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SHORT COMMUNICATION

Untargeted Gas Chromatography–Mass Spectrometry Analysis and Evaluation of Antimicrobial and Antioxidant Activity of *Zingiber nimmonii* (J. Graham) Dalzell Rhizome Extracts

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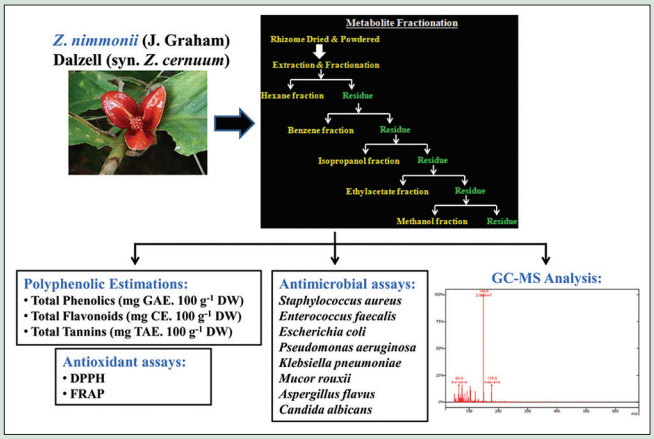
ABSTRACT

Background: *Zingiber nimmonii* (J. Graham) Dalzell (syn. *Z. cernuum*) endemic to peninsular India have been documented to be in use since ancient times in several traditional systems of medicine. *Z. nimmonii* with ethnomedicinal applications constitute a rich source of secondary metabolites toward identifying potential bioactive constituents with antioxidant and antimicrobial properties. **Objective:** Rhizomes of *Z. nimmonii* (J. Graham) Dalzell, endemic to the Western Ghats, were analyzed for bioactivity and phytochemical composition. **Materials and Methods:** Polyphenolic contents, namely total phenolics (TPs), total flavonoids (TFs) and total tannin (TT), were determined and expressed using gallic acid (GA), catechin (C) and tannic acid (TA) as standards. Antibacterial and antifungal activities were evaluated against two Gram-positive, three Gram-negative bacteria and three fungi by agar well diffusion method. Antioxidant activity was determined by 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) and Ferric Reducing Antioxidant Power (FRAP) assay, and volatile metabolite composition was determined by gas chromatography–mass spectrometry (GC-MS) analysis. **Results:** THE highest TF and TT contents were detected in ethyl acetate (557.64 ± 41.74 mg CE. 100 g^{-1} DW) and isopropanol (63.995 ± 2.062 mg TA equivalent. 100 g^{-1} DW) extract, respectively. High TP content in isopropanol extract (112.80 ± 10.99 mg GA equivalent. 100 g^{-1} DW) contributed to antimicrobial activity against Gram-positive *Staphylococcus aureus* (16.3 ± 0.6 mm) and antifungal activity against *Aspergillus flavus* (11.7 ± 0.6 mm). Methanol extracts showed high antioxidant activity as determined by DPPH (67.4 ± 12.5 µg/ml) and FRAP (127.8 ± 12.4 µg/ml) assays. Major bioactive phytochemical constituents in *Z. nimmonii* rhizome following GC-MS analysis included heptanediamide, N, N′-di-benzoyloxy (arachidonic acid inhibitor), n-hexadecanoic acid (antibacterial, cytotoxic, and antioxidant), and oleic acid (antitumor). **Conclusion:** The present study demonstrates potential of *Z. nimmonii* rhizomes as a rich source of secondary metabolites which can be exploited toward developing anti-infective formulations and free radical quenchers.

Key words: Antimicrobial, antioxidant, gas chromatography–mass spectrometry, polyphenolics, *Zingiber nimmonii*

SUMMARY

- Solvent extracts of *Zingiber nimmonii* (J. Graham) Dalzell obtained following sequential extraction subjected to determination of antimicrobial and antioxidant activity
- Isopropanol extract that yielded the highest total phenolic (TP) content (112.80 ± 10.99 mg gallic acid equivalent. 100 g^{-1} DW) also showed the highest antimicrobial activity against Gram-positive *Staphylococcus aureus* (16.3 ± 0.6 mm) and highest antifungal activity against *Aspergillus flavus* (11.7 ± 0.6 mm)
- Methanol extract of *Z. nimmonii* rhizomes exhibited high antioxidant activities determined by 1,1-diphenyl-2-picrylhydrazyl radical assay as 67.4 ± 12.5 µg/ml and following FRAP assay as 127.8 ± 12.4 µg/ml
- Major bioactive metabolites identified by gas chromatography–mass spectrometry analysis included arachidonic acid inhibitor, namely heptanediamide, N, N′-di-benzoyloxy; antibacterial, cytotoxic, and antioxidant, namely n-hexadecanoic acid; and antitumor, namely oleic acid.



Abbreviations Used: GC-MS: Gas Chromatography- Mass Spectrometry, DPPH:1,1- diphenyl-2-picrylhydrazyl radical, FRAP: Ferric Reducing Antioxidant Power.

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DOI: 10.4103/pr.pr_19_20

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INTRODUCTION

Genus *Zingiber* distributed throughout tropical Asia[1] comprises rhizomatous plants known for their aromatic constituents and extensive ethnomedicinal applications. The rich repertoire of secondary metabolites of *Zingiber* species constitutes potential source for identifying bioactive constituents with antioxidant and antimicrobial properties toward developing anti-infective formulations.[2] Among wild *Zingiber* taxa, *Z. nimmonii* (J. Graham) Dalzell (syn. *Z. cernuum*) is endemic to the Western Ghats, a biodiversity hotspot in the southern part of Indian peninsula.[3] *Z. nimmonii* is a perennial herb with aromatic, fleshy rhizome that is purplish-lilac inside. Besides propagating vegetatively

Cite this article as: Nair AR, Ganapathy G. Untargeted gas chromatography–mass spectrometry analysis and evaluation of antimicrobial and antioxidant activity of *Zingiber nimmonii* (J. Graham) Dalzell rhizome extracts. Phcog Res 2020;12:466-70.

Submitted: 14-Mar-2020 **Revised:** 29-May-2020
Accepted: 18-Aug-2020 **Published:** 23-Jan-2021

from rhizomes, *Z. nimmonii* also profusely set seeds under natural conditions.[4] The plant grows both at low and high altitudes in moist areas under the shades of trees.[3] Due to high rate of habitat loss, the species has been included in the International Union for Conservation of Nature Red List of Threatened Species.[5] Earlier studies characterizing volatile oil composition of *Z. nimmonii* rhizomes[6,7] had detected β-caryophyllene (42.2%) and α-humulene (α-caryophyllene, 27.7%) as major metabolites.[6]

Essential oil from rhizome of *Z. cernuum* (syn. Z. *nimmonii*) has been studied for antimicrobial activity,[6,7] larvicidal and repellent activity against *Anopheles stephensi* (malaria vector), *Aedes aegypti* (dengue vector), and *Culex quinquefasciatus* (lymphatic filariasis vector).[8] Choice of extraction solvent greatly influences the yield and type of metabolite extracted and identified from a particular plant.[9,10] Hence, sequential extraction using solvents of increasing polarity would provide more insight into the bioactive potential of *Z. nimmonii* solvent extracts. Except for one study wherein *Z. nimmonii* rhizome extracts prepared using hexane, chloroform, and methanol revealed high antioxidant potential of hexane extracts,[11] not many studies have explored the antioxidant and antimicrobial activity of *Z. nimmonii* rhizome extracts prepared by sequential fractionation using solvents of increasing polarity. Hence, the present study was undertaken to evaluate: (i) polyphenolic content, (ii) volatile metabolite (s) by gas chromatography–mass spectrometry (GC-MS) analysis, and (iii) antimicrobial activity and antioxidant activities.

MATERIALS AND METHODS

Plant material

Z. nimmonii was collected from their natural habitat from Thirunelly hills, Western Ghats, Kerala (Latitude: 11.9117°N, Longitude: 75.9958°E), India. The rhizomes were planted in pot containing mixture of sand, soil, and cow dung in 1:1:1 and watered every 2–3 days with tap water. Plants were grown and maintained under natural light conditions (~12:12 h) and temperature (28°C ± 5°C).

Polyphenolic extraction and profiling

Rhizomes were sliced and air dried at room temperature (37°C) for 3 weeks, after which it was ground to a uniform powder of 40 mesh size. Powdered rhizome samples (100 g) were sequentially extracted with hexane, benzene, isopropanol, ethyl acetate, and methanol using a Soxhlet extractor. Extracts were filtered through Whatman filter paper no. 42 (125 mm) to remove unextractable matter. Extracts were concentrated to dryness using rotary evaporator under reduced pressure. The dried samples were used for estimation of polyphenolics, namely TPs, total flavonoids (TFs), and total tannins (TTs) as described in Aswati *et al.*[12] TP content was determined by incubating equal volume of sterile water-diluted solvent extract and Folin–Ciocalteu reagent at room temperature for 6 min. To this, 1.25 ml of 7% (w/v) sodium carbonate was added, and after incubating samples at 30°C for 1.5 h, absorbance was measured at 760 nm using UV spectrophotometer (UV/Vis, UV 3000, LabIndia). Quantitative measurements were made from a standard calibration curve prepared using gallic acid (GA) as standard at varying concentrations (20–120 mg/L). TP content was expressed as GA equivalents (GAEs) in mg. 100 g^{-1} dry weight (DW) and determined in triplicates. TF was determined by incubating an aliquot of diluted solvent extract with 1/3rd volume of 5% (w/v) $NaNO_2$ for 5 min at 30°C followed by further incubation after addition of 150 µl of 10% (w/v) $AlCl_3$ at room temperature for 6 min. Absorbance is measured against reagent blank at 510 nm after stopping the reaction by adding 0.5 ml of 1M NaOH. TF content is quantified in extracts using catechin (20–120 mg/L) as standard and expressed as mg catechin equivalents (CE). 100 g^{-1} DW. To determine TT content, the assay mixture comprising 0.1 ml aliquot of solvent extract is mixed with sterile water and made up to 7 ml to which 1 ml of potassium ferricyanide (0.008 M) followed by 1 ml of ferric chloride (0.02 M in 0.1 M HCl) is added. After measuring absorbance at 700 nm, TT content in extracts is quantified from standard curve prepared using tannic acid as standard (20–120 mg/L). Results are expressed as mg TA equivalent (TAE). 100 g^{-1} DW and determined in triplicates.

Antimicrobial and antioxidant assays

The five solvent extracts were screened for their antibacterial and antifungal activities against Gram-positive bacteria (*Staphylococcus aureus* and *Enterococcus faecalis*); Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*); and fungi (*Mucor rouxii*, *Aspergillus flavus*, and *Candida albicans*) by agar well diffusion method.[13] Nutrient agar plates are seeded with indicator bacterial and fungal strains. A well of 6 mm diameter was made using a sterile cork borer and 50 µg/ml of respective solvent extracts placed in it. Ampicillin was used as positive control. Zones of growth inhibition were measured after 18 h of incubation at 37°C for bacteria and 48 h for fungi at 28°C. Sensitivity of the tested micro-organisms to the extracts was determined by measuring the diameter of inhibitory clear zones on the agar surface around the well. Values ≤6 mm were considered as not active against tested microorganisms. All experiments were done in triplicate.

Free radical scavenging activity of *Z. nimmonii* extracts were determined by 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) method and FRAP assay as described in Aswati *et al.*[12] Increasing concentration of the solvent extracts (20–120 µg/ml) was diluted with methanol and made up to 1 ml, followed by addition of 1 ml of methanolic solution of DPPH (100 µM) and incubated in dark for 30 min. Reduction of DPPH radical was determined by measuring decrease in absorbance at 517 nm. Ascorbic acid at varying concentrations (20–100 µg/ml) was used as standard. IC_{50} values were estimated using each of the extracts to determine the amount required to scavenge 50% of DPPH-free radicals. For FRAP assay, varying concentration of solvent extracts (20–120 µg/ml) diluted using deionized water to 1.0 ml was mixed with 2.5 ml of phosphate buffer (0.1 M; pH – 6.6) and 2.5 ml potassium ferricyanide (1% w/v). After incubating the mixture at 50°C for 20 min, reaction was stopped by addition of 2.5 ml of trichloroacetic acid (10% w/v) and centrifuged at 3000 rpm for 10 min. The upper layer of 2.5 ml was mixed with equal volume of deionized water, and finally, 0.5 ml of freshly prepared ferric chloride solution (0.1% w/v) was added. Amount of Fe (II) was monitored by recording absorbance after 10 min at 700 nm with higher absorbance indicative of higher reducing power. Ascorbic acid at varying concentrations (20–100 µg/ml) was used as standard. Antioxidant activity was determined as: scavenging effect (SE) (%) (for DPPH assay) and/or increase in reducing power (%) (FRAP assay) = $(A_{test} - A_{blank})$ $A_{blank}^{-1} \times 100$, where A_{test} is absorbance of test solution and A_{blank} is absorbance of blank.

Gas chromatography–mass spectrometry analysis

Methanolic extracts were shaken for 90 min at 25°C, centrifuged, and filtered using a Whatman filter paper (No. 2). Filtered solvent extract was analyzed qualitatively by GC-MS on Hewlett-Packard HP 6890 GC (injector temperature, 220°C; 1 µL splitless injection) coupled to a HP MS-5973 mass-selective detector in a DB5MS column (30 m × 0.32 mm × 0.25 µm) with 1.4 ml min − 1 helium as carrier gas. The oven temperature was programmed from initial temperature of 60°C with an increase of 3°C/min to 240°C. Identification was based on

matching mass spectra in Wiley and National Institute of Standards and Technology (NIST) Mass Spectral Library.

RESULTS

Rhizome of *Z. nimmonii* were sliced, dried, powdered, and sequentially extracted with solvents, hexane, benzene, isopropanol, ethyl acetate, and methanol using a Soxhlet extractor. Extracts after filtration were concentrated and used for estimation of polyphenolics, namely TP, TF, and TT. TP, TF, and TT contents were expressed using GA, catechin (C), and TA as standards. Analysis revealed the highest TP content (112.80 ± 10.99 mg GAE. 100 g^{-1} DW) in isopropanol and methanol extract (110.26 ± 15.35 mg GAE. 100 g^{-1} DW) followed by ethyl acetate extract (103.66 ± 18.29 mg GAE. 100 g^{-1} DW). Ethyl acetate extract also showed the highest TF content (557.64 ± 41.74 mg CE. 100 g^{-1} DW) while the highest TT content was observed in isopropanol extract (63.995 ± 2.062 mg TAE. 100 g^{-1} DW) [Table 1].

Of the five *Z. nimmonii* sequentially fractionated solvent extracts, the highest antibacterial and antifungal activities were shown by isopropanol extract [Figure 1]. Isopropanol extract exhibited the highest antimicrobial activity against Gram-positive *S. aureus* (16.3 ± 0.6 mm) while the highest antifungal activity was observed against *A. flavus* (11.7 ± 0.6 mm). Phytoconstituents with natural redox properties were determined in *Z. nimmonii* rhizome extracts by DPPH and FRAP assays. DPPH radical quenching and FRAP assays revealed methanol extract of *Z. nimmonii* rhizomes to exhibit high antioxidant activities. The free radical SE determined by DPPH assay was 67.4 ± 12.5 µg/ml while the reducing power determined following FRAP assay was obtained as 127.8 ± 12.4 µg/ml [Table 1].

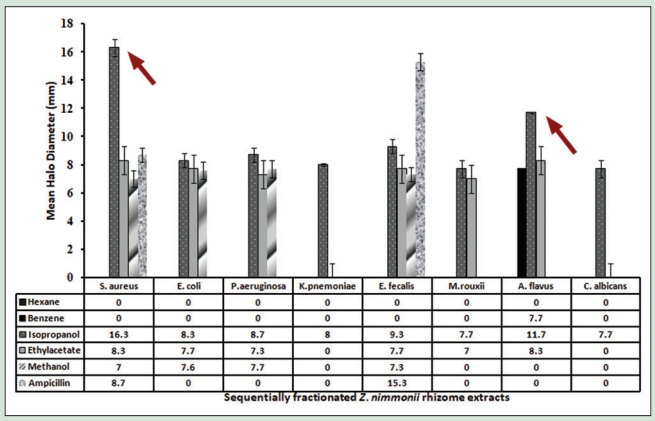

Figure 1: Antibacterial activity of solvent extracts of *Zingiber nimmonii* rhizome determined by agar well diffusion method. Zone of inhibition (in mm) observed for tested microbes obtained for each of the solvent fractions is shown with arrows indicating the maximal activity obtained for isopropanol fraction against *S. cereus* and *Aspergillus flavus*

GC-MS analysis followed by mass spectral matching in Wiley and NIST Mass Spectral Library identified various metabolites of therapeutic significance in *Z. nimmonii* rhizome [Table 2]. Major bioactive metabolites identified included arachidonic acid inhibitor, heptanediamide, N, N'-di-benzoyloxy (area: 7.104e+9; Rt: 22.278), antibacterial, cytotoxic and antioxidant, n-hexadecanoic acid (area: 4.187e+9; Rt: 23.442), and antitumor, oleic acid (Area: 3.983e+9; Rt: 24.080).

DISCUSSION

A comprehensive extraction using solvents of increasing polarity was undertaken in the present study due to the influence of extraction solvent on the type of metabolite extracted. Polyphenolic contents of *Z. officinale* (ginger)[14] and *Z. zerumbet*[15] have been studied, however, that for *Z. nimmonii* has not been determined, till date. Several studies have shown the significance of plant extracts with high phenolic content as antioxidant and antimicrobial agents.[13,16] Plant extracts and essential oils have been studied by many for antimicrobial activity against Gram-reactive bacteria and fungi.[16] High phenolic and tannin content could be attributed to the antimicrobial and antifungal activity exhibited by the isopropanol fraction in the present experiment. Phenolic compounds are known to exert inhibitory effect by disrupting membrane integrity leading to leakage of intracellular content.[17] Tannins exert antimicrobial effect by inducing damages to cell membrane by forming complexes with permease enzymes and porins found on outer bacterial membrane.[18] The present study had evaluated antimicrobial effect of *Z. nimmonii* rhizome solvent extracts on microbes that cause nosocomial infections[19] and found the highest inhibitory activity against Gram-positive bacteria. Earlier studies had reported less sensitivity of Gram-negative bacteria against various plant extracts[20] due to inability of metabolites in extracts to penetrate the complex outer membrane of Gram-negative bacteria.[21,22] *Zingiber* species are rich sources of polyphenolics with studies showing their antioxidant properties exceeding many currently used natural and synthetic antioxidants.[2] Methanolic and aqueous extracts of *Z. officinale* rhizome has been previously reported to possess high antioxidant activity.[14] The presence of kaempferol and zerumbone in *Z. zerumbet* has been ascribed to its strong radical scavenging activity.[23] Flavonoids and phenolics are known to exert antioxidant activity by scavenging free superoxide radicals.[24] In *Z. nimmonii*, earlier studies had reported flavonoids in hexane and chloroform extracts which were proposed to contribute to antioxidant activity activity.[11] Non-polar solvents generally tend to extract more of aglycone flavonoids.[25] Contrastingly, glycoside flavonoids are extracted more in polar solvents and are of

Table 1: Polyphenolic estimation and antioxidant activity of solvent extracts of *Zingiber nimmonii* rhizomes

Estimations	Hexane	Benzene	Isopropanol	Ethyl acetate	Methanol
Polyphenolic profiling					
TP (mg GAE/100/g DW)	21.85±2.33	77.24±19.06	112.80±10.99	103.66±18.29	110.26±15.35
TF (mg CE/100/g DW)	57.64±16.97	6.94±3.18	53.47±17.72	557.64±41.74	25.00±4.17
TT (mg TAE/100/g DW)	0.679±0.036	1.693±0.386	63.995±2.062	1.699±0.109	2.191±0.224
Antioxidant activity (IC_{50} values; µg/ml)					
DPPH assay	172.4±15.1	147.4±10.4	68.6±8.3	69.4±7.3	67.4±12.5
FRAP assay	427.9±54.4	165.7±24.5	211.9±4.7	163.8±10.6	127.8±12.4

TP content expressed as GAE; TF content expressed as CE; and TT content expressed as TAE. Results are expressed as mean±SD (*n*=5). For antioxidant activity assay, ascorbic acid used as positive control (IC_{50} value of 24.6±2.3 µg/ml) and the antioxidant activity is expressed as AAE (µg AAE/ml extract). TP: Total phenol; GAE: Gallic acid equivalent; TF: Total flavonoid; CE: Catechin equivalent; TT: Total tannin; TAE: Tannic acid equivalent; SD: Standard deviation; DPPH: 1,1-diphenyl-2-picrylhydrazyl radical; FRAP: Ferric reducing antioxidant power; AAE: Ascorbic acid equivalent; DW: Dry weight

Table 2: Metabolites identified by gas chromatography-mass spectrometry analysis of *Zingiber nimmonii* rhizome extracts with retention time, composition (in %), molecular weight, and nature of metabolite with bioactivity

Retention time	Percentage of composition	Molecular weight	Nature of compound; activity
8.496	0.494	229	Benzeneethanamine, 2-fluoro-.beta.,3,4-trihydroxy-N-isopropyl; anti-asthmatic
15.463	0.099	484	2-Myristynoyl pantetheine; biosurfactant
16.127	1.568	260	Z, Z, Z-1,4,6,9-Nonadecatetraene
16.817	1.346	256	1-Hexadecanol, 2-methyl; antimicrobial
17.853	8.146	324	3-Trifluoroacetoxypentadecane
18.536	9.925	278	10-Heptadecen-8-ynoic acid, methyl ester, (E)
19.126	13.630	594	Hexadecane, 1,1-bis (dodecyloxy)
19.634	14.316	363	1-Nitro-.beta.-d-arabinofuranose, tetraacetate
20.066	9.092	213	9-Oxabicyclo[3.3.1]nonane-2,6-dione, 2-oxime-6-ethylene ketal
20.743	11.765	205	2-Bromo-4-chloroaniline
22.278	14.814	398	Heptanediamide, N, N'-di-benzoyloxy; arachidonic acid inhibitor
22.861	5.545	160	1-(2-ethoxyethoxy)-2-(vinyloxy)-ethane
23.442	2.882	256	n-Hexadecanoic acid; antibacterial, cytotoxic, antioxidant
24.080	3.268	282	Oleic acid; antitumor

greater interest as vital phytochemicals with antioxidant properties.[25,26] It was with this rationale that solvents of different polarity indices were used in the present study which identified methanol extracts with the highest antioxidant activity as determined following DPPH and FRAP assays. Earlier studies evaluating antimicrobial activity of *Z. nimmonii* essential oil had identified significant inhibition of fungi such as *Candida glabrata*, *C. albicans,* and *Aspergillus niger* and bacteria such as *Bacillus subtilis* and *Pseudomonas aeruginosa*.[6] The present studies had identified the TP-rich isopropanol extract to exhibit antimicrobial activity against Gram-positive *S. aureus* and antifungal activity against *A. flavus*. GC-MS analysis identified various metabolites of therapeutic significance in *Z. nimmonii* rhizome. Detection of metabolites such as heptanediamide, N, N'-di-benzoyloxy (arachidonic acid inhibitor), n-hexadecanoic acid (antibacterial, cytotoxic, antioxidant), and Benzeneethanamine, 2-fluoro-.beta.,3,4-trihydroxy-N-isopropyl (anti-asthmatic) is indicative of *Z. nimmonii* rhizome to be rich source for bioactive phytoconstituents and natural antioxidants that could serve as potential source for developing lead compounds with therapeutic benefits.

CONCLUSION

Sequential fractionation was followed to determine the antimicrobial and antioxidant activities of *Z. nimmonii* rhizome. The isopropanol extracts with high TP content showed antimicrobial activity to Gram-positive bacteria, *S. aureus,* and antifungal activity against *A. flavus.* GC-MS analysis identified chemical constituents of pharmaceutical significance in *Z. nimmonii* rhizome. Bioactive properties and chemical constituents documented in the present study demonstrate the potential of *Z. nimmonii* as a rich source of secondary metabolites which can be exploited toward developing anti-infective formulations and free radical quenchers.

Acknowledgements

The authors sincerely acknowledge the research facilities extended at NITC and CUK for undertaking the present work. GG also thanks MHRD (Ministry of Human Resource and Development, Govt. of India) for the research fellowship received.

Financial support and sponsorship

The authors are also thankful to the Department of Science and Technology-Science and Engineering Research Board (DST-SERB) for the research grant (No. EMR/2016/002229) received to undertake the present work.

Conflicts of interest

There are no conflicts of interest.

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