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An insight on micro propagation of *Myrica* species for improvement in cultivation practices of nutraceutically important fruits

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ABSTRACT

Myrica is one of the dioecious genera of the *Myricaceae* family, which is widely distributed and 97 species of this genus are reported globally. The fruits of *Myrica* are extremely nutritive and are used in the manufacturing jams, syrups, juices, and can be also consumed raw, whereas the bark is used to manufacture paper and ropes. In Ayurveda, the roots and bark of *Myrica* extracts have been reported to have carminative, astringent, and antiseptic properties, whereas the role of bark decoction is documented in the treatment of cough and fever, diarrhea, and dysentery, toothache, lung infection, chronic bronchitis, and asthma. One of the biggest problems within *Myrica* species is their poor regeneration in their natural habitats because of their dioecious nature and less availability of mature seeds due to over exploration. This review is a small effort to provide a comprehensive account of the tissue culture-mediated investigation made on the *Myrica* genus (*Myrica esculenta*, *Myrica gale*, *Myrica nana*, *Myrica rubra*, and *Myrica cerifera*) and aimed for improvement of this nutraceuticals important fruit species.

1. INTRODUCTION

Myricaceae is considered to be the most ancient Actinorrhizal family by taxonomists having predominantly evergreen shrubs and drupaceous fruits [1]. The family has shown wide distribution from tropical to temperate regions [2]. The taxonomy of the family *Myricaceae* is highly controversial. Spach [3] and Chevalier [4] divided the *Myricaceae* family into three genera, viz., *Comptonia*, *Myrica*, and *Gale*, based on morphological studies of leaf, inflorescence, flower, ovary, fruit, and wood. Furthermore, in 1994, Wilbur [5] divided this family into three genera *Comptonia*, *Myrica*, and *Morella*, and one sub-genus *Cerothamnus*, and this division was further supported by cytological analyses carried out by Baird [6]. Later on, Elias [7] divided *Myricaceae* into two genera (*Comptonia* and *Myrica*) and one sub-genus (*Morella*) based on pollen morphology, and in 1985 this classification was supported by Sundberg [8]. According to the modern taxonomist, the *Myricaceae* family comprises only three genera, viz., *Comptonia*, *Myrica,* and *Morella* [5,9].

Myrica is widely distributed around the globe and 97 species of this genus are reported globally. In the Indian subcontinent, the geographical distribution of the *Myrica* genus can be seen in the sub-tropical Indian Himalayas [10]. The most common and economically important species of *Myrica* are *M. esculenta* Bunch. -Ham., *M. rubra Sieb*, *M. gale* L., *M. cerifera* L., *M. nana* Cheval, and *M. adenophora* Hance. The fruits of *Myrica* are highly nutritive and are used in making jams, syrups, juices, and can be also consumed raw, whereas the bark is used to making paper and ropes [11]. In traditional Ayurveda, *Myrica* root and bark extracts are used as carminative, astringent, and antiseptic, while the bark decoction is useful in the treatment of cough and fever, diarrhea and dysentery, toothache, lung infection, chronic bronchitis, and asthma [12,13].

The National Food/Nutrition Security of developing countries cannot be improved by the national agricultural production alone. It must be combined with the selection, evaluation, domestication, and utilization of wild edible plants of local importance. This could be possible by improving the linkage between production

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and utilization of traditional biodiversity crops that could have the importance to promote the cereal staples more sustainably and also spread the nutrition and food security at the household level in a better way [14–16]. Due to increased biotic and abiotic stresses on wild edible species, their regeneration is becoming poor in their natural habitats [13]. Another reason for less regeneration may be the less availability of mature seeds of wild edibles plants as all approachable locations are mostly searched by the local people for collection of fruits to earn money, therefore facing the problem of over exploration. However, for the synchronization of artificial regeneration of such wild plant species, the extra knowledge of the maturity time of seeds (mature and viable) is important [18].

Like other wild fruiting plants, the regeneration power of *Myrica* species is very poor [19] which might be due to hard seed coat that shows physical dormancy as well as various other biotic and abiotic factors like overharvesting, grazing, and compaction of soil and fire. In addition to these, *Myrica* species have shown very poor response in germination trials at nursery conditions so micropropagation through tissue culture technique is the only alternative and now becoming the boon for the regeneration of economically important plants. The other problem with the *Myrica* genus is its dioecious nature; therefore, female plants are cultivated and studied extensively due to higher economic value for fruit production as compared to male plants [20–22]. Therefore, micropropagation appears to be the only way for mass propagation of the species [23]. In addition to this, micropropagation avoids the problem associated with the propagation of species and gives a high rate of multiplications and a high number of plants in aseptic conditions [24]. Because *Myrica* plant parts are rich in phenolic content, they create a problem in explant activation on tissue culture media, but still, various attempts have been made by researchers to regenerate *Myrica* species through micropropagation techniques. The present review is therefore an attempt to provide a comprehensive on the tissue culture techniques nodal segment, epicotyl, seeds or seedling, somatic embryogenesis, and multiple shoots made on *Myrica* species and aimed at helping nutraceutical important *Myrica* species.

2. TISSUE CULTURE ON GENUS *MYRICA*

Tissue culture is an *in vitro* technique for the culturing of sterilized cells, organs, or tissue of plants. This is a significant tool for both basic and applied studies as well as in commercial applications [25]. The initial step in the direction of plant tissue culture was taken by Bloch [26], who did his pioneer studies on wound healings in plants through callus formations [27]. Later, several researchers have tested the idea but Tuskan *et al.*'s [28] work on the formation of callus was purposed to be most important. Thorpe [29] proposed the theoretical basis for plant tissue culture and addressed his experiments on the single-cell culture of the German Academy of Science.

The process of micropropagation is completed in three major stages: initiation phase, multiplication phase, and root formation (Fig. 1). The initiation phase is the first phase of tissue culture which is achieved by the introduction of tissue of interest in culture, followed by its sterilization to make it free from any microbial contamination. The second stage of tissue culture is the multiplication stage, which is achieved by redividing and introducing the *in vitro* plant material into the medium supplemented with growth regulators and nutrients. This stage is repeated several times to obtain the number of desired plants and characterized by tissue propagation and numerous roots production. The third stage also called the root formation stage is characterized by the formation of roots and dependent on essential hormones to achieve proper rooting and come plantlets [30].

For large-scale production of plants and cryopreservation purposes, the most widely used technology is tissue culture or micropropagation [31]. Different kinds of medicinal, fruiting,

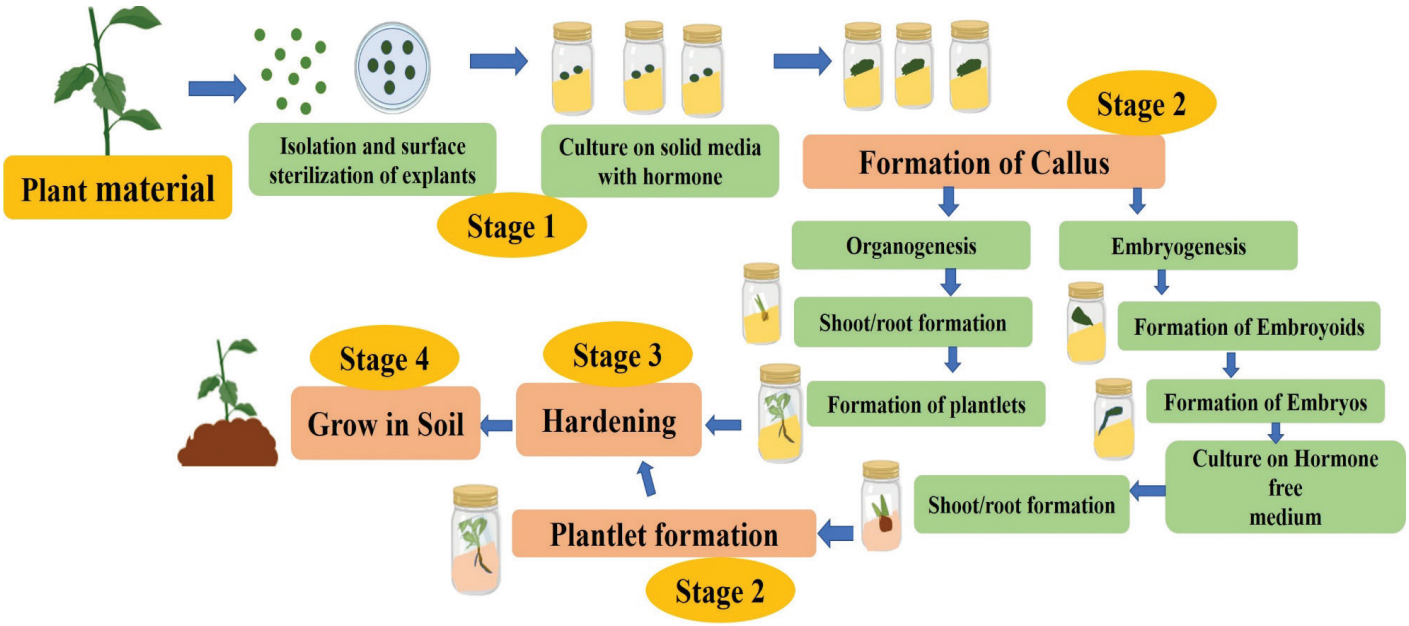

Figure 1: General methodology followed for tissue culture.

as well as ornamental plants have been propagated with this technology [32]. The easiest method for culturing of plants is by using the meristem of shoot tip or nodes as explants. Several protocols have been used for the multiplication of plants from shoot cells or tissues. The three ways through which micropropagation can be achieved are direct or indirect adventitious buds production via callus, enhancing axillary bud-breaking, and direct and indirect somatic embryogenesis on explants [33].

2.1. *Myrica esculenta*

On *M. esculenta* which is a modest-sized fruiting tree mostly distributed in the sub-tropical Himalayan region, mass multiplication and regeneration were first studied by Nandwani [34], Bhatt and Dhar [20], and Rawat *et al*. [35]. Nandwani [34] carried out tissue culture of *M. esculenta* trees (15–20 years old) by introducing nodal stem segments (1–2 cm) on Murashige and Skoog (MS) media, including various amounts of naphthalene acetic acid (NAA), 6-Benzylaminopurine, benzyl adenine (BAP), indole-3-acetic acid, 6-Benzylaminopurine, and Kinetin. The authors observed multiple shoot regeneration on media by using low amount of Auxin (0.05–0.2 mg/l) and high Cytokinin (2.5–5.0 mg/l) concentrations, respectively. The highest (7–8 shoots/explant) number of shoots were obtained from the explant when MS media was supplemented with NAA (0.05 mg/l) and kinetin (5.0 mg/l) in 4 weeks of culture. In addition to this, for root formation, maximum (4–6/explant; 30%) roots were obtained when explant was cultured in three-quarter strength MS media containing IBA (5.0 mg/l) (Table 1). Hence, authors proved that the clonal propagation technique of tissue culture is very useful for the large-scale production *M. esculenta* trees.

Similarly, Bhatt and Dhar [20] studied the role of various factors, such as season, browning, plant growth regulators, and media type, which influence the micropropagation of female trees of *M. esculenta* from the nodal segments. The result showed that Poly vinyl propyl (PVP-0.5%) was most effective to obtained maximum (79.16%) percent survival of explants and removal of partial phenolic compounds. The Woody Plant Medium (WPM) supplemented with 0.1 µM NAA and 10 µM kinetin showed the highest number of shoots (4–5/explant) under suitable photoperiod. The maximum rooting (33.3%) was obtained when half of the WPM was supplemented with 20 µM NAA (Table 1). Among all the media types used, the WPM was observed to be the best for micropropagation. Likewise, Kinetin was found to be best for the explant establishment and multiplication. The authors observed that maximum aseptic cultures at 0.1% $HgCl_2$ (10 minutes) and stated that duration of more than 10 minutes is deleterious to explants, whereas a concentration lower than 0.1% is less effective. According to the authors, this method is very effective to produce a large number *M. esculenta* female trees of higher economical value.

On the other hand, Rawat *et al*. [35] worked on seed dormancy of *M. esculenta*. The authors observed that mechanical scarification supplemented with GA_3 (100 and 50 ppm) improves seed germination up to 85% and 73.33%, respectively. The authors also observed that 48 hours soaking treatment of hot water also increased the survival rate (60%) of the seedlings (Table 1). From their study, the authors concluded that seeds soaking for 48 hours in hot water is best and easy method to remove the damage to the seeds.

2.2. *Myrica gale*

Myrica gale is a small deciduous shrub, native to high latitudes of the northern hemisphere, including North America, Northern and Western Europe, and East Asia [36,37]. Traditionally, due to aromatic properties, the fruits of *M. gale* are used for Scandinavian snaps and flavoring beer in Northern and Western Europe [38]. The bark of the plant is used for relieving itching, whereas the leaves and branches have been reported with diuretic, styptic, anti-cathartic, and anti-helminthic properties [39].

On *M. gale*, only Tavares *et al*. [40] worked on epicotyl culture using MS media containing sucrose, glycine, and BAP. The authors observed that and high light intensity (25–30 µmol $m^{-2}s^{-1}$) and reduced medium strength (1:3 MS) promotes the microshoots, root induction, and elongation, respectively. In addition to this, IBA (1.23 µM) in MS medium (1:3) showed a high rooting rate (73.3%) under high light intensity whereas, MS with BAP at 2.5 or 5 µM induced the maximum multiplication shoot rate (20 shoots/explant) (Table 1). In another study, Torrey and Callaham [41] observed the seeds of *M. gale* treated with 500 ppm gibberellic acid for 24 hours were studied for nodule development. The result of this study showed five nodule lobes in 3-month-old seedlings, but in at this age majority of nodules were still one-to-three lobed. Vanden Bosch and Torrey [42] observed the seeds of *M. gale* treated with water culture containing culture isolated of Frankia were studied for the development of endophytic Frankia sporangia. The authors observed that in June, the sporangia began to differentiate, whereas after in late summer, vesicles were formed. Therefore, the study concluded that pronounced seasonality was exhibited by *M. gale* nodules on sporangial formation.

2.3. *Myrica nana*

Myrica nana is a common ecological and the economically important tree of southern China and Yunnan-Guizhou Plateau [43]. Traditionally, the plant bark of *M. nana* has been reported for treatment of dysentery, diarrhea, stomach ache, and rheumatism [44].

The work on tissue culture on *M. nana* was carried out by only Lin-lin [45]. The author used different explants like roots, stem, and leaves derived from sterile seedlings of *M. nana* for the generation of callus. For this purpose, they used MS medium with different concentrations of growth regulators 6-BA, NAA, 2,4-D, and PVP was added at the time of rooting induction. Their results showed 100% of optimal root callus induction in culture medium supplemented with MS, 2,4-D (1 mg/l) and 0.2 mg/l 6-BA, whereas 78.67% optimal stalk callus induction in culture medium with MS, and 2,4-D (1 mg/l), and 6-BA (0.6 mg/l). Likewise, 21.3% of stalk callus induction was observed in culture medium supplemented with MS media containing 2,4-D (3 mg/l), 6-BA (0.2 mg/l) and NAA (0.2mg/l) (Table 1).

Table 1: Micro-propagation of different species of *Myrica* genus.

Species		Explant	Media + growth regulators		Results	Reference
			Shoot multiplication/Seed germination	Root multiplication		
M. esculenta	Female elite	Nodal segments	WPM + 3% sucrose + 0.5% PVP + NAA (0.1 µM) + Kinetin (10 µM)	WPM + NAA 0.1 µM + PVP 0.5%+ + IBA (20 µM)	Multiple shoot/root formation, and root/ shoot elongation.	[20]
	Female elite	Nodal segments	MS medium + NAA (0.5 mg/l) + Kinetin (5.0 mg/l) + Cytokinin (5.0 mg/l) + Auxin (0.2 mg/l)	¾ MS medium + IBA (5.0 mg/l)	Multiple shoot/root formation, and root/ shoot elongation.	[34]
	Female elite	Seeds	Mechanical scarified seeds + GA_3 100 ppm (85.00%)	Mechanical scarified seeds + GA_3 100 ppm (85.00%)	Increase germination of seeds and produced greatest root length in field conditions.	[35]
M. rubra	Female elite	Seeds	BW medium + TDZ (0.2 mg/l) + IBA (1.0 mg/l) + BA (0.5 mg/l) + NAA (0.1 mg/l)	BW medium + IBA (0.5 mg/l)	Multiple shoot/root formation, and root/ shoot elongation.	[48]
	–	Shoot tips	BW medium + vitamins + sucrose (30 g) + TDZ (0.6 mg/l) + 2,4-D (0.075 mg/l)	¼ BW medium + IBA (0.3 mg/l)	Multiple shoot/root formation, and root/ shoot elongation.	[49]
M. cerifera	Female elite	Seeds	–	¼ Hoagland's solution + KNO_3 (126.25 mg/l) + $MgSO_4$ $7H_2O$ (123 mg/l) + Ca $(NO_3)_2.4H_2O$ (295 mg/l) + KH_2PO_4 (34 mg/l) + H_3BO_3 (0.715 mg/l) + $MnCl_2.4H_20$ (0.4525 mg/l) + $ZnSO_4.7H_2O$ (0.055 mg/l) + $CuSO_4.5H_2O$ (0.02 mg/l) + $Na_2MoO_4.2H_20$ (0.0062 mg/l) + $CoCL_2.6H_2O$ (0.0062 mg/l) + $FeSO_4.7H_2O$ (6.95 mg/l) + Na_2EDTA (9.31 mg/l) + KCL (18.75 mg/l)	Clusters roots formed significantly ($p < 0.05$)	[52]
	Female elite	Seeds	I0 % Hoagland's solution + 50 µM NaCl	–	The plant height significantly increases ($p < 0.05$)	[53]
M. nana	–	Robust root	MS medium +2, 4- D (1 mg/l) + 6-BA (0.3 mg/l)	–	Formation of root callus occurred with 100% induction rate	[45]
	–	Stem	MS medium + 2,4-D (1 mg/l) + 6-BA (0.6 mg/l)	–	Formation of stem callus occurred with 78.76% induction rate	
	–	Robust leaves	MS medium + 6-BA (0.4 mg/l) + 2, 4-D (3 mg/l) + NAA (0.2 mg/l)	–	Formation of leaves callus occurred with 21.33% induction rate	
M. gale	Female elite	Seeds	½ MS medium + sucrose (10 g/l) + BAP (2.5 mM)	1/3 MS medium + IBA (1.23 µM) + Light intensity (25–30 µmol $m^{-2} s^{-1}$)	Multiple shoot/root formation, and root/ shoot elongation.	[40]

2.4. *Myrica rubra*

Myrica rubra tree is native to China, and largely cultivated in southern China since 2000 years [46]. In Chinese traditional medicine all parts of *M. rubra* plant are used for various medicinal purposes (asthma, fever, dyspnea,throat, and lung infections) [47]. Asghar *et al.* [48] worked on the somatic embryogenesis of *M. rubra* from cotyledons using WPM (WB) supplemented with [Thidiazuran (TDZ); synthetic cytokine] alone or in combination with 2,4-D. The authors observed 3.34% of embryogenesis (7 embryo/explant) with TDZ (0.05 mg/l) alone, and 22% of somatic embryogenesis and adventitious shoots from cotyledons explant in TDZ in combination with 2,4-D (0.1 mg/l). In addition to this, the authors also observed repetitive embryogenesis in the media with TDZ (0.05 mg/l) with of 2, 4-D and with BA (0.5 mg/l). The media supplemented with TDZ (0.2 mg/l) plus IBA (1.0 mg/l) gave rise the maximum number (8.5/explant) of shoots whereas media supplemented with NAA (0.1 mg/l) and BA (0.5 mg/l) showed shoot elongation. In addition to this, BW medium fortified with IBA (0.5 mg/l) produced highest rooting of microshoots (Table 1).

In another experiment, Asghar *et al*. [49] used BW medium supplemented with TDZ and 2,4-D for the micropropagation of *M. rubra* from shoot tip and nodal explants. The maximum (5.75/ explant) number and length of shoots was obtained with TDZ (0.6 mg/l) and with 2,4-D (0.075 mg/l) in different concentrations. However, in media supplemented with IBA (0.3 mg/l) maximum rooting quarter and half) was obtained. The authors from their study concluded that BW medium leads better roots/shoot generation resulting in the formation of the maximum number of long roots/shoots in cultivars *M. rubra* (Table 1).

2.5. *Myrica cerifera*

Myrica cerifera, also known as "bayberry" or "wax myrtle," is mostly grows in the northern and central parts of America, Bermuda, and the Caribbean [50]. Ethno medicinally, leaves and fruits are used to treat stomachache, constipation, dental problems, and also as a skin cleanser, whereas the root bark is used as an astringent [51]. Louis *et al*. [52] observed the effect of mineral nutrients on cluster root formation and plant growth of *M. cerifera* seedling. The authors observed that with increasing phosphorus supply the formation of cluster root decreased whereas solutions containing ≥ 1 mg p^{-1} completely suppressed the roots formation Therefore, from their study, the authors concluded that the external phosphorus level in the soil has no role in the initiation of cluster root formation and is can only be determined by internal phosphorous concentration. In another study, Sande and Young [53] observed the effect of NaCl on the growth and nitrogenase activity in seedlings of *M. cerifera.* The authors concluded that NaCl in higher concentration decreased photosynthesis as well as nitrogenase activity in *M. cerifera*, leading to slower growth.

3. CONCLUSION

Myrica species are very common and economically important species distributed globally. The economic importance of the plants is due to their nutritious fruits. *M. esculenta*, *M. rubra*, *M. gale*, *M. cerifera*, *M. nana*, and *M. adenophora* are the species of *Myrica* that have been reported in the literature for their medicinal values (bark has carminative, astringent, antiseptic, and other medicinal properties). For local villagers the species are being used as an income-generating source. Although the species has ecological and economic importance, the natural regeneration of the *Myrica* species is very poor due to its hard seed coat and unavailability of the mature seed of *Myrica* in its natural habitat. On the other hand, these species are dioecious; therefore, for cultivation purposes, only female plants are preferred over male plants. Therefore, it is important to develop new strategies for plant regeneration and cultivation purposes that can collectively overcome these two limitations of the plants. Today's tissue culture techniques are playing a very important role in sustaining the pure quality of the plants. But on *Myrica* species, very little effort has been made by the researchers on the development of rapid, reproducible, and reliable *in vitro* plant regeneration. On *Myrica* genus, clonal propagation was only conducted from shoot, roots and nodal stem segments (*M. esculenta* and *M. rubra*), seeds (*M. esculenta*, *M. rubra*, *M. cerifera*, and *M. gale*), the mature somatic embryo (*M. rubra*), and root cuttings (*M. nana*). Further research work is needed to refine the somatic embryogenesis and protoplast culture of the plant parts. The best micropropagation or plant tissue culture techniques would facilitate the mass propagation of *Myrica* plants and therefore will add new ways in importing these plants at the single-cell level.

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5. ABBREVIATIONS

%: Percentage; µM: Micromolar; µmol m^{-2} s^{-1}: Micromole per second and square meter; 2,4-D: 2,4 dichloro-phenoxy acetic acid; BA: Benzyl adenine; BAP: 6-Benzylaminopurine, benzyl adenine; BW: Basal medium; $Ca(NO_3)_2.4H_2O$: Calcium nitrate tetra hydrate, cm: Centimeter; $CoCL_2.6H_2O$: Cobalt chloride hexahydrate; $CuSO_4.5H_2O$: Copper sulfate pentahydrate; $FeSO_4.7H_2O$: Iron sulfate heptahydrate: g: Gram; g/l: Gram per liter; GA_3: Gibberellin acid: H_3BO_3: Boric acid; IBA: Indole 3-butyric acid; KCL: Potassium chloride; KH_2PO_4: Potassium dihydrogen phosphate; KNO_3: Potassium nitrate; mg/l: Milligram per liter; $MgSO_4.7H_2O$: Magnesium sulfate heptahydrate; Mm: Mill molar; $MnCl_2.4H_2O$: Manganese chloride tetra hydrate; MS: Murashige and Skoog; Na_2EDTA: Disodium ethylenediaminetetraacetic acid; $Na_2MoO_4.2H_2O$: Sodium molydate dehydrate; NAA: Naphthalene acetic acid; NaCl: Sodium chloride; PVP: Poly vinyl propyl; TDZ: Thidiazuran; WPM: Woody plant medium; $ZnSO_4.7H_2O$: Zinc sulfate heptahydrate

6. AUTHORS' CONTRIBUTION

Sohan Lal and Amita Kumari: conception and design the manuscript; Sohan Lal, Amita Kumari, and Ishita Guleria: wrote the manuscript; Amita Kumari: drafting, interpretation, and editing of manuscript; Jyoti Dhatwalia, Shabnam Thakur, Shailja Kumari, and Subhash Sharma: data collection (table, figures, and references). All authors approved to submit the manuscript in the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work.

7. ETHICAL APPROVAL

Not applicable.

8. CONFLICTS OF INTEREST

The authors declare no conflicts of interest in this work.

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