Haleshappa et al., 2020, 2021). They are Benzo-Ɣ-Pyrone derivates broadly found in various fruits and vegetables (Karasava and

Mohan, 2018). The outcome of antioxidant functions is through scavenging of free radicals and other oxidizing intermediates. Flavonoids are involved in protection melanocytes from oxidative stress, protecting them and preserving them for the normal functions to be carried out. Polyphenols are considered as potent antioxidants; they are also considered as important cell metabolism regulators (31. Polyphenols such as resveratrol, anthocyanins, terpenes like Taxol have gained a significant importance in the treatment of diseased conditions such as Alzimers, Parkinson's and Diabetes Many studies have revealed that polyphenols inhibit chemical carcinogen or ultra-violet radiation induced skin tumorigenesis in different animal models. Dietary carotenoids a lot in the skin yellowness that contributes to the attractive appearance (Lefevre an Perrett, 2015). Carotenoids protect skin and eyes from photodamage and also provides many health benefits. ß-carotene plays a very important role in the contribution to human nutrition. Carotenoids protect the skin against UV-irradiation damage. Carotenoids are found widespread in animal species, they play a vital role in coloration of skin in birds, insects, fish and crustaceans (Tapiero et al., 2004, Slifka et al., 1999). It has been proved that carotenoids of fruits and vegetables adds to more than 70% of vitamin A intake in third World countries. Carotenoids play a major and vital role in cell-to-cell communication, particularly the stimulatory effect exerted on gap junction communication (Milani et al., 2017). Zebra fish danio is a fresh water species that is used worldwide as vertebrate model organism, due to its ful sequence genome it has been majorly used in many major research works (Teame et al., 2019). The embryonic development stages have been studied and monitored to relate their way of development that is somewhere similar to the humans. Zebra fish danios have been majorly utilized in the studies of neurological diseased condition like Alzhamer's and Parkinson's. Zebra fish was the first model organism to be cloned (Best and Alderton, 2008, Wang et al., 2021).

Conclusion

The study confirm the efficacy of plant extract which show repigmnetaion activity in Albino Zebra fish with the presence of essential phytochemicals in the extract of *Moringa olifera* leaves and it is observed depends on processing methods to isolate good amount of metabolites. This finding emphasis on physiological issues such as Vitiligo and Leucoderma remediation using the isolated plant metabolite as an enzyme alone or processed crude extract of the plant. Hence our pre-clinical studies evidenced with remarkable experimental outcome as potent novel molecules are found in the plant extract of *M. olifera* leaves and may further studies will helpful to go for various applications and make it as new drug discovery.

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CONFLICT INTEREST

The authors declare that there is no conflict of interest.

REFERENCES

1. Agarwal, B.B. and Shishodia, S. (2006). Molecular Targets of Dietary Agents for Prevention and Therapy of Cancer. *Biochemical Pharmacology*, *71*(10), 1397-1421.
2. Ahmadiani, A., Javan, M., Semnanian, S., Barat, E. and Kamalinejad, M. (2001). Anti-Inflammatory and Antipyretic Effects of *Trigonella foenum-graecum* Leaves Extract in the Rat. *Journal of Ethnopharmacology*, *75*(2-3), 283-286.
3. Ainsworth, E. and Gillespie, K. (2007). Estimation of total phenolic and other oxidation substrates in plant tissues using Folin–Ciocalteu reagent. Nature protocols. 2. 875-7. 10.1038/nprot.2007.102.
4. Alkhateeb, A., Fain, P.R., Thody, A., Bennett, D.C., and Spritz, R.A. (2003).

Epidemiology of Vitiligo and Associated Autoimmune Diseases in Caucasian Probands and their Families. *Pigment Cell Research*, *16*(3), 208-214.

5. Alzoreky, N.S. and Nakahara, K. (2003). Antibacterial Activity of Extracts from Some Edible Plants Commonly Consumed in Asia. *International Journal of Food Microbiology*, *80*(3), 223-230.
6. Anwar, F., Latif, S., Ashraf, M. and Gilani, A. H. (2007). *Moringa oleifera*: A Food Plant with Multiple Medicinal uses. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*, *21*(1), 17-25.
7. Aswar, U., Bodhankar, S. L., Mohan, V., and Thakurdesai, P. A. (2010). Effect of Furostanol Glycosides from *Trigonella foenum-graecum* on the Reproductive System of Male Albino Rats. *Phytotherapy Research*, *24*(10), 1482-1488.
8. Baltas, E., Nagy, P., Bonis, B., Novak, Z., Ignacz, F., Szabo, G. and Kemeny, L. (2001). Repigmentation of Localized Vitiligo with the Xenon Chloride Laser. *British Journal of Dermatology*, *144*(6), 1266-1267.
9. Baquer, N. Z., Kumar, P., Taha, A., Kale, R. K., Cowsik, S. M., and Mclean, P. (2011). Metabolic And Molecular Action of *Trigonella foenum-graecum* (Fenugreek) And Trace Metals in Experimental Diabetic Tissues. *Journal of Biosciences*, *36*, 383-396.
10. Best, J. D., & Alderton, W. K. (2008). Zebrafish: An in vivo model for the study of neurological diseases. *Neuropsychiatric Disease and Treatment*, *4*(3), 567–576. https://doi.org/10.2147/ndt.s2056
11. Best, J.D., Berghmans, S., Hunt, J.J., Clarke, S.C., Fleming, A., Goldsmith, P. and Roach, A.G. (2008). Non-Associative Learning in Larval Zebrafish. *Neuropsychopharmacology*, *33* (5), 1206-1215.
12. Bhanger, M. I., Bukhari, S. B., and Memon, S. (2008). Antioxidative Activity of Extracts from a Fenugreek Seeds (*Trigonella foenum-graecum*). *Pakistan Journal of Analytical and Environmental Chemistry*, *9*(2), 6.
13. Bin-Hafeez, B., Haque, R., Parvez, S., Pandey, S., Sayeed, I. and Raisuddin, S. (2003). Immunomodulatory Effects of Fenugreek (*Trigonella foenum graecum* L.) Extract In Mice. *International Immunopharmacology*, *3*(2), 257-265.
14. Boersma, B. R., Westerhof, W. and Bos, J. D. (1995). Repigmentation in Vitiligo Vulgaris by Autologous Minigrafting: Results In Nineteen Patients. *Journal of the American Academy of Dermatology*, *33*(6), 990-995.
15. Boulton, M. and Marshall, J. (1985). Repigmentation of Human Retinal Pigment Epithelial Cells in Vitro. *Experimental Eye Research*, *41*(2), 209-218.
16. Brysk, M.M., Newton, R.C., Rajaraman, S., Plott, T., Barlow, E., Bell, T. and Smith, E.B. (1989). Repigmentation of Vitiliginous Skin by Cultured Cells. *Pigment Cell Research*, *2*(3), 202-207.
17. Cui, J., Shen, L. Y. and Wang, G. C. (1991). Role of Hair Follicles in the Repigmentation of Vitiligo. *Journal of Investigative Dermatology*, *97*(3), 410-416.
18. Difran, A. J. M., Abhinaya, H. V., Verma, S., Sampath, S., Umaima, U., Swetha, B. N., and Guruprasad, R. (2016). Extraction of Tyrosine Ammonia Lyase (Tal) from *Murraya koenigii* Plant for the Treatment of Hyperpigmentation-involves in-Vivo Analysis on Zebrafish Embroy's and Molecular Docking. *World Journal of Pharmacology and Pharmaceutical Science*, 5, 1769-1780.
19. Haleshappa R., Patil S.J. and Siddalinga Murthy K. R. (2021). Phytochemical Analysis, In Vitro Evaluation of Antioxidant and Free Radical Scavenging Activity of *Simarouba glauca* Seeds. *Advances in Pharmacology and Pharmacy*, *9*(1), 1 - 8. DOI: 10.13189/app.2021.090101.

20. Haleshappa R., Patil. S.J., Usha. T. and Murthy KRS. (2020). Phytochemicals, Antioxidant Profile and GCMS Analysis of Ethanol Extract of *Simarouba glauca* Seeds. *Asian Journal of Biological and Life Science, 9*(3):379-85.
21. Islam, Z., Islam, S. M. R., Hossen, F., Mahtab-Ul-Islam, K., Hasan, M. R., & Karim, R. (2021). *Moringa oleifera* is a Prominent Source of Nutrients with Potential Health Benefits. *International Journal of Food Science, 2021*, 6627265.
22. Karasawa, M. M. G. and Mohan, C. (2018). Fruits as Prospective Reserves of bioactive Compounds: A Review. *Natural Products and Bioprospecting, 8*(5), 335–346. https://doi.org/10.1007/s13659-018-0186-6
23. Kolgi, R. R, Haleshappa, R., Sajeeda, N., Keshamma, E., Karigar, C.S. and Patil, S.J. (2021). Antioxidant Studies, *in vitro* Cytotoxic and Cell Viability Assay of Flavonoids and Alkaloids of *Leucas aspera* (Wild.) Linn Leaves. *Asian Journal of Biological and Life Science, 10*(1):165-71. DOI:10.5530/ajbls.2021.10.24
24. Laksmiani, N.P., Widiantara, I. and Pawarrangan, A. (2022). Potency of moringa (Moringa oleifera L.) leaves extract containing quercetin as a depigmentation agent inhibiting the Tyrosinase enzyme using in-silico and in-vitro assay. *Pharmacia*, *69*, 85-92. 10.3897/pharmacia.69.e73132.
25. Lefevre, C. E., & Perrett, D. I. (2015). Fruit over sunbed: carotenoid skin colouration is found more attractive than melanin colouration. *Quarterly Journal of Experimental Psychology, 68*(2), 284–293. https://doi.org/10.1080/17470218.2014.944194
26. Martín-Sánchez, A., Cherif, S., Ben-Abda, J., Xavier, B., Pérez-Álvarez, J.A. and Sayas-Barberá, E. (2014). Phytochemicals in date co-products and their antioxidant activity. *Journal of Food Chemistry*, 158(1): 513-520.
27. Mbikay M. (2012). Therapeutic Potential of Moringa oleifera Leaves in Chronic Hyperglycemia and Dyslipidemia: A Review. *Frontiers in Pharmacology, 3*, 24. https://doi.org/10.3389/fphar.2012.00024
28. Moreiras, H., Seabra, M. C., & Barral, D. C. (2021). Melanin Transfer in the Epidermis: The Pursuit of Skin Pigmentation Control Mechanisms. *International Journal of Molecular Sciences*, *22*(9), 4466. https://doi.org/10.3390/ijms22094466
29. Narkhede, C. K., Sonar, S.S., Chaudhari, H.R. and Gajare S. M. (2013). Phytosterol quantitation of some marketed medicinal plant samples. In - A Compendium of Research Articles by Budding Researchers, 4(85): 85-97.
30. Saini, R. K., Shetty, N. P., Prakash, M., and Giridhar, P. (2014). Effect of dehydration methods on retention of carotenoids, tocopherols, ascorbic acid and antioxidant activity in Moringa oleifera leaves and preparation of a RTE product. *Journal of Food Science and Technology*, *51*(9), 2176–2182. https://doi.org/10.1007/s13197-014-1264-3
31. Salehi, B., Quispe, C., Sharifi-Rad, J., Cruz-Martins, N., Nigam, M., Mishra, A. P., Konovalov, D. A., Orobinskaya, V., Abu-Reidah, I. M., Zam, W., Sharopov, F., Venneri, T., Capasso, R., Kukula-Koch, W., Wawruszak, A.and Koch, W. (2021). Phytosterols: From Preclinical Evidence to Potential Clinical Applications. *Frontiers in Pharmacology, 11*, 599959. https://doi.org/10.3389/fphar.2020.599959
32. Slifka, K.A., Bowen, P.E., Stacewicz-Sapuntzakis, M. and Crissey, S. (1999). A Survey of Serum and Dietary Carotenoids in Captive Wild Animals. *The Journal of Nutrition*, *129*(2), 380-390. https://doi.org/10.1093/jn/129.2.380.
33. Tapiero, H., Townsend, D. M.. and Tew, K. D. (2004). The role of carotenoids in the prevention of human pathologies. *Biomedicine and Pharmacotherapy = Biomedecine and Pharmacotherapie*, *58*(2), 100–110.

https://doi.org/10.1016/j.biopha.2003.12.006

34. Teame, T., Zhang, Z., Ran, C., Zhang, H., Yang, Y., Ding, Q., Xie, M., Gao, C., Ye, Y., Duan, M. and Zhou, Z. (2019). The use of Zebrafish (*Danio rerio*) as biomedical models. Animal frontiers, *The Review Magazine of Animal Agriculture*, *9*(3), 68–77. https://doi.org/10.1093/af/vfz020
35. Tief, K., Hahne, M., Schmidt, A. and Beermann, F. (1996). Tyrosinase, the key enzyme in melanin synthesis, is expressed in murine brain. *European Journal of Biochemistry*, *241*(1), 12–16. https://doi.org/10.1111/j.1432-1033.1996.0012t.x
36. Wang, X., Zhang, J. B., He, K. J., Wang, F., & Liu, C. F. (2021). Advances of Zebrafish in Neurodegenerative Disease: From Models to Drug Discovery. *Frontiers in Pharmacology*, *12*, 713963. https://doi.org/10.3389/fphar.2021.713963
37. Wissal, K., Fatma, A., Christophe, B., Hamadi, A. and Souhail B. (2013). Effects of extraction solvents on phenolic contents and antioxidant activities of Tunisian date varieties (*Phoenix dactylifera* L.) *Journal of Industrial Crops and Products*, 45, 262-269.
38. Zaidi, K. U., Ali, A. S., Ali, S. A. and Naaz, I. (2014). Microbial Tyrosinases: Promising Enzymes for Pharmaceutical, Food Bioprocessing, and Environmental Industry. *Biochemistry Research International, 2014*.
39. Zaidi, K. U., Ali, A. S., and Ali, S. A. (2014). Purification and Characterization of Melanogenic Enzyme Tyrosinase from Button Mushroom. *Enzyme Research, 2014*.