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GREEN SYNTHESIS OF ZNO NANOPARTICLES USING PEELS OF CITRUS LIMETTA AND EVALUATION OF THEIR ANTIBACTERIAL ACTIVITY

J R Thenmozhi, Shahanaz S, Suba G A Manuel*

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ABSTRACT

Background: The fruit peel wastes produced during the processing of different agriculture-related products after production are not utilized to their full potential, and their environmental impact has become a significant global issue. Citrus peel waste is rich in nutrients and has biopotential activity. **Objective:** The main objective of the current study is to analyze peels of *Citrus limetta* qualitatively and quantitatively and synthesize Zinc oxide Nanoparticles using the green synthesis method. **Methodology:** The synthesized ZnO NPs were characterized by UV-visible spectroscopy (UV-Vis), X-ray diffraction (XRD), and Scanning electron microscopy (SEM). The antibacterial activity of *C. limetta* peels ZnO NPs against *Pseudomonas aeruginosa* was evaluated by the Agar Disc Diffusion method. **Results:** The UV-Vis spectrum was measured in the 200 – 400 nm range, and the crystalline structure was analyzed via XRD. SEM/EDS analysis confirmed the nano-spherical structures and the agglomeration of the synthesized ZnO NPs. A minimal inhibitory effect against the *Pseudomonas aeruginosa* by *C. limetta* peels ZnO NPs was observed. **Conclusion:** Hence, the green synthesized ZnO NPs offer an effective and economical way to utilize citrus peel waste in both food and non-food sectors.

INTRODUCTION

Fruits and vegetables are fundamentally important diets that ensure human health and wellness [1]. They significantly improve the amount of nutrients and fiber in our everyday diet [2]. These plant-based foods can be eaten fresh, cooked, or combined with other foods. Worldwide, fruits and vegetables are one of the most widely utilized goods, rendering up over forty-two percent of all food waste [3]. Throughout the whole manufacturing process phase, the food preparation and production industry generate food losses and waste due to factors like damage during transit or poor transportation facilities, issues during storage, losses during preparation or contamination, and improper packaging [4]. These wastes are adequately discarded in rivers or landfills, leading to an undesirable effect on the health of the planet [5]. Demand for food has risen as a result of the recent rapid growth in the global population, causing a significant challenge for food producers in increasing productivity [6]. For these public concerns, finding

*Department of Life Science, Mount Carmel College, Vasanth Nagar, Bengaluru, Karnataka 560052 India

***For Correspondence:** **subamanuel@mccblr.edu.in**

the source and beneficial use for fruit remains is vital. Recovery of the bioactive elements from fruit and vegetable waste is one of the most effective methods. Fruit wastes like citrus fruit peels, pineapple remains, sugar cane bagasse, and other fruit remnants (primarily peels and seeds) are produced in large amounts [7]. Some fruit skin and seed components are also found to exhibit more significant antioxidant potential compared to their pulp components [8]. Many bioactive substances have antimicrobial, antitumor, antiviral, antimutagenic, and cardioprotective properties [9]. The development of vitamin and mineral supplements, dietary additives, and therapeutic foods can benefit from bioactive compounds [10]. On the other hand, it is now well-known that one of the most significant and promising inventions in all of science is nanotechnology [11].

Nanotechnology has established the basis for incredible industrial uses and rapid advancement [12]. Nanoparticles range in size between one and one hundred nanometers and are composed of biological material, metal, metal oxides, or carbon [13]. Metal and metal oxide nanoparticles show different physiochemical properties and reducing or oxidizing agents are used during their synthesis [14]. Nanoparticle production is accomplished through a variety of physical, chemical, and biological processes. Chemical methods typically involve colloidal synthesis, chemical precipitation, and sol-gel methods. Physical methods are generally referred to as a top-down approach and employ techniques like thermal decomposition, arc discharge and diffusion etc [15]. However, most of these techniques are still under research, and instability of the nanoparticle preparations, manipulation of crystal growth and particle aggregation are common challenges [16]. Hence, biological synthesis has become a compelling substitute for conventional synthesis techniques to produce nanoparticles. Actinomycetes, bacteria, fungi, plants, viruses, and yeast are just a few examples of unicellular and multicellular living things that are used in the environmentally benign, green chemistry-based process of biosynthesis [17]. Synthesis of nanoparticles using a green method is easy, affordable, and less toxic. By preserving the natural balance, this strategy reduces pollution, maintains the quality of the ecosystem, and uses the fewest resources that can be found naturally [18]. Green chemistry can be used to create biocompatible nanoparticles that are less harmful to both the ecosystem and people's health. A broad range of biocompatible secondary metabolites derived from plants or fruits can be used to generate and stabilize metal nanoparticles. These metabolites have powerful antibacterial properties, making them ideal chemical agents in the synthesis of nanoparticles [19]. There is great promise for the use of bioactive, small molecular compounds from a range of fruits to treat cancer, microbial infections, inflammatory disorders, and other medical issues. Additionally, there have been initiatives to employ naturally existing bioactive substances as delivery agents [20]. Bioactive compounds extracted from medicinal plants and fruit peel wastes are also progressively being used in the therapeutic field [21].

Hence, in the current study, an effort was made to estimate the phytochemical constituents from the peels of *C. limetta* by using methanol as a solvent. Further being aware of the advantages of green synthesis, zinc oxide nanoparticles are synthesized from the peels of *C. limetta* by the green synthesis method. The *In-vitro* antibacterial activity was also analyzed. The characterization of the obtained ZnO NPs was done by UV-Visible spectroscopy, X-ray diffraction and Scanning electron microscopy.

METHODOLOGY

Collection of Peels

The peels of *Citrus limetta* were collected from the canteen at Mount Carmel College, Vasanth Nagar, Bengaluru.

Preparation of peel powder

The peels were washed with distilled water and cut into pieces. These pieces were dried in a hot air oven at 60°C for a day. The dried peels were then ground into a coarse powder. This powder was then stored in an air-tight container at room temperature for further analysis.

Preparation of methanol extract

Methanol extraction was done based on the method described by Parekh and Chanda [22], with slight modifications. The crude methanol extract was obtained by extracting dried peel powder (10 g) in methanol (100 mL) and kept on a rotary shaker for 48 hours at room temperature. The solution was filtered through the Whatman filter paper. The filtrate was stored at 4ºC in airtight containers for further use.

Qualitative analysis of phytochemical constituents in the methanol extract of *Citrus limetta*

Various phytochemicals like alkaloids, anthocyanin, anthraquinone, carbohydrates, cardiac glycosides, flavonoids,

phenols, phlorotannins, proteins, saponins, terpenoids, and quinones were identified in the methanolic extracts obtained from *Citrus limetta* peels following the standard tests [23].

Quantitative analysis of phytochemical constituents in the methanol extract of *citrus limetta* peels

Estimation of total flavonoid and total phenol content

Total flavonoid content was determined by Aluminium chloride method as described by Chang *et al*., [24]. Quercetin was used as a standard and the absorbance was measured at 510 nm using a spectrophotometer (Thermo Scientific™ GENESYS™ 180). The results were expressed in mg QE/g dry weight. Total phenol content was determined by the Folin-Ciocalteau method following the procedure of Singleton [25] with slight modifications. Gallic acid is used as a standard. The absorbance was measured at 760 nm (Thermo Scientific™ GENESYS™ 180). Total phenolic content was expressed in mg GAE/g dry weight.

Green synthesis of ZnONPs

The green synthesis of ZnO NPs was done based on the method described by Thi *et al.,* [26]. Maceration of the dried peel powder was done by adding the powder (1g) to deionized water (50 mL) and stirring for 3 hours. Once macerated, the mixture was kept in a water bath at 60°C for 1 hour. After cooling, the mixture was filtered with Whatman No.1 filter paper. Zinc nitrate (2 g) was added to the filtrate (42.5 mL) and stirred for 1 hour. Then, the mixture was placed in the water bath at 60°C for 1 hour. Subsequently, the mixture was dried at 150°C in a hot air oven and then calcinated at 400°C in a muffle furnace. A white powder that forms, indicates the ZnO NPs formation.

Characterization of green synthesized ZnONPs

UV-Visible spectrophotometry

The maximum absorbance of the ZnO NPs was determined by UV-Visible spectrophotometry using Thermo Scientific™ GENESYS™ 180 spectrophotometer in the range of 200-400 nm.

X-Ray diffraction (XRD) analysis

X-Ray diffraction analysis was carried out at CENSE Lab, IISC, Bengaluru using Rigaku, Smart lab X- Ray Diffractometer. X-Ray diffraction is explained by Debye-Scherrer formula.

$$D = \frac{K\lambda}{\beta \cos\theta}$$

where, K=0.9 is Scherrer's constant, λ is the wavelength of X-rays, θ is the Bragg diffraction angle, and β is the full width at half-maximum (FWHM) of the diffraction peak corresponding to plane [27].

Scanning Electron Microscopy and Energy–dispersive X-ray Spectroscopy (EDS) analysis of green synthesized ZnO NPs

Scanning electron microscopy (Zeiss Ultra 55 Field Emission SEM) working at 5KV was used to determine the morphology of the green synthesized ZnO NPs. EDS was performed in conjugation with SEM to estimate the composition of NPs.

***Invitro* anti-bacterial activity using disc diffusion method**

Maintenance of bacteria culture and culture media

A common human pathogenic Gram-negative bacteria, *P. aeruginosa* (Strain MTCC 1688) was used for the assay. Pure cultures were revived and maintained on nutrient agar plates on a regular basis in sterile conditions. The cultures were streaked on sterile nutrient agar plates and kept in an incubator for 18 hours at 37°C and stored at 4°C. A loop-full containing the test organism was inoculated in Luria-Bertani broth for 18 hours at 37°C in sterile conditions. The bacterial cultures obtained were stored at 4°C and used for further analysis [28].

Agar disc diffusion assay of the synthesized ZnO NPs

The disc diffusion method for antimicrobial susceptibility testing was performed according to the standard method by Bauer *et al.,* [29]. The test bacteria culture was inoculated on sterile Muller Hinton agar plates evenly using spread plate technique. The plates were allowed to dry for 5-10 minutes. The sterile discs are impregnated with a series of different concentrations of NPs (100,200,600 mg/mL) and were placed on the agar surface along with a positive control disc (Pencillin G 10 units-Standard disc), negative control disc (Deionized water) and a disc with methanolic extract of the *C. limetta* peels equidistant from each other over the culture plate and incubated at 37°C for 24 hours. The inhibition zones around the discs were measured in mm.

RESULTS AND DISCUSSION

Qualitative analysis of phytochemical components using methanolic extract of *C. limetta* peels

The study of qualitative analysis of phytochemicals done with the methanol extract of *C. limetta* peels has indicated the occurrence of various phytochemicals. It has also been

illustrated that several of these phytochemicals exhibit therapeutic properties. For example, constituents like alkaloids have antibacterial and analgesic properties, phlobatannins have been proven to have anti-inflammatory, analgesic, antioxidant and wound-healing activities and terpenoids are also found to have anti-inflammatory, anti-viral, anti-malarial, cholesterol synthesis inhibition and anti-bacterial activities [30]. *C. limetta* indicates the existence of alkaloids, carbohydrates and essential phyto nutrients like cardiac glycosides, flavonoids, phenols, terpenoids and the absence of anthraquinones, anthracyanine, phlobatannins, saponins (Table 1). Phytochemical profiling of *C. limetta* by Martínez-Cano *et al.,* [31] showed similar results except for cardiac glycosides which is indicated as negative in their studies.

Table1: Phytochemical screening tests for methanolic extracts of *C. limetta peels*

S No	Phytochemical constituent	Methanol extract of *C. limetta peels*
1	Alkaloids	+
2	Anthraquinones	-
3	Anthocyanin	-
4	Carbohydrates	+
5	Cardiac glycosides	+
6	Flavonoids	+
7	Phenols	+
8	Phlobatannins	-
9	Protein	-
10	Saponins	-
11	Terpenoids	+
12	Quinones	+

*:+Indicates the presence;-Indicates the absence.

Quantitative analysis of phytochemical components using methanolic extract of *C. limetta* peels

Total flavonoid and phenolcontent of *C. limetta* peels

Flavonoid has been depicted to show analgesic and anti-inflammatory properties [32]. The estimation of the total flavonoid content in the methanolic extract of *C. limetta* peels done using quercetin as standard was found to be 10.887 mg QE/g **(Table 2).** In a previous study conducted by Olfa*et al.,*[33], the total flavonoid content in the ethanolic peel extract of *C. limetta* estimated using catechin as a standard, was 1.08 ± 0.02 mg EC g−1DW. The estimated amount of flavonoids could vary due to the use of different standards and organic solvents used for extraction.

Citrus fruit peels have a high concentration of phenolic chemicals [34]. Phenols play a key role in oxidative stability and antimicrobial defence [35]. The total phenolic content in *C. limetta* peels is estimated to be 3.940mgGE/g **(Table 2).** According to a study conducted by Padilla-Camberos*et al.,* [36], in a sample concentration of 20 mg/mL, the aqueous *C. limetta* extract had a total phenolic content of 19±1.6 mg GAE/g. Thus, it can be inferred that the total phenol content can vary depending upon the extracts used.

Table 2: Estimation of total content of flavonoid and Phenol in *C. limetta peels*

Methanolic extract of peels	Total content of flavonoids (mgQE/g)	Total content of phenol (mgGE/g)
C. limetta	10.887	3.940

ZnO NPs GREEN SYNTHESIS OF *C. limetta* PEELS

The ZnO NPs green synthesis was done based on the method described by Doan Thi *et al*., [37]. From 1g of *C. limetta* peel powder, approximately 0.5 g of *C. limetta* ZnO NPs was produced in the form of white powder**.**

Characterization of green synthesized *C. limetta* peels ZnO NPs

UV-visible spectrophotometry analysis

UV–Vis absorption of ZnO nanoparticles was observed to have a wavelength range of 250-400nm [38]. The absorption wavelength for bulk ZnO is reported to be around 385 nm [39]. In the current study, the UV – Vis spectra of *C. limetta* ZnO NPs had maximum absorbance of 0.211 at 369nm.

X-ray diffraction (XRD) analysis

XRD analysis of the synthesized NPs was done using Rigaku, Smartlab X- Ray Diffractometer (CeNSE, IISC, Bengaluru). Crystal structure, crystallite size, and lattice parameters can all be assessed through XRD [40]. **Figure 1** represents the pattern of XRD of ZnO NPs synthesized by the green synthesis method using *C. limetta* peels. The sample exhibited diffraction peaks at 2θ ≈ 31.73°, 34.40°, 36.21°, 47.49°, 56.52°, 62.80°, 66.28°, 67.87°,68.99°,72.52°,76.86°,81.31°,89.50° corresponding to lattice planes of (100), (002), (101), (102), (110), (103), (200), (112), (201), (004), (202), (104) and (203) respectively. The diffraction peak at (101) plane corresponding to zinc oxide had the highest intensity**.** The average size of the synthesized ZnO NPs was calculated using Debye-Scherrer formula and was found to be 102.86 nm.

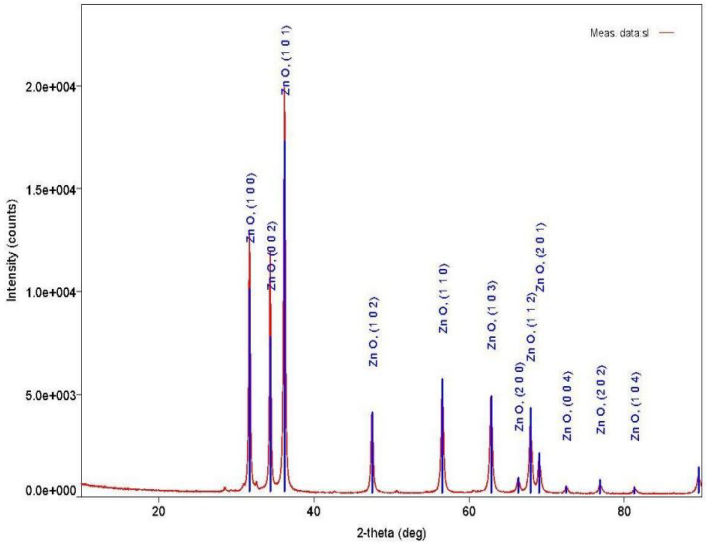
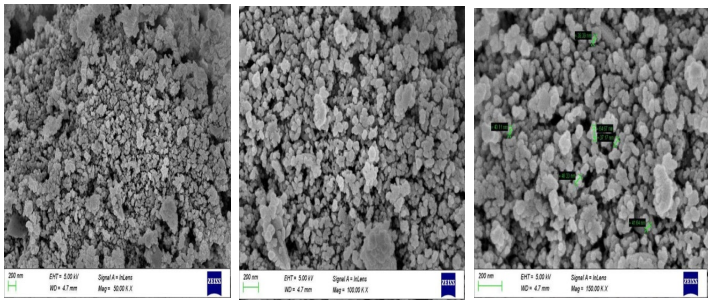


Figure 1: XRD spectrum of green synthesized C.limetta peels ZnONPs

Scanning Electron Microscopy (SEM) analysis of green synthesized ZnONPs OF *C. limetta* peels

Scanning electron microscopy (SEM) in conjugation with energy dispersive X-ray spectroscopy (EDS) gives more information about core/shell structure [41]. Scanning electron micrographs of the ZnONPs obtained by green synthesis using C. *limetta* peels indicated the spherical shape of NPs. The micrographs also illustrated the agglomeration of nanospheres along with individual ZnO NPs



(A) **(B)** **(C)**

Figure 2: Plate 1 Scanning electron micrographs obtained for green synthesized C.limetta peels ZnONPs at different magnifications (A) 50.00 KX (B) 100.00 KX (C) 150.00 KX

Energy dispersive X-ray spectroscopy (EDS) analysis of green synthesized ZnO NPs of *C. limetta* peels

EDS is generally used to identify and estimate the elemental composition of the nanoparticles [42]. The EDS spectrum of ZnO NPs obtained by green synthesis from *C.limetta* peels, the ZnO NPs exhibited the occurrence of zinc and oxygen, indicating that the synthesized nanoparticles are in a pure state **(Figure 2)**. The weight percentage of zinc, oxygen and carbon was observed to be 71.68 %, 20.56 % and 7.76 % respectively, while the atomic percentage of zinc, oxygen, and carbon was observed to be 36.21 %, 42.45 % and 21.33% respectively in *C.limetta* ZnO NPs **(Table 3)**. A trace element of carbon was also observed which is due to the coating of the sample on carbon tape which was used as a sample holder for the analysis.

Table 3: EDS analysis with weight and atomic percentage of zinc and oxygen elements in the green synthesized *C. limetta* peels ZnO NPs

Element	Weight percentage	Atomic percentage
Zn	71.68	36.21
O	20.56	42.45
C	7.76	21.33
Total	100.00	

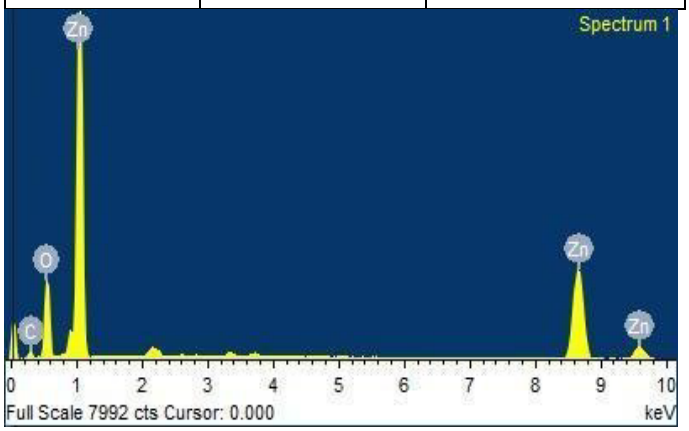


Figure 3: EDS spectrum of green synthesized ZnONPs of *C. limetta* peels

Evaluation of *in vitro* antibacterial analysis of green synthesized ZnO NPs of *C. limetta* peels

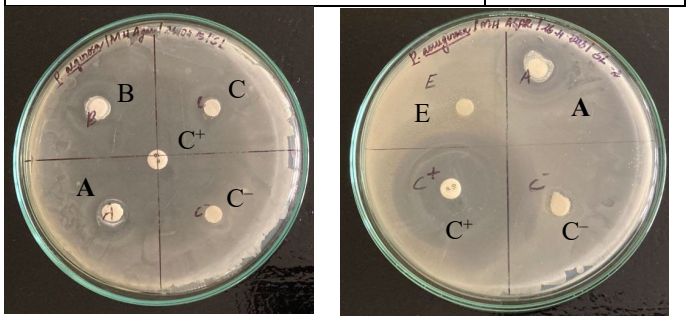
In the present study, the antibacterial property of *C. limetta* peel extract (methanolic) and the green synthesized ZnO NPs of *C. limetta* peels were performed against a pathogenic bacterium, *Pseudomonas aeruginosa*. It has been reported to generate a lot of acquired b-lactamases and aminoglycoside-modifying enzymes and it has been demonstrated that the expression of strong aminoglycoside-modifying enzymes ultimately results in penicillin resistance [43].

P. aeruginosa showed sensitivity to the green synthesized ZnO NPs of *C. limetta* peels. The diameter of zone of inhibition was measured in mm. The anti-bacterial activity increased with the

increase in concentration of nanoparticles. The inhibition shown at the concentration of 600 mg/ml was the highest with a zone of inhibition of 14 ±.14 mm. The least inhibition was shown at the concentration of 100 mg/ml with a zone of inhibition of 8 ± 0.28 mm. It was also observed that *P.aeruginosa* did not exhibit sensitivity to methanol extract of *C.limetta* peels **(Plate 2).** Hence, it can be inferred that minimal anti-bacterial activity was exhibited by the green synthesized ZnO NPs of *C. limetta* peels. The results are indicated in the following **(Table 4).** These findings imply that using *C.limetta* ZnO NPs could inhibit the growth of Gram negative pathogen, *P.aeruginosa* to some extent. According to the study conducted by Hungund *et al.,*[44] AgNPs from sweet lime showed zone of inhibition of 5 mm and 6 mm against pathogens *E. coli, K. pneumoniae* respectively, thus showing considerable microbicidal activity on Gram negative bacteria.

Table 4: *In vitro* antimicrobial activity of *C. limetta* peels ZnO NPs against *P. aeruginosa*

Samples	Zone of inhibition (mm)
Positive control (Penicillin G 10 units-Standard disc)	39 ± 1.41
Negative control (Deionizedwater-20µl)	Nil
Methanolic extract (1%)	Nil
C. limetta peel ZnO NPs(100mg/ml)	8 ±0.28
C. limetta peel ZnO NPs(200mg/ml)	10 ±0
C. limetta peel ZnO NPs(600mg/ml)	14 ±0.14



(A) **(B)**

Figure 4: Plate 2 Zone of inhibition observed for the green synthesized *C. limetta* peels ZnO NPs against *P. aeruginosa* *(*A) Plates with different concentrations o*f C. limetta* peels ZnO NPs, C^+ and C^-; (B) Plates with *C. limetta* peels methanolic extract and the highest concentration o*f C. limetta* peels ZnO NPs, C+ and C-. *C+- Positive control; C -- Negative control; E- Methanol extract; A -600mg/mL; B-200mg/mL; C-100mg/mL

CONCLUSION

In the current study, the qualitative analysis of the methanolic extract of *C. limetta* peels indicated the presence of various phytochemicals and the total content of essential phytochemicals like flavonoids and phenols in the methanolic extract were estimated. The *C. limetta* peels ZnO NPs were successfully synthesized by using eco-friendly green synthesis method and their characterization was carried out by UV-visible spectrophotometry, XRD, and SEM-EDS.

The green synthesized *C. limetta* ZnO NPs showed minimal antibacterial activity against *P. aeruginosa.* The waste residues like peels and seeds of various fruits not only serve as a potential source of bioactive compounds but are also eliminated from our environment due to efficacious solid waste management. Thus, the current study showed that value-added products of varied applications may be obtained from the peels of *C. limetta* that can be utilized in both food and non-food sectors.

Further work can be focused to optimize pectinase production. The green synthesized *C. limetta* ZnO NPs can be used as an effective and alternative low-cost substrate for the pectinolytic microbes growth due to the presence of abundant pectin in the peels. Besides, antioxidant potential determination and cytotoxicity analysis of the green synthesized ZnO NPs to ascertain its safety properties for therapeutic applications can be performed.

FINANCIAL ASSISTANCE

Nil

CONFLICT OF INTEREST

The authors declare no conflict of interest

AUTHOR CONTRIBUTION

Suba G A Manuel designed the research work, J R Thenmozhi, Shahanaz S carried out the experiments and analysed the results. J R Thenmozhi prepared the manuscript with review by all the co – authors.

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