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Research Article

AN *IN VITRO* INVESTIGATION OF ANTIMICROBIAL EFFICACY OF *EUPHORBIA HIRTA* AND *MURRAYA KOENIGII* AGAINST SELECTED PATHOGENIC MICROORGANISMS

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ABSTRACT

Objective: To investigate the solvent-dependent antimicrobial activity and phytochemical analysis of extracts of *Euphorbia hirta* (leaves and flowers) and *Murraya koenigii* (leaves), as well as to evaluate the synergistic activity of these medicinal extracts with suitable antibiotic discs and antibiotics susceptibility of selected pathogenic microorganisms.

Methods: The antimicrobial activity of the medicinal extracts was screened through agar well diffusion method and antibiotics susceptibility of selected microorganisms was investigated using disc diffusion method. A combined agar well diffusion and disc diffusion methods were used for the determination of synergistic activities of the extracts with antibiotic discs.

Results: Among the different solvents, ethanol had maximum zone of inhibition against the test pathogens. Ethanolic leaf extracts of *E. hirta* exhibited the highest inhibitory activity against *Candida albicans* and *Staphylococcus aureus* with minimum inhibitory concentration value of 12.5 mg/mL and 25.0 mg/mL, respectively. Antimicrobial assay revealed that *E. hirta* extracts were active against all tested Gram-negative bacteria. However, none of the plant extracts had inhibitory activity against Gram-positive bacterium *Propionibacterium acnes*. Phytochemical screening for both the extracts from *E. hirta* revealed the presence of steroid, tannin, terpenoids, carbohydrates, alkaloid, flavonoid, diterpene, and glycoside, whereas *M. koenigii* extract was rich in saponins, protein, steroid, tannin, carbohydrates, alkaloid, flavonoid, and glycoside.

Conclusion: The present study proposes that *E. hirta* and *M. koenigii* extracts are excellent sources of natural bioactive compounds that could be used as potent antimicrobial drugs to counter the emerging problem of antibiotic resistance of pathogenic microorganisms.

Keywords: *Euphorbia hirta*, *Murraya koenigii*, Antimicrobial activity, Phytochemical analysis, Minimum inhibitory concentration.

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INTRODUCTION

In the present world, there are many infectious and re-emergent diseases that led to the development of a number of antibiotics. The literature reveals that infectious diseases represent the second leading cause of death worldwide. At present, more than 100 types of antibiotics are being widely used to treat various microbial infectious diseases [1]. However, irregular use of these antibiotics has caused the emergence of antibiotic-resistant bacteria, particularly *Staphylococcus aureus* (MRSA). Moreover, the side effects associated with the widespread use of these antibiotics may have deleterious effects on human organs. Therefore, due to the rising incidences related to multidrug resistance among pathogenic microorganism and the antibiotic side effects, there is a need to find out new antimicrobial sources for the treatment of human diseases [2]. The natural products derived from medicinal plants represent an immense and practically unexploited source of potentially useful bioactive compounds. Plants have been used globally in the treatment of various infectious diseases as a traditional medicine for thousands of years. Currently, a number of pharmaceutical companies are spending a lot of money and time to develop cost-effective plant-derived natural drugs [3]. Plants are the rich source of many secondary plant metabolites, such as tannins, terpenoids, alkaloids, flavonoids, phenols, and quinines [4-6]. These phytochemicals are thought to play an important role in the treatment of urinary tract infections, gastrointestinal disorders, respiratory diseases, and cutaneous infections [7]. There are about 500,000 plant species around the world, and only one percent of their phytochemicals have been evaluated [8]. Therefore, the plants hold great potential for the discovery of novel bioactive compounds that could prove alternative to the synthetic drugs or antibiotics.

Euphorbia is the largest genus of the family *Euphorbiaceae*, which includes about 1600 species. Many species of this group are used in traditional medicines and have been intensively used for phytochemical investigation and antimicrobial properties. These species are known to contain various antimicrobial compounds, namely, flavonoids, triterpenoids, alkanes, amino acids, and alkaloids [9]. *Euphorbia hirta* is a pantropical weed, possibly native to India. It is a hairy herb that grows in open grasslands, roadsides, and pathways. *E. hirta* is broadly used as a medicinal herb as it possesses antibacterial, anthelmintic, antiasthmatic, sedative, antispasmodic, antifertility, antifungal, and antimalarial properties [10].

Murraya koenigii, commonly known as curry leaf or Kari patta in Indian dialects, belongs to Family *Rutaceae* that includes more than 150 genera and 1600 species [11]. *M. koenigii* is a small evergreen tree native of India and also found in Sri Lanka and other South Asian countries. Different parts of *M. koenigii* are used in folkloric medicine for the treatment of various diseases. This plant is known to possess antioxidative, cytotoxic, antimicrobial, antibacterial, antiulcer, positive inotropic, and cholesterol-reducing activities [12,13]. In view of the significant medicinal importance of these plant species, the present study was undertaken with an objective of analysis of phytochemicals and antimicrobial properties of *E. hirta* and *M. koenigii* against selected pathogenic microorganisms, which might be a promising source of new isolates capable of combating the problem of antibiotic resistance among the microorganisms in the future.

METHODS

Collection of plant samples

Medicinal plant samples of *E. hirta* (leaves and flowers) and *M. koenigii* (leaves) were collected from different areas of Ambala district, Haryana,

India. After the collection, the plant material was washed with distilled water and shade-dried on paper towels in the laboratory at 37°C.

Test microorganisms

The microorganisms used in the present study, *Candida albicans*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Propionibacterium acne*, and *S. aureus,* were obtained from IMTECH, Chandigarh, India. The microbial cultures were maintained in culture broth (Himedia) at 37°C and on agar (Himedia) plates at 4°C.

Extract preparation

The extraction was performed according to the method of Cheesbrough [14]. The collected plant materials were finely powders using a blender. Five gram of powdered plant material was taken in four 100 mL conical flasks and 50 mL of each solvent, namely, water, ethanol, petroleum ether, and chloroform:methanol (1:1) was added separately in the concerned flasks. The sealed flasks were kept in a rotary shaker for 2 days, after which the extract was filtered through muslin cloth followed by Whatman No. 1 filter paper. The solvent from aqueous extract was removed through lyophilization, while the ethanol, petroleum ether, and chloroform:methanol (1:1) extracts were kept in a water bath at 65°C to evaporate the solvent. Finally, the residues were collected and dissolved in sterile distilled water (for aqueous extract) and 70% acetone (others). Each extract was stored at 4°C in the refrigerator until use. Further, all the plant extracts were screened for their antimicrobial activity.

Determination of antimicrobial activity

The antimicrobial activity of each crude extract was determined using the agar well diffusion method Rojas *et al*. [15]. After autoclaving, the medium was cooled at 45-50°C and poured into flat-bottomed Petri dishes (90 mm in diameter) to obtain a depth of nearly 4 mm. The agar media was allowed to cool and solidify at room temperature, and the plates were pre-incubated at 35°C for 18-20 h to confirm sterility. About 100 µL of the test inoculum was uniformly spread on the surface of the solidified agar using a sterile spreader. Four equidistant wells of 8 mm in diameter and 3 mm in depth were made on the agar plate. About 100 µL of the plant extracts was filled in the wells. The bacterial agar plates were incubated aerobically for 24 h at 37°C except for *P. acne* which was incubated under anaerobic condition. The fungal *C. albicans* plates were incubated for 48 h at 30°C. Antimicrobial activity was determined by measuring the diameters of the zones of inhibition in mm. The test was performed in triplicates with controls (70% acetone).

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC)

MIC of *E. hirta* and *M. koenigii* extracts was determined through broth dilution method using 96 well plates [16,17]. The wells of each row were filled with 0.5 mL sterilized nutrient broth for *E. coli* and *P. aeruginosa*, Mannitol salt broth for *S. aureus* and malt extract broth for *C. albicans* followed by the addition of 0.5 mL of a mixture of culture medium. Each well-received plant extract, serially diluted to create a concentration ranging from 50 to 3.125 mg/mL. The plates were incubated under aerobic conditions at 37°C for 24 h (for bacteria) and 25°C for 48 h (for fungus). The lowest concentration (highest dilution) of the extract that resulted in no visible growth (no turbidity) in the first 24 h as compared with the control tubes was considered as an initial MIC. The dilutions that had no turbidity were further incubated at 37°C for 24 h. The lowest concentration that exhibited no visible turbidity after a total incubation period of 48 h was considered as the final MIC. MBC/MFC value was determined by subculturing the test dilution that showed no visible turbidity on to freshly prepared respective agar media. The plates were incubated further at 37°C for 42 h. The highest dilution that yielded no single bacterial colony on the nutrient agar plates was taken as MBC.

The antimicrobial susceptibility pattern of tested microorganisms

The antimicrobial susceptibility pattern of tested microorganisms was determined using disc diffusion method [18]. The freshly prepared and cooled medium was poured into flat-bottomed Petri dishes, and 100 µL of the tested inoculum was evenly spread on the surface of the solidified agar media using a sterile spreader. Suitable antibiotics/antifungal discs were placed on the agar plates and incubated for 24 h. The antimicrobial susceptibility was determined by measuring the diameters of inhibition zone (mm).

Synergistic antimicrobial activities of plant extracts with antibiotics

For the synergistic activities of plant extracts with antibiotics (exhibiting the largest zone of inhibition against respective microorganism), levofloxacin (for Gram-negative bacteria) and lincomycin (for Gram-positive bacteria) were used with extracts. About 0.1 mL of the tested inoculum was evenly spread on the surface of the solidified agar media using a sterile spreader. After a few minutes, a well of 8 mm (diameter) and 3 mm (depth) was made in the mid of agar plate and was filled with 100 µL of the plant extract. The antibiotic disc was placed on the top of the well and the plates were incubated at 35°C for 18–20 h. The synergistic activity was obtained by measuring the diameters of inhibition zones (mm) and further comparing with inhibition zones of the extracts and antibiotics against respective microorganisms [19].

Phytochemical analysis

Phytochemical analysis of the tested plants was carried out by dissolving the extracts in respective solvents. The extracts were analyzed for the presence of alkaloids, flavonoids, saponins, tannins, carbohydrates, terpenoids, protein, glycosides, diterpenes, and steroids using the standard procedures [20-22].

Statistical analysis

The data were analyzed by using simple arithmetic means of the different extracts and the SE was compared with the controls.

RESULTS AND DISCUSSION

Determination of antimicrobial activity of different extracts

In the present study, the antimicrobial activity of *E. hirta* and *M. koenigii* extracts prepared in different solvents was determined against *E. coli*, *P. aeruginosa* (Gram-negative), *P. acnes*, *S. aureus* (Gram-positive), and fungus *C. albicans*. The results presented in Table 1 revealed that among the four solvents, ethanol proved to be the best one with the strongest and broadest action spectrum against the test microorganisms. The differential behavior of solvents on antimicrobial activity might be due to differences in their polarities. Antimicrobial compounds such as flavonoids and terpenoids are polar constituents and are difficult to be extracted using non-polar solvent systems. Ethanol being the most polar of the solvents used would have facilitated better release of these antimicrobial compounds from the extracts. The present results are also corroborated by the previous reports suggesting ethanol as a preferential solvent in determining the antimicrobial potential of medicinal plants [7,23-25]. Because of the maximum inhibitory potential of the ethanol, all further experiments were conducted with ethanolic extracts.

Both ethanolic extracts of *E. hirta* displayed significant antibacterial activity against all test microorganisms except *P. acnes*. Maximum activity was observed against *C. albicans* (17 mm) and *S. aureus* (13 mm), whereas moderate activity was obtained against Gram-negative bacteria. The results obtained in this study are in close proximity to the findings of previous reports related to the antimicrobial properties of the *E. hirta* against various microorganisms [26-30]. In comparison, the ethanolic extract of *M. koenigii* inhibited the growth of only *E. coli*, *C. albicans*, and *S. aureus,* whereas no antibacterial activity was displayed against *P. aeruginosa* and *P. acnes*. Maximum inhibition was observed against *C. albicans* (12 mm) followed by *S. aureus* (9 mm). There are also previous reports supporting the current results regarding the antimicrobial potential of *M. koenigii* [24,31]. Since all the extracts exhibited maximum activity against *C. albicans*, it could be concluded that these extracts may find one of the major applications in the treatment of skin and diaper rash, genital infection, and other *Candida*-associated diseases.

Determination of MIC and MBC/MFC

The inhibitory activity of all ethanolic extracts varied significantly against the tested isolates with MIC value ranging from 12.5 to 50 mg/mL. The least MIC was produced by the leaf extract of *E. hirta* on *C. albicans* (12.5 mg/mL), while maximum value (50 mg/mL) was exhibited by the same extract against *P. aeruginosa*. The extracts of *E. hirta* (flower) and *M. koenigii* produced the least MIC (25 mg/mL) against *C. albicans* and *S. aureus* while maximum MIC (50 mg/mL) was against *E. coli* and *P. aeruginosa* (Table 2). Following the MIC, the MBC/MFC was also determined and the results are depicted in Table 3. The MBC of all the extracts was found to be 50 mg/mL for *E. coli*, *P. aeruginosa,* and *S. aureus*. MIC value equal to MBC value represents their bactericidal activity against respective microorganisms. The MIC and MBC values for *E. coli* (*E. hirta* flowers and *M. koenigii*) and *P. aeruginosa* (both extracts of *E. hirta*) were found to be equal. The MFC of *E. hirta* (leaves) for *C. albicans* was found to be 25 mg/mL which is 2 times higher than its MIC value, whereas it was 50 mg/mL for *E. hirta* (flowers) and *M. koenigii* extracts.

Antimicrobial susceptibility pattern of test microorganism

The antimicrobial susceptibility pattern of Gram-positive bacteria is given in Table 4. The strain *S. aureus* exhibited maximum zone of inhibition against antibiotic lincomycin (29 mm) and the minimum against tetracycline (21 mm). On the contrary, *P. acnes* was found to be resistant to cloxacillin and lincomycin. Its growth was inhibited maximally against cefotaxime (30 mm), whereas the lowest zone of 20 mm was observed against co-trimoxazole. In case of Gram-negative bacteria, *E. coli* was found to be susceptible maximally to levofloxacin and minimally to aztreonam exhibiting inhibition zones of 35 mm and 15 mm, respectively. *P. aeruginosa* was found to be resistant to

Table 1: Antimicrobial activity of *E. hirta* and *M. koenigii* extracts against tested microorganisms

Extract nature and solvent system			Gram-negative bacteria		Fungus	Gram-positive bacteria	
Plants	**Parts used**	**Solvent used**	***E. coli* (mm)**	***P. aeruginosa* (mm)**	***C. albicans* (mm)**	***P. acnes* (mm)**	***S. aureus* (mm)**
E. hirta	Leaves	Aqueous	6±0.4	NA	12±0.3	NA	3±0.3
		C:M	NA	NA	NA	NA	NA
		Ethanol	9±0.2	4±0.3	17±0.1	NA	13±0.2
		Petroleum ether	NA	NA	NA	NA	NA
E. hirta	Flower	Aqueous	NA	NA	NA	NA	NA
		C:M	NA	NA	NA	NA	NA
		Ethanol	6±0.3	4±0.1	15±0.4	NA	11±0.5
		Petroleum ether	NA	NA	NA	NA	NA
M. koenigii	Leaves	Aqueous	NA	NA	5±0.3	NA	2±0.3
		C:M	NA	NA	4±0.2	NA	4±0.3
		Ethanol	6±0.4	NA	12±0.2	NA	9±0.4
		Petroleum ether	NA	NA	3±0.2	NA	2±0.2

NA: No activity exhibited by extract against microorganism, C:M: Chloroform: methanol (1:1). *mm: Diameter of zone of inhibition in millimeter. *E. hirta: Euphorbia hirta, M. koenigii: Murraya koenigii, E. coli: Escherichia coli, P. aeruginosa: Pseudomonas aeruginosa, C. albicans: Candida albicans, P. acnes: Propionibacterium acnes, S. aureus: Staphylococcus aureus*

Table 2: MIC of the different plant extracts against tested microorganisms

Microorganism/plants	Concentration (mg/mL)	*C. albicans*	*E. coli*	*S. aureus*	*P. aeruginosa*
E. hirta (leaves)	50.0	NG	NG	NG	NG
	25.0	NG	NG	NG	G
	12.50	NG	G	G	G
	6.25	G	G	G	G
	3.125	G	G	G	G
E. hirta (flowers)	50.0	NG	NG	NG	NG
	25.0	NG	G	NG	G
	12.50	G	G	G	G
	6.25	G	G	G	G
M. koenigii	50.0	NG	NG	NG	-
	25.0	NG	G	NG	-
	12.50	G	G	G	-
	6.25	G	G	G	-

MIC: Minimum inhibitory concentration, *E. hirta: Euphorbia hirta, M. koenigii: Murraya koenigii*

Table 3: MBC/MFC of different plant extracts against tested microorganisms

Microorganism/plants	Concentration (mg/mL)	*C. albicans*	*E. coli*	*S. aureus*	*P. aeruginosa*
E. hirta (leaves)	50.0	NG	NG	NG	NG
	25.0	NG	G	G	G
	12.50	G	G	G	G
	6.25	G	G	G	G
E. hirta (flowers)	50.0	NG	NG	NG	NG
	25.0	G	G	G	G
	12.50	G	G	G	G
	3.125	G	G	G	G
M. koenigii	50.0	NG	NG	NG	-
	25.0	G	G	G	-
	12.50	G	G	G	-
	3.125	G	G	G	-

MBC: Minimum bactericidal concentration, MFC: Minimum fungicidal concentration, *E. hirta: Euphorbia hirta, M. koenigii: Murraya koenigii*

aztreonam, ceftazidime, and cefotaxime. However, the maximum inhibition zone of 30 mm of *P. aeruginosa* was observed against levofloxacin (Table 5). On the contrary, the fungus *C. albicans* was resistant to all the tested antibiotics (Table 6).

Synergistic antimicrobial activity of different extracts with antibiotics

Synergistic activity of all extracts (*E. hirta* and *M. koenigii*) with selected antibiotics (with maximum inhibition) was determined to observe any effect of the collective action on the antimicrobial efficiency. The results presented in Table 7 revealed that combination of leaf extract of *E. hirta* with levofloxacin instigated a slight increase in the inhibition zone (38 mm). However, there was no synergistic effect of the combination of *E. hirta* (flower) and *M. koenigii* extracts with the antibiotic levofloxacin on Gram-negative bacteria *E. coli* and *P. aeruginosa*. On the other hand, the combination of antibiotic lincomycin with all extracts, particularly *E. hirta* had much higher effect on the growth of Gram-positive bacterium *S. aureus* as witnessed by the increase in the diameter of the zone of inhibition (36 mm).

Table 4: Antibiotic susceptibility pattern of Gram-positive bacteria

Antibiotics	Concentration (µg)	*S. aureus* (mm)	*P. acne* (mm)
Co-trimoxazole	25.0	27	20
Cloxacillin	1.0	24	NA
Lincomycin	2.0	29	NA
Cefuroxime	30.0	23	22
Cefotaxime	30.0	25	30
Tetracycline	30.0	21	27

NA: No activity, *S. aureus: Staphylococcus aureus, P. acnes: Propionibacterium acnes*

Table 5: Antibiotic susceptibility pattern of Gram-negative bacteria

Antibiotics	Concentration (µg)	*E. coli* (mm)	*P. aeruginosa* (mm)
Levofloxacin	5.0	35	30
Aztreonam	30.0	15	NA
Amikacin	30.0	26	28
Imipenem	10.0	27	26
Ceftazidime	30.0	16	NA
Cefotaxime	30.0	21	NA

NA: No activity, *E. coli: Escherichia coli, P. aeruginosa: Pseudomonas aeruginosa*

Table 6: Antibiotic susceptibility pattern of fungus

Antibiotics	Concentration (µg)	*C. albicans*
Nystatin	50.0	NA
Clotrimazole	10.0	NA
Miconazole	30.0	NA
Ketoconazole	50.0	NA

NA: No activity, *C. albicans: Candida albicans*

Phytochemical analysis of the different extracts

Freshly prepared ethanolic extracts of *E. hirta* and *M. koenigii* were subjected to phytochemical analysis for the presence of various constituents (primary and secondary metabolites) responsible for the antimicrobial properties. Phytochemical screening of *E. hirta* extracts revealed the presence of steroid, tannin, terpenoid, carbohydrate, alkaloid, flavonoid, diterpene, and glycoside, while saponin and protein were present only in case of *M. koenigii*. However, this extract was devoid of terpenoid and diterpene (Table 8). The phytochemicals observed in these extracts have been reported to possess medicinal properties and physiological activity. Flavonoids are characterized by the presence of antibacterial, antioxidant, antidiarrheal, anti-inflammatory, anti-allergic, antimutagenic, and vasodilatory properties [31,32]. Saponins are known to possess hypocholesterolemic and antidiabetic properties, while steroids and triterpenoids exhibit analgesic properties [33- 35]. The presence of biologically important phytochemicals in the *E. hirta* and *M. koenigii* as reported in this study contribute toward their medicinal relevance, and therefore, suggests potential sources for valuable natural drugs.

CONCLUSION

Evaluation of antimicrobial properties and phytochemical analysis of medicinal plant species possessing pharmacological properties is essential to exploit them as a source of potent natural drugs. In the present study, three medicinal plant species were analyzed for their antimicrobial properties against selected pathogenic microorganisms in different solvents and were screened for the presence of various phytochemical compounds. On the basis of the results obtained, the present work concludes that the highest inhibitory activity of all the plant species was found in ethanol extract as compared to other solvents. On the basis of the antimicrobial study, it was revealed that leaf extract of *E. hirta* exhibited maximum antimicrobial activity against all tested pathogens except *P. acnes.* The other extracts were also found to be inhibitory, but the effect was less pronounced as compared to the leaf extract of *E. hirta*. Further, both the extracts of *E. hirta* had similar phytochemical constituents, but *M. koenigii* had variation in its phytochemical compounds with the presence and or absence of some compounds. The occurrence of different secondary metabolites, namely, steroid, tannin, terpenoid, carbohydrate, alkaloid, flavonoid, diterpene, glycoside, and saponin were supposed to be responsible for the antimicrobial properties of *E. hirta* and *M. koenigii* and confirmed their antimicrobial efficiency against selected pathogens. Hence, the results of this study apparently specify that *E. hirta* and *M. koenigii* could be used as potent antimicrobial drugs of natural origin capable of

Table 7: Synergistic activity of the different plant extracts with selected antibiotics

Plant	Z.O.I of plant (mm)	M.O.	Antibiotic	Z.O.I of antibiotic (mm)	Concentration of antibiotic (µg/disc)	Z.O.I as synergistic activity (mm)
Gram-negative bacteria						
E. hirta (leaves)	9±0.2	*E. coli*	LE	35	5	38±0.2
	4±0.3	*P. aeruginosa*		30		30±0.3
E. hirta (flowers)	6±0.3	*E. coli*	LE	35	5	35±0.2
	4±0.1	*P. aeruginosa*		30		30±0.3
M. koenigii	6±0.4	*E. coli*	LE	35	5	35±0.2
	-	*P. aeruginosa*		30		30±0.1
Gram-positive bacteria						
E. hirta (leaves)	13±0.2	*S. aureus*	L	29	2	36±0.4
E. hirta (flowers)	11±0.5	*S. aureus*	L	29	2	36±0.2
M. koenigii	9±0.4	*S. aureus*	L	29	2	32±0.3

Z.O.I: Zone of inhibition, LE: Levofloxacin, *L: Lincomycin, *E. hirta: Euphorbia hirta, M. koenigii: Murraya koenigii, E. coli: Escherichia coli, P. aeruginosa: Pseudomonas aeruginosa, S. aureus: Staphylococcus aureus*

Table 8: Phytochemical analysis of various constituents present in the different extracts

Plant/components	*E. hirta* (leaves)	*E. hirta* (flowers)	*M. koenigii*
Steroid	+	+	+
Saponin	-	-	+
Tannin	+	+	+
Protein	-	-	+
Terpenoids	+	+	-
Carbohydrate	+	+	+
Alkaloids	+	+	+
Flavonoid	+	+	+
Diterpenes	+	+	-
Glycosides	+	+	+

battling the problem of antibiotic resistance among the microorganisms in the future.

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AUTHORS CONTRIBUTION

Planning and designing of study: Mukesh Kumar, Vishal Gupta; Experimentation: Divya Gupta; Result Analysis: Mukesh Kumar, Divya Gupta; Manuscript Drafting: Mukesh Kumar, Divya Gupta, Vishal Gupta. All authors contributed in the final approval of manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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