DOI: 10.7860/JCDR/2024/72643.20188

Original Article

Dentistry Section

Phytochemical Profiling and Cytotoxicity Evaluation of *Wrightia tinctoria* Extracts on Vero Cell Lines: A Comparative Analysis and Insight into the Bioactive Compounds

KT MAGESH[1], DEEPAK CHANDRASEKARAN[2], RAMYA MAHALINGAM[3], R ARAVINDHAN[4], M SATHYA KUMAR[5], A SIVACHANDRAN[6]

ABSTRACT

Introduction: The utilisation of plant-based medicine is a continuing practice that remains prevalent even with modern times. Some therapeutic modalities have a long history, while others are quite recent. The lack of sufficient reporting on the utilisation of medicinal plants has resulted in an ongoing deficit in the understanding of their therapeutic benefits, as well as, their potential short-term and long-term side-effects. The Sweet Indrajao, commonly known as Pala Indigo Plant or Dyer's Oleander, and scientifically identified as *Wrightia tinctoria* R. Br, is a member of the Apocynaceae family. It is known as the "Cure for jaundice tree" in southern India. Wrightia is a plant commonly found in India and Burma. The *Wrightia tinctoria* plant has been chosen for this study due to its long-standing medicinal usage and the variety of pharmacologically active compounds it contains. Despite of the fact, it is been widely used for medicinal uses, the information concerning its safety mentioned in the literature is scarce, particularly its cytotoxic effects on normal cellular systems, which this research aims to address.

Aim: The study was aimed to analyse the therapeutic potential of *Wrightia tinctoria* plant extracts by evaluating the phytochemical composition and to assess the effect of the *Wrightia tinctoria* plant extracts on Vero cell lines, for a clearer understanding of its medicinal application and its safety profile.

Materials and Methods: This in-vitro study was conducted at SRM Institute of Science and Technology, Chennai and the duration of the study was eight months (Jan to August 2023). For the phytochemical analysis of *Wrightia tinctoria*, dried plant material (leaves) was collected shade dried and extracted using Soxhlet solvent extraction methods followed by fractionation using techniques like column chromatography. Ethanol, ethyl acetate and aqueous solvents were used for the in-vitro study.

The various standard protocols were employed to identify phytoconstituents. The cytotoxicity analysis on Vero cell line (Kidney epithelial cells of African green Monkey) was performed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay, where vero cells were cultured and seeded into 96-well microtitre plates. Different concentrations of *Wrightia tinctoria* extract were added to the wells, followed by incubation. After incubation, MTT solution was added, and formazan crystals formed by viable cells were solubilised using a suitable solvent. Absorbance was measured at a specific wavelength using a microplate reader to assess cell viability and cytotoxic effects.

Results: Phytochemical analysis revealed the presence of phenols, steroids, terpenoids, alkaloids, flavonoids, and glycosides. Of the three solvents used in the study, ethanolic extract yielded maximum amount of phytochemicals when compared with the other solvents (aqueous and ethyl acetate) that were investigated. MTT assay was used to determine the cytotoxicity and the *Wrightia tinctoria* ethanolic extract demonstrated Half maximal Inhibitory concentration (IC50) >100, ensuring the safety profile of the extract on normal cells.

Conclusion: *Wrightia tinctoria* was a significant plant with highly potent phytochemicals with therapeutic properties. Moreover the extract was also safe against the normal cell line (vero cell line).

Keywords: MTT assay, Phytochemical analysis, Sweet indrajao

INTRODUCTION

Wrightia tinctoria (*W.tinctoria*) commonly called as vetpalai in Tamil, sweet Indarjao or Inderjo meetha in Hindi, Pala indigo plant, was a well-known medicinal plant commonly found in countries like India and Burma [1]. This little deciduous tree is a member of the Apocyanaceae family. Traditional medicine has made use of several parts of it [2]. The bark yields milky white latex and was smooth with a yellowish brown hue.

The *Wrightia tinctoria* tree has opposite simple leaves that are very briefly petioled. The flowers are white, becoming soft cream yellow when mature. Its fruits are cylindrical and become dark green to blackish in August with flat, brown but lustrous seeds having delicate white hair-like fibres. Furthermore, the seeds are dispersed by wind while their life cycle is maintained by insects which help in pollination [3]. *W.tinctoria* can be found growing in a variety of soil types, including rocky, semi-arid, dry, and moist areas. The fruits, leaves, and blossoms are edible. A blue dye or dye resembling indigo is produced by glucoside, which is found in leaves, flowers, and roots [4]. For every kilogram of dye, 100-200 kg of leaves are required. This plant species has gathered significant attention due to its diverse pharmacological properties attributed to its rich phytochemical composition. Literature shows the presence of various bioactive compounds of medicinal values in the different parts of *W.tinctoria* [5]. However, there remains a gap in understanding the underlying mechanisms and safety profile of its cytotoxic effects on normal mammalian cells. Vero cell line is a mammalian continuous cell line studied in many cell biology research and molecular studies.

Further to evaluate a drug for any oral disease its cytotoxic potential is very much essential. The cytotoxicity of the extracts can be assessed using the vero cell line is the primary step in formulating any drug for oral diseases [6,7]. In this context, the present study aimed to comprehensively characterise the phytochemical profile of *W.tinctoria* and evaluate its cytotoxicity on normal Vero cell lines. Understanding the cytotoxic effects of *W.tinctoria* on normal cells is crucial for elucidating its pharmacological profile and assessing its suitability for therapeutic use.

MATERIALS AND METHODS

This in-vitro study was designed to analyse both qualitative and quantitative analysis of the *Wrightia tinctoria* extracts, its effects over cell viability that gives an idea about toxicity, and their therapeutic values. The study was conducted at SRM Institute of Science and Technology, Chennai and the duration of the study was eight months. Ethical approval was obtained from the Institutional Ethical Committee Letter No. 2441/IEC/2021 dated 27/05/2021.

Collection of plant and authentication: *Wrightia tinctoria* was collected from the Araku valley in Eastern Ghats of Visakhapatnam, Andhra Pradesh, India. The taxonomic identification of *Wrightia tinctoria* was confirmed by the morphological analysis and authenticated by a qualified botanist. Unwanted dust particle from fresh plant material (leaves and stems) was removed by washing under running tap water and with distilled water then shade dried for 14 days. The shade dried plant material blended to a fine powder using the domestic blender, stored in airtight containers at 4°C until further used [8].

Preparation of extracts from Wrightia tinctoria: Dried powder of *Wrightia tinctoria* was subjected to solvent extractions by using a Soxhlet apparatus. Extract was prepared with three solvents (ethyl acetate, ethanol and aqueous). A thimble containing about 30 g of *Wrightia tinctoria* powder was placed in a Soxhlet apparatus flask with 300 mL of solvent, and extraction was carried out employing different solvents in the following order of increased polarity: ethyl acetate, ethanol and aqueous until the solvent in the separator syphon tube turned transparent. For extract filtering, Whatman No. 1 filter paper was employed. The filtered extracts were concentrated at 40°C in a vacuum oven, and then stored in a cold, dark environment until use.

Preliminary phytochemical analysis [9]: Preliminary phytochemical analysis was performed for all three solvent extracts of *Wrightia tinctoria*. All three extracts were qualitatively analysed for identification of various phytoconstituents such as alkaloids, saponins, quinines, tannins, glycosides, flavonoids, phenolic compounds, proteins, terpenoids and steroids by using standard protocols [Table/Fig-1] [9].

S. No.	Phytochemicals	Inference		
		Ethyl acetate	Ethanol	Aqueous
1.	Alkaloids	(-)	(+)	(+)
2.	Saponins	(-)	(++)	(+)
3.	Quinines	(-)	(-)	(-)
4.	Tannins	(-)	(+++)	(+)
5.	Glycosides	(-)	(++)	(+)
6.	Flavinoids	(-)	(+++)	(+)
7.	Phenolic compounds	(-)	(++)	(+)
8.	Proteins	(-)	(+)	(+)
9.	Terpenoids	(+)	(+)	(+)
10.	Steroids	(+)	(+)	(+)

[Table/Fig-1]: Phytochemical analysis of *Wrightia tinctoria*.
The signs "-", "+", "++", and "+++" are used to indicate the relative presence or abundance of specific phytochemicals in the plant extract. The indicators are qualitative in nature where the signs "-", "+", "++", and "+++" represents negative, positive, moderately positive and strongly positive presence of the phytochemicals in the extract

Mayer's test (Alkaloids Test) [9]: A 2 mL of 1% HCl was added to 5 mL of extract. The mixture was then mixed with Mayer's reagent. Alkaloids are indicated by turbidity.

The foam test (saponin test) [9]: After mixing 5 mL of extract with 5 mL of distilled water, the mixture was heated. The presence of saponins is indicated by the production of stable foam.

Quinine testing [9]: A few drops of strong sulfuric acid were poured along the test tube's edges to 1 mL of extract. Quinines are indicated by their red appearance.

Tannin test (a lead acetate test) [9]: A few drops of $FeCl_3$ were added after 2 mL of the extract and 2 mL of distilled water were mixed together. Tannins are indicated by green precipitate.

Glycosides test [9]: A 1 mL of Fehling's solution was boiled and added to 1 mL of extract. Glycosides are indicated by orange precipitate.

Ferric chloride test: a flavonoid test [9]: One millilitre of 10% lead acetate solution was added to one millilitre of extract. The presence of flavonoids is shown by the production of a yellow precipitate.

Phenolic substances [9]: A tiny amount of the extract was diluted with water. A solution of ferric chloride was used the dilute. Phenolic substances are indicated by a violet colour.

Protein test (Biuret test) [9]: Two drops of 0.1% copper sulphate and 10% sodium hydroxide were used to treat the extract. Proteins are indicated by a violet or pink tint.

Terpenoids [9]: A 5 mL of extract was mixed with 2 mL of chloroform and 3 mL of strong sulfuric acid in that order. Terpenoids are indicated by a reddish-brown interface in the solution.

Steroid usage [9]: Two millilitres of acetic anhydride and one millilitre of extract were combined with 2 mL of sulfuric acid. Steroids are present if the colour changes from violet to blue or green.

MTT assay for assessment of cytotoxicity against vero cell line

- MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) -the solution is filtered through a 0.2 µm filter and kept frozen for long periods of time or at 2-8°C for frequent usage.
- The DiMethylsulfoxide (DMSO).
- CO_2 incubator
- Reader for microplates
- An inverted microscope
- Cooling centrifuge

Preparation of test solutions: Serial two-fold dilutions ranging from 3.125 to 100 µg were produced for the MTT test.

Culture medium and cell lines: The National Centre for Cell Sciences (NCCS), Pune provided the Vero cell line, which was cultivated in Dulbecco's Modified Eagle Medium (DMEM) media supplemented with 10% inactivated Foetal Bovine Serum (FBS), penicillin (100 IU/mL), and streptomycin (100 µg/mL) at 37°C in a humidified atmosphere with 5% CO_2 until confluent.

Procedure

Using the appropriate media containing 10% FBS, the monolayer cell culture was trypsinised and the cell count was adjusted to 1.0×10^5 cells/mL. A volume of 100 µL of the diluted cell suspension (1×10^4 cells/well) was introduced into every well of the 96-well microtiter plate. Once a partial monolayer had developed after 24 hours, the supernatant was discarded, the monolayer was once again washed with medium, and 100 µL of various test sample concentrations were applied to the partial monolayer in microtiter plates. After that, the plate was incubated for 24 hours at 37°C in an environment 5% CO_2.

Following the incubation period, each well received 20 µL of MTT (2 mg/1 mL of MTT in PBS), with the test solutions in the wells

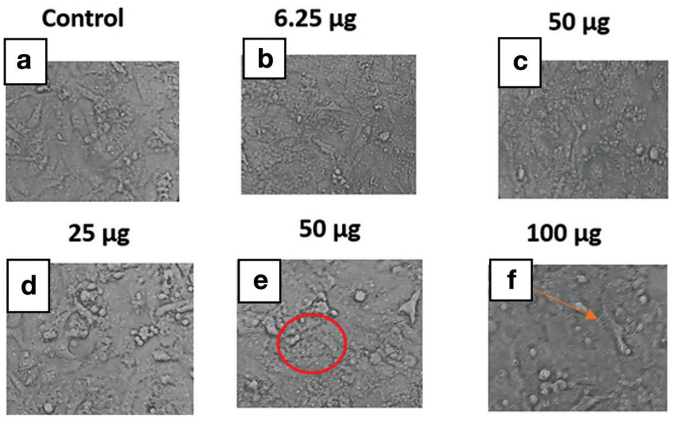
being removed. The plate was incubated in a 5% CO_2 environment at 37°C for four hours. After removing the supernatant, 100 µL of DMSO was added, and the plate was gently shaken to dissolve the formazan that had formed. At 570 nm, the absorbance was determined with a microplate reader. The formula used to compute the percentage of viability was % viability=Sample abs/Control abs×100 [10].

RESULTS

The phytochemical analysis of the extract on various solvents revealed presence of many bioactive compounds as tabulated in [Table/Fig-1].

Of the three extracts ethyl acetate, ethanol and aqueous, the ethanolic extract showed higher quantifiable bioactive compounds namely the tannins, flavonoids, saponins, glycosides and phenolic compounds when compared to the other two extracts. The presence of this various bioactive compounds is responsible for the pharmacological actions against oral disorders.

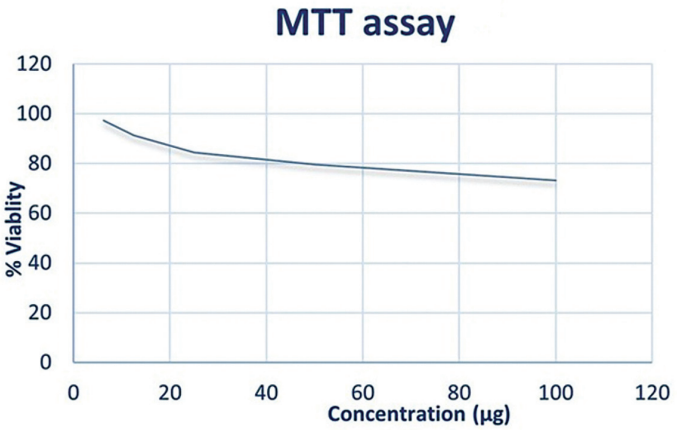
Microscopic images of Vero cells cultured with varying concentration of *Wrightia tinctoria* extract, showing normal cell morphology and confluence are described in [Table/Fig-2].



[Table/Fig-2]: Effect of *Wrightia tinctoria* ethanolic extract at varying concentrations on Vero Cell lines. a) Represents normal vero cells; b-d) Shows no change in the morphology as compared to the of control group cells; e) Shows strong disintegration, condensation and loss in morphology showing branch like shape and elongation of the cells (Circle); f) Reveals dose dependent cytotoxic response of *Wrightia tinctoria* extract on vero cell line characterised, and loss of adherence (Arrow).

Ethanolic extract of *wrightia tinctoria* was used at varying concentrations. The concentration required for 50% inhibition (IC50) was calculated. The assay was performed and triplicated and the % of cell viability was calculated.

Graphs were plotted with various concentration of the ethanolic extract of *Wrightia tinctoria* sample on the x-axis and the percentage of viability of the Vero cells at various concentrations on the y-axis [Table/Fig-3]. With the ethanolic extract demonstrated the cytotoxicity indices IC 50 >100 which indicates even with the concentration of 100 µg/mL more than 70% of the cells were viable.



[Table/Fig-3]: Concentration versus cell viability.

DISCUSSION

The plant extract and the phytochemical analysis were performed in accordance previously established standard methods [11,12]. The current study observed the presence of large number of bioactive compounds in the ethanolic extracts of the *Wrightia tinctoria* including steroids, phenolic compounds, flavonoids, glycosides, saponins and tannins. These compounds are normally present in many regular food items and hold great potential to be used as drugs for various diseases due to their safety and less cytotoxicity which is in accordance with the study done to assess the bioactive components [13]. These phytoconstituents have been extensively studied for their diverse pharmacological activities, including antioxidant, anti-inflammatory, and anticancer properties. These properties can be utilised in treating oral conditions like oral ulcers, promotion of wound healing and various inflammatory conditions affecting the gingiva and the oral mucosa. Alkaloids, for instance, have been reported to exhibit cytotoxic effects against cancer cells by inducing apoptosis and cell cycle arrest [14]. Similarly, flavonoids have shown promising anticancer potential through various mechanisms such as inhibition of cell proliferation and angiogenesis, and induction of apoptosis [15,16].

The cytotoxicity evaluation of *Wrightia tinctoria* on normal Vero cell lines using the MTT assay demonstrated dose-dependent effects. While cytotoxicity against cancer cells is desirable for therapeutic purposes, it is equally crucial to ensure the safety of natural products on normal cells. The observed cytotoxic effects may be attributed to the presence of bioactive compounds in *Wrightia tinctoria*, which could interfere with cellular processes and viability [17,18]. The MTT assay is a commonly used method for evaluating cell viability and cytotoxicity in various biological and pharmaceutical studies [19]. The present study with the ethanolic extract demonstrated the cytotoxicity indices IC 50> 100 (half maximal Inhibitory concentration) which indicates even with the concentration of 100 µg/mL more than 70% of the cells were viable [Table/Fig-3]. This is in accordance with study done by Chaudhary S et al., where petroleum ether and ethyl acetate extracts of Wrightia tinctoria doesn't have lethal effects on non-tumour fibroblast [20]. Assessing the cytotoxicity of *Wrightia tinctoria* using the MTT assay can provide valuable insights into its potential therapeutic benefits and help in understanding role in promoting overall health and wellness [21,22].

Limitation(s)

In the current study, the crude extract of the plant was used to study the cytotoxicity in the cell line; however, further studies should be conducted with specific bioactive compounds present in the plant extract as the toxic profiles of the individual biomolecules may have different effects on the cell line. Also, studies on animal models should be conducted to ensure the safety of the extract in humans.

CONCLUSION(S)

According to the current research, *Wrightia tinctoria* was a significant plant with highly potent therapeutic properties. The plant exhibits varied phytochemical elements and the results of this investigation demonstrated a noteworthy level of IC50 >100 when various concentrations of ethanolic extracts of *Wrightia tinctoria* leaf in normal cell line which confirms the safety of the use of the plant extract. Also, further studies pertaining to various components present in the plant extract for assessment of their potential in treating various oral diseases can be conducted done in the future.

REFERENCES

[1] Srivastava R. A review on phytochemical, pharmacological, and pharmacognostical profile of Wrightia tinctoria: Adulterant of kurchi. Pharmacogn Rev. 2014;8(15):36-44.

[2] Deventhiran M, Wyson JW, Marimuthu S, Padmanabhan AS, Saravanan P, Anand D, et al. Phytochemical analysis of leaf extracts from Wrightia tinctoria R. Br. and its antidermatophytic activity. Int J Pharm Sci Rev Res. 2016;40:24-27.
[3] Babu M, Hareeshbabu E. Pharmacological evaluation of Wrightia Tinctoria- a review. Immunology. 1983;65:55-63.
[4] Moorthy K, Aravind A, Punitha T, Vinodhini R, Suresh M, Thajuddin N. In vitro Screening of antimicrobial activity of Wrightia tinctoria (Roxb.) R Br. Asian J Pharm Clin Res. 2012;5:54-58.
[5] Mahadevan N, Moorthy K, Perumal P, Raju SV. Pharmacognosy of leaves of wrightia tinctoria R. Br. Anc Sci Life. 1998;18(1):78-83. PMID: 22556873; PMCID: PMC3331058.
[6] Ammerman NC, Beier-Sexton M, Azad AF. Growth and maintenance of Vero cell lines. Curr Protoc Microbiol. 2008;4:04-10.
[7] Nahapetian AT, Thomas JN, Thilly WG. Optimization of environment for high density Vero cell culture: effect of dissolved oxygen and nutrient supply on cell growth and changes in metabolites. J Cell Sci. 1986;81:65-103.
[8] Abubakar AR, Haque M. Preparation of medicinal plants: basic extraction and fractionation procedures for experimental purposes. J Pharm Bioallied Sci. 2020;12(1):1-10. Doi: 10.4103/jpbs.JPBS_175_19. Epub 2020 Jan 29. PMID: 32801594; PMCID: PMC7398001.
[9] Trease EC, Evans WC. Pharmacognosy. 16th Edition. Philadelphia: WB Saunders; 2009.
[10] Ghasemi M, Turnbull T, Sebastian S, Kempson I. The MTT assay: Utility, limitations, pitfalls, and interpretation in bulk and single-cell analysis. Int J Mol Sci. 2021;22(23):12827. Doi: 10.3390/ijms222312827. PMID: 34884632; PMCID: PMC8657538.
[11] Jensen WB. The origin of the Soxhlet extractor. Journal of Chemical Education. 2007;84:1913.
[12] Nortjie E, Basitere M, Moyo D, Nyamukamba P. Extraction methods, quantitative and qualitative phytochemical screening of medicinal plants for antimicrobial textiles: A review. Plants (Basel). 2022;11:2011.
[13] Hemavathy A, Shanthi P, Sowndharya C, Thiripura SS, Priyadharshni K. Extraction and isolation of bioactive compounds from a therapeutic medicinal plant-Wrightia tinctoria (Roxb.) R Br. Int J Pharmacogn Phytochem Res. 2019;11:199-204.
[14] Gupta SC, Patchva S, Koh W, Aggarwal BB. Discovery of curcumin, a component of golden spice, and its miraculous biological activities. Clin Exp Pharmacol Physiol. 2012;39:283-99.
[15] Imran M, Rauf A, Abu-Izneid T, Nadeem M, Shariati MA, Khan IA, et al. Luteolin, a flavonoid, as an anticancer agent: A review. Biomed Pharmacother. 2019;112:108612.
[16] Cragg GM, Pezzuto JM. Natural products as a vital source for the discovery of cancer chemotherapeutic and chemopreventive agents. Med Princ Pract. 2016;25(Suppl 2):41-59.
[17] Antony J, Saikia M, Vinod V, Nath LR, Katiki MR, Murty MS, et al. DW-F5: A novel formulation against malignant melanoma from Wrightia tinctoria. Sci Rep. 2015;5:11107.
[18] Krishnamurthi JR, Kalaimani S, Veluchamy G. Clinical study of Vetpalai (Wrightia tinctoria) oil in the treatment of Kalanjagapadai (Psoriasis). Journ Res Ayur Siddha. 1981;2:58-66.
[19] Khyade MS, Vaikos NP. Pharmacognostical and physio-chemical standardization of leaves of Wrightia tinctoria R. Br. Int J Pharm Res Dev. 2009;8:1-1.
[20] Chaudhary S, Devkar RA, Bhere D, Setty MM, Pai KS. Selective cytotoxicity and pro-apoptotic activity of stem bark of Wrightia tinctoria (Roxb.) R. Br. in cancerous cells. Pharmacogn Mag. 2015;11:481-87.
[21] Ramalakshmi S, Edaydulla N, Ramesh P, Muthuchelian K. Investigation on cytotoxic, antioxidant, antimicrobial and volatile profile of Wrightia tinctoria (Roxb.) R. Br. flower used in Indian medicine. Asian Pacific Journal of Tropical Disease. 2012;1:S68-75.
[22] Yadala KP, Asha S. Characterization and evaluation of antibacterial, antioxidant and cytotoxicity of synthesised silver nanoparticles (AgNps) using chloroform crude callus extracts of Wrightia tinctoria (Roxb.). Current Trends in Biotechnology and Pharmacy. 2018;12:275-85.

PARTICULARS OF CONTRIBUTORS:
1. Vice Principal, Department of Oral Pathology and Microbiology, SRM Kattankulathur Dental College, Chennai, Tamil Nadu, India.
2. Professor and Head, Department of Orthodontics and Dentofacial Orthopedics, SRM Kattankulathur Dental College, Chennai, Tamil Nadu, India.
3. PhD Scholar, Department of Oral Pathology and Microbiology, SRM Kattankulathur Dental College, Chennai, Tamil Nadu, India.
4. Associate Professor, Department of Oral Pathology and Microbiology, SRM Kattankulathur Dental College, Chennai, Tamil Nadu, India.
5. Professor, Department of Oral Pathology and Microbiology, SRM Kattankulathur Dental College, Chennai, Tamil Nadu, India.
6. Associate Professor, Department of Oral Pathology and Microbiology, SRM Kattankulathur Dental College, Chennai, Tamil Nadu, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. KT Magesh,
Vice Principal, Department of Oral Pathology and Microbiology,
SRM Kattankulathur Dental College, SRM Institute of Science and Technology,
Chennai-603203, Tamil Nadu, India.
E-mail: magesht@srmist.edu.in

PLAGIARISM CHECKING METHODS: [Jain H et al.]
- Plagiarism X-checker: May 06, 2024
- Manual Googling: May 24, 2024
- iThenticate Software: Sep 07, 2024 (14%)

ETYMOLOGY: Author Origin

EMENDATIONS: 7

AUTHOR DECLARATION:
- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? No
- For any images presented appropriate consent has been obtained from the subjects. NA

Date of Submission: **May 06, 2024**
Date of Peer Review: **May 22, 2024**
Date of Acceptance: **Sep 08, 2024**
Date of Publishing: **Oct 01, 2024**