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RESEARCH ARTICLE

LINEAR DISCRIMINANT FUNCTION ANALYSIS OF TWO MUTANT VARIETIES OF *PLANTAGO OVATA* FORSSK. USING CHROMOSOME LENGTH MEASUREMENTS

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SUMMARY *Plantago ovata* var. mayuri and niharika mutants have been subjected to linear discriminant function analysis. By conventional root tip squash technique, metaphase chromosomes of both the mutants were obtained and measured using KaryoType software. For data analysis, chromosome measurements, numeric or place of development and string version or type of plant material were used. Data was analysed statistically and results suggested that linear discriminant function analysis of *P. ovata* var. niharika and mayuri differs from each other in their chromosome measurements and their time duration of development.

Keywords: *Plantago ovata*, var. niharika, var. mayuri, chromosomes, karyotype, linear discriminant function.

INTRODUCTION

Plantago ovata (Plantagenaceae) commonly known as Ispaghula is an ancient medicinal herb. Its seeds and mucilage have various medicinal, nutritional, cosmetics and food industries applications (Dybka-Stepien et al. 2021). The demands of seed husk in the market for certain purpose lead the development of mutant variety. Two mutant varieties of *P. ovata* were developed for commercial production at Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow. The mutant varieties, var. mayuri and niharika of *P. ovata* were developed in 1998 and 2004 respectively through mutation breeding (irradiation with gamma rays) (Lal et al. 1998, 2004). The time duration was 5–6 y for the development of both varieties. They were marked as A and B and based on their place of development as LN-1 and LM-2 respectively for linear discrimination function (LDF) analysis. The aim of the paper was to find out differences if any between the 2 mutant varieties through chromosomal characteristics.

MATERIALS AND METHODS

P. ovata var. mayuri and niharika mutant seeds were obtained from CIMAP. For germination, seeds were sprinkled over the water-soaked filter paper in petri dish. Root tips (1–2 cm) were cut and kept in refrigerator at 4^0 C for 12 h (pre-treatment) followed by fixation in acetic-alcohol

(3:1) solution for 24 h and preserved in 70% ethanol at 4^0 C for further analysis. Root tips were hydrolyzed with 1N HCl for 1 min at 37^0 C and washed with water and squashed in 2% aceto-carmine. The metaphase chromosomes were photographed with Magnus MX21iLED microscope attached with digital camera. The photomicrographs were used to measure the chromosome lengths using KaryoType software.

For data analysis, chromosome measurements (C1-C8), numeric or place of development (LN-1 and LM-2) and string version or type of plant material (mutant variety) were used. Data was analysed statistically using SPSS ver. 20.

OBSERVATIONS

The diploid chromosome number of *P. ovata* is 8. Chromosome length measurements were used to analyse the linear discrimination function of 2 mutant varieties, niharika and mayuri (Figs 1–3, Tables 1–5).

DISCUSSION

Chromosome measurement data were analyzed and preliminary information obtained in scatter plot matrix for 2 mutants A (niharika) and B (mayuri). Preliminarily, scatter plot matrix separated mutants A and B into 2 groups and discriminated visibly (Udoudo et al. 2021).

Mean values of chromosome length measurements suggested shorter chromosome length for B than A. Longest and smallest chromosome lengths were measured as 3.39 µm and 2.92 µm for B respectively. Similarly, 5.41 µm and 3.97 µm were measured for A in the same pattern. The difference between longest and smallest chromosome length is negligible for B than A thus suggesting not much of difference in the chromosome length of B and found similar to A. It suggested that A and B differ from each other for minute differences of chromosome length measurements (Table 1) (Herliana et al. 2023).

Covariance matrix showed similar trend of difference to some extent and partitioned them in 2 different groups of A and B, although Box's test of equality of covariance of the difference was not significant (Table 2) (Dhamnetiya et al. 2022).

Four chromosomes, chromosomes 5, 6, 7 and 8 failed for tolerance test at tolerance level of 0.001. Canonical correlation (0.998) indicated the place or location of mutant variety development of A and B (LN-1 and LM-2) were associated. Also, variance per cent was recorded

TABLE 1: Mean chromosome length measurements of mutants, LN-1 and LM-2.

Mutant	C1	C2	C3	C4	C5	C6	C7	C8
LM-2	3.26 ± 0.65	3.00 ± 0.44	3.39 ± 0.56	3.33 ± 0.54	3.39 ± 0.74	2.97 ± 0.73	2.92 ± 0.89	3.20 ± 1.12
LN-1	4.45 ± 1.79	5.41 ± 0.78	4.64 ± 1.36	4.47 ± 1.48	3.97 ± 0.78	4.40 ± 1.63	4.64 ± 1.47	5.14 ± 1.93
LM-2+LN-1	3.85 ± 1.37	4.21 ± 1.43	4.01 ± 1.15	3.90 ± 1.18	3.68 ± 0.75	3.68 ± 1.37	3.78 ± 1.44	4.17 ± 1.76

LM-2, Lucknow mayuri-2; LN-1, Lucknow niharika-1; C1–C8, Chromosomes 1–8.

TABLE 2: Covariance matrix of mutants A and B (LN-1 and LM-2).

Mutant		C1	C2	C3	C4	C5	C6	C7	C8
LM-2	C1	0.42	0.28	0.36	0.32	0.46	0.47	0.56	0.70
	C2	0.28	0.19	0.24	0.22	0.31	0.32	0.38	0.47
	C3	0.36	0.24	0.32	0.30	0.37	0.40	0.45	0.56
	C4	0.32	0.22	0.30	0.29	0.32	0.37	0.40	0.49
	C5	0.46	0.31	0.37	0.32	0.55	0.51	0.66	0.83
	C6	0.47	0.32	0.40	0.37	0.51	0.53	0.63	0.78
	C7	0.56	0.38	0.45	0.40	0.66	0.63	0.80	1.00
	C8	0.70	0.47	0.56	0.49	0.83	0.78	1.00	1.25
LN-1	C1	3.21	1.35	1.27	2.65	1.18	2.89	2.63	3.46
	C2	1.35	0.60	0.76	1.10	0.58	1.26	1.14	1.45
	C3	1.27	0.76	1.85	0.96	0.97	1.40	1.24	1.35
	C4	2.65	1.10	0.96	2.20	0.94	2.37	2.16	2.86
	C5	1.18	0.58	0.97	0.94	0.62	1.16	1.04	1.27
	C6	2.89	1.26	1.40	2.37	1.16	2.66	2.41	3.11
	C7	2.63	1.14	1.24	2.16	1.04	2.41	2.18	2.83
	C8	3.46	1.45	1.35	2.86	1.27	3.11	2.83	3.73

TABLE 3: Summary of canonical discriminant function of A and B.

Eigen Values				Wilk's Lambda			
Eigen value	Percentage of variance	Cumulative percentage	Canonical correlation	Wilk's Lambda	Chi-square	Df	Sig.
199.72	100	100	0.99	0.005	10.60	4	0.03

TABLE 4: Canonical discriminant function coefficients of mutants, A and B.

Chromosome	Unstandardized	Standardized
C1	6.09	8.21
C2	–13.08	–8.30
C3	2.36	2.47
C4	–1.55	–1.74
(Constant)	28.19	

TABLE 5: Classification of mutants, A and B based on chromosome measurement data.

Predicted group membership				
		LM-2	LN-1	Total
Original count %	LN-1	3	0	3
	LM-2	0	3	3
	LN-1	100	0	100
	LM-2	0	100	100
Cross-validated count %	LN-1	2	1	3
	LM-2	1	2	3
	LN-1	66.70	33.30	100
	LM-2	33.30	66.70	100

Fig. 1: *P. ovata* var. niharika, Somatic chromosomes.

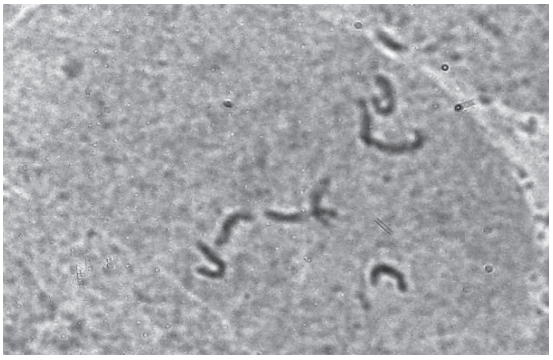

Fig. 2: *P. ovata* var. mayuri, Somatic chromosomes

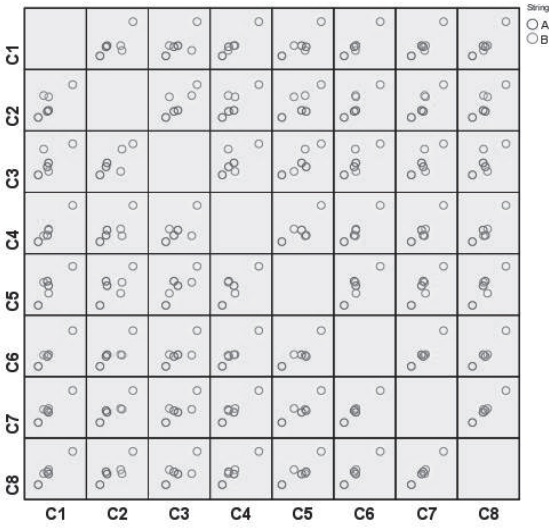

Fig. 3: Scatter plot matrix of mutant varieties A and B based on chromosome length measurements.

100% for A and B with Eigen value of 199.73. Wilk's lamda test corroborated with significant small difference between A and B mutants based on the chromo-some length measurements developed in 1998 (LN-1) and 2004 (LM-2) with chi-square (10.60) and level of significance (0.031) (Table 3) (Okoli & Eze-Golden 2023).

Fisher's linear discriminant function was recorded (28.19) based on the pooled correlation of standardized and unstandardized canonical discriminant function (Table 4). Linear discriminant function classified A and B 66.70% correctly on the basis of chromosome measurements (Table 5) (Adebiyi et al. 2022). Linear discriminant function analyses of *P. ovata* var. niharika and mayuri (mutants) differ from each other in their chromosome measurements and their time duration of mutant development collected from LN-1 and LM-2.

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RESEARCH ARTICLE

METALLOTHIONEIN EXPRESSION IN EARTHWORM *EUDRILUS EUGENIAE* EXPOSED TO HEAVY METALS AND TEMPERATURE STRESS

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SUMMARY Metallothioneins (MTs) are metal-rich proteins with low molecular weight that consist of cysteine and sulphur-based clusters. Earthworms have demonstrated the ability to manage stress by stimulating MTs responsible for maintaining metal balance and aiding in detoxification processes. In the current study, the earthworm *Eudrilus eugeniae* was exposed to zinc (Zn^{2+}) and chromium (Cr^{6+}) at various temperatures to investigate how temperature influences the stress induced by metals for 7, 14, 21, and 28 d. The exposure to Zn^{2+} spiked soils at 18°C resulted in a noteworthy modulation of MT levels, showing a percentage increase of +11.11, +113.33, and +166.66 at 7, 14, and 21 d respectively. However, at 24°C, the expression decreased until day 21 with observed percentage decrease of −38.46, −58.33, and −85.71; yet a remarkable increase of +906.66% was observed on day 28. Similarly, exposures at 28°C led to a significant exacerbation in MT expression on days 7 (+825.0%) and 21 (+150.95%), with a decrease on days 14 (−70.83%) and 28 (−08.92%). Exposure to Cr^{6+} spiked soils at 18°C resulted in an initial decrease in MT expression for the first 7 d (−66.66%); however, an increase was observed on days 14, 21, and 28 with percentage increases of +20.0, +150.0 and +19.40 respectively. When the earthworms were exposed to Cr^{6+} spiked soils at 24° C, the total MT expression decreased on day 7 (−76.92%) and 14 (−75.0%), but exhibited an exacerbated increase on days 21 (+78.57%) and 28 (+420.0%); notably, no animals survived at 28° C following exposure to Cr^{6+}, indicating severe toxic stress leading to mortality. Hence, MT expression in earthworms can be considered as a biomarker to assess metal and temperature stress.

Keywords: Metallothioneins, earthworm, biomarker, metal stress, temperature.

INTRODUCTION

Metallothionein (MT) is a ubiquitous metal-inducible protein involved in direct metal sequestration, detoxification and accumulation in eukaryotic organisms. It is considered as environmental biomarker in invertebrates and often

correlated with heavy metal pollution in the environment. MTs are low molecular weight (2–16 k Da) proteins with unique abundance of cysteine residues (>30%). They are single-chain proteins with varied amino acid number in different organisms. Almost one-third of cysteine residues occurring in conserved sequences cys-x-cys, cys-x- y-cys a cys-cys where x and y represent other amino acids. High cysteine content and identified motifs, such as C–C, C–X–C and C–X–Y–C, are required for MTs to coordinate with metal ions (Duncan & Stillman 2006). MTs exhibit the highest affinity for metals in the order Cu>Cd>Zn. Of the 18 metal ions known to be bonded by MT, only Cu^{2+}, Cd^{2+}, Pb^{2+}, Hg^{2+}, Ag^{+} and Bi^{2+} shown to be replaced by Zn^{2+} in MT structure. The main function of MTs in organism is to transport metal ion and to maintain oxidative-reducing conditions. They act either as scavenger of radicals or zinc donors for enzymes participating in repairing processes. Tolerance mechanisms to heavy metals in organisms are partly due to metal chelating gene products, which include low molecular weight proteins and MTs involved in detoxification and homeostasis. These mechanisms are differentially expressed within heavy metal-exposed populations, with transcription initiated by metal ions (Andrews 2000).

Ecosystem residents must navigate various stressors, because of human activities, pollution caused by humans, and fluctuations in seasons. Therefore, it is crucial to comprehend the combined impacts of multiple stressors such as pollutants and temperature and their underlying mechanisms to accurately predict tolerance levels and adaptations. The interactions between environmental temperature and metal pollution have been demonstrated to influence physiological tolerance towards both stressors, potentially constraining the survival and range of species. (Sokolova & Lanning 2008).

High temperatures have also been shown to increase metal availability in soils (Si et al. 2006) thereby enhancing the toxic effects of metals (Holmstrup et al. 2010) due to increase in metabolic rates (Šustr & Pizl 2010), thus enhancing the rate of metal uptake and faster metal circulation within the organism (Sokolova & Lannig 2008). Liang et al. (2011) studied the extent of MT induction upon exposure to Cd contaminated soils in different earthworm species while Calisi et al. (2011, 2013) reported changes in *Lumbricus terrestris* exposed to cadmium, copper and mercury. Localisation of MT immunoperoxidase in the major organs and tissues of the earthworm *L. rubellus* from a heavily polluted mine soil with Pb, Zn and Cd was reported by Morgan et al. (2004).

Congregating the earlier studies, evidence on induction of MT by Zn^{2+} is well established while no attempt has been made to study MT induction upon Cr^{6+} exposure coupled with temperature variations. Although much is known about the induction of MTs and its expression in variety of organisms as a stress response, it is not clearly

known, to what extent variations in habitat temperatures can influence the MT expression in earthworms exposed to heavy metals such as Zn^{2+} and Cr^{6+}. Since MTs are considered as biomarkers of heavy metal exposures, and earthworms being the most sensitive and sentinel species for ecotoxicological assessments this study was undertaken to address the interactive effects of temperature and metal toxicity and the role of habitat temperatures minimum (18° C) and maximum (28° C) in modulating MT induction in earthworm species *Eudrilus eugeniae.*

MATERIAL AND METHODS

Earthworms were collected from pollution-free areas of Bengaluru and were maintained in the laboratory in culture boxes and were used for experimentation. Ten earthworms were cultured in each plastic box and 10 replicates for each treatment were maintained. MTs were estimated spectrophotometrically by adopting the method given by Viarengo et al. (1997). Tissues were homogenised in 3 volumes of homogenization buffer (0.5 M sucrose, 20 mM Tris-HCl buffer, pH 8.6, containing protease inhibitors 6 µM leupeptine, 0.5 mM PMSF and 0.01% β-mercaptoethanol) in a tissue homogenizer. Homogenate was centrifuged at 30000 × *g* for 20 min to obtain a supernatant containing MT. To the resulting supernatant, 1.05 ml of cold (−20° C) absolute ethanol and 80 µl of chloroform were added per 1 ml and centrifuged in cold (0–4° C) at 6000 ×g for 10 min. Three volumes of cold ethanol were added to the resulting supernatant and stored at −20° C for 1h.

The samples were centrifuged at 6000 × g for 10 min, washed with ethanol-chloroform homogenization buffer (87:1:12) and dried under a nitrogen gas stream. The resuspended MT fraction was added to a solution of DTNB and phosphate buffer. The absorbance of reduced sulfhydryl concentration was measured. The amount of MT in the samples was estimated using the GSH standard, assuming 1 mol of MT contains 20 mol of cysteine.

OBSERVATIONS

Data shown in Table 1, illustrate the effect of temperature stress (18° C, 24° C, 28° C) on the modulation of the heavy metal toxicity (Zn^{2+} and Cr^{6+}) in terms of changes occurred in the level of total MTs in earthworm, *E. eugeniae* exposed for different durations i.e., 7, 14, 21 and 28 d (as per OECD guidelines).

In *E. eugeniae* the exposure of Zn^{2+} spiked soils at 18° C for 7, 14, 21 and 28 d was found to have a significant modulatory effect in augmenting the level of MTs and observed percentage increase found to be +11.11, +113.33 and +166.66 respectively, while exposures carried out at 24° C found to decrease the expression up to day 21 and the observed percentage decreases were −38.46, −58.33 and −85.71 respectively; further, an exacerbation in expression (+906.66%) was observed on day 28. When exposures made at 28° C, a similar exacerbation in MT expression was observed on days 7 (+825.0 %) and 21 (+150.95 %), while a decreased expression was evident on days 14 (−70.83 %) and 28 (− 08.92 %).

TABLE 1: The effects of heavy metal exposure (Zn^{2+} and Cr^{6+}) on MT levels in earthworm *E.eugeniae:* Influence of temperature.

Temperature	Duration of exposure (in d)	Experimental groups		
		Control	Zinc (350 ppm)	Chromium (8 ppm)
18° C	7	0.09 ± 0.02[a]	0.10 ± 0.02 (+11.11)[a]	0.03 ± 0.01(−66.66)[b]
	14	0.15 ± 0.01[a]	0.32 ± 0.01 (+113.33)[c]	0.18 ± 0.01(+20.0)[b]
	21	0.06 ± 0.0[a]	0.16 ± 0.02 (+166.66)[b]	0.15 ± 0.02(+150.0)[b]
	28	4.69 ± 0.05[a]	4.67 ± 0.03 (−0.42)[a]	5.6 ± 0.03(+19.40)[b]
24° C	7	0.13 ± 0.01[a]	0.08 ± 0.01(−38.46)[b]	0.03 ± 0.01(−76.92)[c]
	14	0.12 ± 0.01[a]	0.05 ± 0.01(−58.33)[b]	0.03 ± 0.01(−75.0)[b]
	21	0.14 ± 0.01[a]	0.02 ± 0.01(−85.71)[b]	0.25 ± 0.01(+78.57)[c]
	28	0.15 ± 0.01[a]	1.51 ± 0.14(+906.66)[c]	0.78 ± 0.08(+420.0)[b]
28° C	7	0.04 ± 0.01[a]	0.37 ± 0.02(+825.0)[b]	—
	14	0.24 ± 0.01[a]	0.07 ± 0.01(−70.83)[b]	—
	21	0.02 ± 0.01[a]	0.05 ± 0.01(+150.95)[b]	—
	28	5.6 ± 0.06[a]	5.1 ± 0.05(−8.92)[b]	—

Values are mean ± standard error of 6 replicates and represented as n moles MT/g protein. Superscript letters (a, b, c) are statistically significant ($p < 0.05$) row-wise as determined by one way ANOVA (SPSS 20.0) Duncan post hoc. Values in parenthesis indicate percentage change,'+' indicates increase and '−'indicates decrease over control.

Exposure of Cr^{6+} spiked soils at 18° C was found to decrease the MT expression initially up to 7 d (− 66.66%), while an augmentation in its expression was found on days 14 , 21 and 28 with % increase of +20.0, +150.0 and +19.40 respectively. When Cr^{6+} spiked soil exposures were carried out at 24°C, the total MT expression decreased on days 7 (−76.92 %) and 14 (−75.0 %), while an exacerbated increase noticed on days 21 (+78.57 %) and 28 (+420.0 %); surprisingly no animal survived at 28°C upon Cr^{6+} exposure indicating severe toxic stress resulting mortality.

Table 2 depicts interactive effects of temperature on metal toxicity which is interpreted by comparing the combined toxicity at 18° C and 28° C with an optimum temperature of 24° C. Exposures to low (18°C) and high (28°C) habitat temperatures showed inconsistent changes in MT expression on day 28. The observed percentage change at 18° C were found to be –30.76, +25.0, –57.14, +3026.0; while exposures at 28°C caused

TABLE 2: Interactive effects of temperature (18°C and 28°C) and heavy metal ($Zn^{2+}$and Cr^{6+}) stress on the modulation of MT expression in earthworm *E. eugeniae.*

Groups	Exposure time in days			
	7	14	21	28
Control	0.13 ± 0.01	0.12 ± 0.01	0.14 ± 0.01	0.15 ± 0.01
Temperature stress at 18° C	0.09 ± 0.02 (−30.76)	0.15 ± 0.01 (+25.0)	0.06 ± 0.0 (−57.14)	4.69 ± 0.05 (+3026.0)
Temperature stress at 28° C	0.04 ± 0.01 (−69.23)	0.24 ± 0.01 (+100.0)	0.02 ± 0.01 (−85.71)	5.6 ± 0.06 (+3633.3)
Zn^{2+} toxicity	0.08 ± 0.01	0.05 ± 0.01	0.02 ± 0.01	1.51 ± 0.14
Zn^{2+} toxicity at 18° C	0.10 ± 0.02 (+25.0)	0.32 ± 0.01 (+540.0)	0.16 ± 0.02 (+700.0)	4.67 ± 0.03 (+209.27)
Zn^{2+} toxicity at 28° C	0.37 ± 0.02 (+362.5)	0.07 ± 0.01 (−40.0)	0.05 ±0.01 (+150.0)	5.1 ± 0.05 (+237.7)
Cr^{6+} toxicity	0.03 ± 0.01	0.03 ± 0.01	0.25 ± 0.01	0.78 ± 0.08
Cr^{6+} toxicity at 18° C	0.03 ± 0.01 (0)	0.18 ± 0.01 (+500.0)	0.15 ± 0.02 (−40.0)	5.6 ± 0.03 (+617.94)
Cr^{6+} toxicity at 28° C	—	—	—	—

Values are mean ± standard error of 6 replicates and represented as n moles MT/g protein. Superscript alphabets (a, b, c) are statistically significant ($p < 0.05$) column-wise as determined by one way ANOVA (SPSS 20.0) Duncan post hoc. Values in parenthesis indicate percentage change, (metal toxicity studied at within (18°C) and above habitat temperature (28°C) compared with toxicity at optimal soil temperature (24°C)), '+' indicates increase and '−'indicates decrease over control.

−69.23 %, +100.0 %, −85.71 %, +3633.3 % changes on days 7, 14, 21 and 28 respectively. Zn^{2+} exposure at 18° C found to cause an exacerbation in MT expression on days 14 (+540.0 %), 21 (+700.0 %) and 28 (+209.27 %) while marginal increase was observed on day 7 (+25.0 %). Likewise, exposures made at 28° C exhibited an augmentation in MT expression on days 7, 21 and 28 and the observed percentage change was found to be +362.5, +150.0 and +237.7 respectively; while a decrease in MT expression was evident on day 14 (−40.0 %).

When Cr^{6+} co-exposures carried out at 18° C showed exacerbation in MT expression on days 14 (+500.0 %) and 28 (+617.94 %) and a decrease in its expression was evident on day 21 (−40.0 %).

DISCUSSION

MTs in earthworms aid in regulating and detoxifying metallic and non-essential metal ions, serving as biomarkers for metal tolerance, stress, and pollution in environmental studies (Aemere et al. 2020). The most important inducible protein in earthworms during toxic

stress is MT, which plays a role in scavenging free radicals in the cell, regulating tissue-specific development (Chan 2004), protecting DNA from pollutant damage (Fourie et al. 2007) and metal sequestration. MTs are ubiquitous in eukaryotic organisms and constitute a superfamily of low-molecular weight MTs rich in cysteines (25–30% of the total protein weight) but lacking aromatic or histidine residues (Kägi & Kojima 1987).

In this study, in *E. eugeniae* exposure to temperature alone at both 18° C and 28° C found to augment the MT levels; while exposure of both metals, Zn^{2+} and Cr^{6+} also expressed metal-specific, time and temperature-dependent responses. Likewise, an increase in the expression of MTs was evident upon co-exposures of both metals, Zn^{2+} and Cr^{6+} at 18° C. Contrarily, at 24° C co-exposures showed suppression in MT expression wherein an exacerbation in expression was evident on day 28; while at 28°C sporadic changes were evident in MT expression.

MT act not only on metal stores, but also as antioxidants (Viarengo et al. 1998). In this context, the levels of MT in earthworms exposed to both Zn^{2+} and Cr^{6+} spiked soils at varied temperatures should account for the absence of responses in the enzymatic antioxidants after the chronic (28 d) exposure to both toxic metals individually.

Thomas et al. (1986) postulated that thiolate clusters of Zn-MT could intercept hydroxyl radical, superoxide anion and initiators of LPO process, emphasizing again the importance of MT as an antioxidant. It is important to note that short duration (7, 14 d) exposures was not enough to change the MT expression significantly, however, co-exposure with within 18° C and above 28° C habitat temperatures was found to have synergistic effect in modulating higher toxicity.

These MT proteins are potential biomarkers for assessing metallic pollution in soil, as demonstrated by the significant induction of MT in *E. fetida* caused by Cd exposure. Similarly, results obtained in the present study suggest that high exposures to metals perhaps lead to a significant increase in the level of MT expression in earthworm cytosolic fractions that could also be considered as a detrimental effect as it represents an increase of metabolic cost. However, increased MT content is likely to play an important regulatory role in Zn homeostasis as outlined by Vašák (2005) thereby the present findings corroborate with those of Vasak (2005). Furthermore, earthworms are shown to accumulate high concentration of heavy metals within their tissues. Laura et al. (2019) reported that MT expression increases in earthworms like *Eisenia andrei* under copper exposure, indicating its role as a stress biomarker and in detoxification processes against environmental stressors. New insights are given into the mechanisms of sub-lethal toxicity of copper as an environmental pollutant and in the identification of novel sub-lethal biomarkers of cellular response to the stressor such as immune response genes. MT gene expression in

earthworms is metal concentration-dependent, stable over time, and species-independent, making it a suitable biomarker for metals (Elmer et al. 2022).

It has been shown that the excess of heavy metals is dealt by at least 2 co-existing intracellular pathways (Morgan & Morgan 1993), the first pathway involves the retention of metals like lead and zinc in granules of insoluble calcium phosphate and the chloragosomes (Morgan & Morris 1982). The second pathway is characterised by the ability of sulphur donating ligands to bind metals like cadmium within vacuoles or cadmosomes (Morgan & Morgan 1993). The chloragosomes and the cadmosome though functionally and morphologically are different thought to be lysosome homologues, representing different lysosomal sub-populations (Morgan & Morgan 1991). In the present study, involvement of such chloragosomes in accumulating Cr which in turn builds up metal stores and related production of exposure free radicals thereby resulting in excess MT production. However, induction appears to be specific and metal-dependent (Thiele 1992), it is also possible that MT induction process is not identical to both metals studied. The data generated from this study allow us to identify differences in response to Zn^{2+} and Cr^{6+} exposures. These differences suggest the existence of adaptation mechanisms in earthworm *E. eugeniae* to contaminated soils.

Exposure of earthworms to metal and temperature stress influences MT expression differently. MT expression in earthworms is strongly metal concentration-dependent, making it a suitable biomarker for metals. However, further research is needed to understand the response of MT gene expression in earthworms. Additionally, MT expression in earthworms from contaminated sites remains consistently high, suggesting a potential loss of inducibility due to adaptation to metal exposure. Hence, MT expression in earthworms is intricately influenced by the type of metal exposure and temperature conditions, highlighting the complexity of their adaptive responses to environmental stressors.

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REVIEW ARTICLE

GENETIC MUTATION SPECTRUM OF GAUCHER DISEASE IN INDIAN POPULATION

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SUMMARY Gaucher disease (GD) is a rare genetic disease with an overall lower prevalence in the Indian population but GD type I has ~1/40,000 and type II and III have <1/50,000 to <1/100,000 incidence among live-births. But when it affects a family member, the lifelong expensive treatment, if feasible, leads to major disturbance and upheaval. GD requires two mutant alleles, one from the father and one from the mother such that the child inherits both mutated alleles, therefore, no copy of a functional allele (so called normal) is present in the child. As the disease is recessive, mostly parents are carriers of the mutant allele but they do not manifest the disease, hence, they remain unaware of their carrier status. Investigations are conducted only when a child is born with the disease. At that stage the only options available are enzyme replacement therapy (ERT) or substrate reduction therapy (SRT) which are expensive and lifelong treatments, not easily affordable by all parents. This results in enormous stress and trauma to the family when children are born with Gaucher disease. Prevention of this situation is possible with increased awareness in the population about the disease, the screening of the mutation/ carrier status of individuals (such that marriage of two carriers is avoided or at least they are aware of the future possibilities) and availability of genetic counselling. This necessitates a well-maintained, regularly updated database of mutations identified in the Indian population. This article attempts to collate all the mutation information present in the published literature from India, updated till date and deduces the frequency of these mutations in the Indian GD cases, along with a general and detailed description about GD to enhance understanding among the readers.

Keywords: Gaucher disease, mutation, Indian population, *GBA1* gene, lysosomal storage disease.

INTRODUCTION

The name Gaucher in Gaucher disease comes from the clinician, Philippe Charles Earnest Gaucher, who first observed and described these disease symptoms (Sheth et al. 2019). Gaucher disease (GD) is a rare genetic disease with a prevalence of 1 in 50,000 worldwide (~1 in 900 among Ashkenazi Jews). Its impact on life ranges

from severe abnormalities among infants, children, including death in utero, to asymptomatic adults even at the later stage of life. GD is a type of more common lysosomal storage disease. Lysosomal storage diseases or commonly known as LSDs (prevalence 1 in 5000 live-births worldwide) are mostly caused by the non-functional lysosomal enzymes which are unable to catalyze their specific substrates, hence, leading to substrate accumulation in the cell. Approximately 50 different types of LSDs have been identified and GD is one of them (Dandana et al. 2016).

Clinical symptoms

Clinically, GD is identified by the presence of enlarged spleen (splenomegaly), enlarged liver (hepatomegaly), haematological abnormalities like anaemia, thrombocytopenia, skeletal disease (bone fractures) which may or may not involve neuropathology (Dandana et al. 2016). The hallmark feature includes Gaucher cells, which are macrophages loaded with lipid due to the deficiency of lysosomal enzyme which catalyzes these lipid molecules. Primarily, GD can be classified as Neuronopathic and Nonneuronopathic but based on the presence of clinical symptoms it has been categorized classically into 3 types (Table 1).

Recent observations envisage GD as a complex of phenotypes, as all the 3 types are not completely mutually exclusive. Its diagnosis needs critical attention as it may get easily misdiagnosed as haematological malignancy, immune thrombocytopenic purpura or as liver disease (Mistry et al. 2017).

Pathology

Lysosomal enzyme, beta-glucocerebrosidase (also k/a beta-glucosidase) catalyzes conversion of glycolipid glucocerebroside to glucose and ceremide. Glycolipids glucocerebroside and glucosyl sphingosine are the common components of cell membranes, which get released when such cells are disintegrated. These glycolipids are then catalyzed by the glucosidase enzyme into their metabolites. In GD, the glucosidase enzyme becomes nonfunctional, thereby blocking the conversion process and leading to substrate accumulation within macrophages. These macro-

TABLE 1: The classification of Gaucher disease with onset age and features.

Disease type	Neuronopathy	Onset	Feature
GD type 1	Non-neuronopathic	Young onset	Most common type
GD type 2	Acute neuronopathic	Early onset	Severe type with survival not beyond two years of age
GD type 3	Chronic neuronopathic	Early to late onset	Slow progress, comparatively milder

phages that have abundant cytoplasm with hydrophobic glucocerebroside in bilayered membranous sheets, give a look of crumpled paper, therefore, referred to as Gaucher cells, the typical feature of this disease. Progressive accumulation and infiltration of these Gaucher cells into different organs like liver, bone marrow and lymph nodes lead to heterogeneous phenotypes (Stirnemann et al. 2017).

Genetic characterization

Normally, conforming to purely Mendelian autosomal recessive inheritance, GD is now envisaged as a complex monogenic disease. Beta-glucocerebrosidase enzyme or glucosidase (GBA; EC 3.2.1.45) is encoded by human gene *GBA 1*, located on chromosome 1p22. The gene comprises 11 exons (Horowitz et al. 1989) which was later corrected to 12 exons, the misperception was due to the presence of 2 start sites in this gene. Human *GBA* gene carries 2 potential initiator sites- ATG codons, with upstream ATG producing 39 amino acid signal peptide and downstream ATG producing 19 amino acid signal peptide (Sorge et al. 1987). Mouse *gba* gene located on chromosome 3 shares 86% identity but it has single ATG start codon (O'Neill et al. 1989). The pseudogene (GBAP1) is located 16 kb downstream (Sorge et al. 1990) with 96% exonic homology (Reiner & Horowitz 1988) and 2 kb shorter than the functional *GBA1* gene (Tayebi et al. 1996). *GBAP* with its own promoter (Reiner & Horowitz 1988) gets transcribed almost similar to *GBA1*. Therefore, the screening of mutations in GD patients can easily be flawed by sequence changes of *GBAP*, as both sequences at the genomic level show identity (Sorge et al. 1990, Tayebi et al. 1996). Mutation identifying strategies therefore, need to be highly specific for *GBA1* to exclude *GBAP1*. Moreover, the identification of recombinant alleles between these 2 genes shows certain type of phenotypic heterogeneity (Tayebi et al. 2003). Comparable amounts of the active gene derived mRNA and pseudogene derived mRNA increases this confusion further. Screening therefore, must exclude the sequence changes of *GBAP* as the cause for GD. Gene conversions and recombination between *GBA1* and *GBAP* could be the source of the disease-causing mutations somewhat similar to *CYP21* gene.

Investigations of mutation spectrum in India

GD is caused by the presence of mutations in *GBA1* gene but the clinical heterogeneity indicates monogenic complex disease pathology. To collect the information about various mutations predominant among Indian cases, all the research articles were screened with the following methodology:

A search was conducted in the NCBI-National Library of Medicine (NLM) with diverse keywords in order to collect all the articles published on the genetics of Gaucher disease till date. Articles were selected on the basis of complete diagnosis and mutation detection methodology along with a clear description of the number of patients (with or without mutation) and healthy

individuals screened for the same mutation(s) along with those which were picked through back references of the selected articles. Majority of articles mentioned 2-stage strategies for screening the genetic mutations. After the complete clinical and biochemical diagnosis of the clear cases of GD, at the first stage the screening of the most predominant mutation (L483P) was conducted in the patients and at the second stage complete *GBA1* gene sequencing was conducted to detect any other known or novel mutation(s) among the affected. In some of the cases where proband genetic profiles were not clear, the parents' samples were analyzed to reach conclusion about the pathology. The reader must also note that some earlier articles used the old nomenclature for reporting mutations whereas recent articles used the new nomenclature therefore, Table 2 describes common mutation in old and new nomenclatures to avoid confusion. The present article also uses the new nomenclature.

TABLE 2: Old and new nomenclatures of selected GBA1 gene mutations.

S. No.	Old nomenclature	New nomenclature
1	Leu444Pro	Leu483Pro
2	Ala448Thr	Ala487Thr
3	S237F	S276F
4	R463C	R502C
5	R496C	R535C
6	R48W/G344D/R120W	R87W/G383D/R159W
7	N188S/R257Q	N227S/R296Q

Present article uses new nomenclature to describe mutations in the text. S: Serine, F: Phenylalanine, R: Arginine, C: Cysteine, W: Tryptophan, G: Glycine, D: Aspartic acid, N: Asparagine, Q: Glutamine.

RESULTS AND DISCUSSION

Each article published data on mixed cases, some with selected mutation screening and some with the earlier mentioned 2-stage strategy. Mutations were observed in a total of approximately 193 cases (all studies combined) and these mutations were also screened among healthy individuals (n = 1260, only from 2 studies) to exclude its chances of being a rare variant or polymorphism. Among 36 (n = 36) genetic mutations, Leu483Pro was found to be the most abundant with an observed frequency of approximately 0.51 which is in line with its abundance worldwide, therefore, it is also referred to as a Pan ethnic mutation (presence in variable populations of the world) (Bisariya et al. 2011, Verma et al. 2012, Ankleshwari et al. 2014, Muranjan & Patil 2016, Sheth et al. 2018, 2019, Singh et al. 2020). The second most frequent mutation observed was Ala487Thr with a frequency of 0.03 among Indian GD patients (Sheth et al. 2018, 2019) but comparatively much less than L483P, followed by S276F (Bisariya et al. 2011, Verma et al. 2012, Sheth et al. 2019), R502C (Bisariya et al. 2011, Ankleshwari et al. 2014, Sheth et al. 2019) and R535C (Verma et al. 2012, Ankleshwari et al. 2014, Sheth et al. 2019) each with a frequency of 0.02. In addition to these 5 comparatively frequent genetic mutations, 31 (n = 31) mutations were observed, with each one present in a single patient either in homozygous condition or compound heterozygous state. Two complex mutations which were reported among Indians include Rec/Nci I with R87W/G383D/R159W in

compound heterozygous condition and complex C with N227S/R296Q in 7 and 3 cases, respectively (Chauhan et al. 2013, Sheth et al. 2019). Data regarding mutations has been collated in this article from various published research articles (mentioned in the methodology) hence data sharing is not applicable to this article, but the collated data is available from the corresponding author upon request.

The larger diversity in the mutation type observed in the Indian patients could be attributed to the heterogeneous nature of the Indian population with some communities practicing consanguineous marriages. The chances of a recessive disease manifestation increase drastically in consanguineous marriages, as it increases the frequency of particular allele(s) in that population. As per global current literature, more than 300 mutations in *GBA1* gene have been identified but in Indian GD cases only 36 genetic mutations have been reported either in simple or complex combinations. This further indicates less diversity of *GBA1* mutations in this population which makes screening programme much easier and efficient.

CONCLUSION

Among the GD cases from India, Leu483Pro was found mostly in the homozygous state with few cases of compound heterozygotes and just 2 cases in heterozygous state. Other frequent mutations also existed mostly in the compound heterozygous state or homozygous state indicating the recessive nature of the manifested disease. All the remaining mutations, also share similar status of either compound heterozygote or homozygous. This information necessitates the screening programme for GD to avoid future trauma to any family. Although Leu483Pro is most abundant but other diverse mutations observed throughout the cases necessitate screening for the complete *GBA1* gene. GD is one of the rare genetic diseases that has a tremendous impact on the life of the affected member as well as the whole family. A screening programme for the at-risk population along with offering more affordable treatments should be in the pipeline for Indian scientists and clinicians working in the field of rare genetic diseases like GD.

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RESEARCH ARTICLE

GC-MS ANALYSIS AND ANTIOXIDANT ACTIVITY OF ROOT EXTRACT OF *GYMNOSTACHYUM FEBRIFUGUM* BENTH.

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SUMMARY The present study was to examine the phytochemical makeup and antioxidant capacity of the root extracts from *Gymnostachyum febrifugum.* Phytochemical screening of chloroform, water and dimethyl sulfoxide (DMSO) extracts revealed the presence of different bioactive substances. Sixtysix phytocompounds were found in the chloroform root extract by gas chromatography-mass spectroscopy (GC-MS) analysis. The main phytoconstituents were, lupeol, octadecanoic acid ethyl ester, hexacosanol, 1-decanol, 2-hexyl- and pentafluoropropionic acid octadecyl ester. Using the DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging test, the antioxidant activity was assessed. Significant antioxidant activity was shown by both the aqueous and DMSO extracts at 6.25 µg/ml and 12.5 µg/ml, respectively. The DMSO extract showed 6.95% and 14.88% radical scavenging activity (RSA). Interestingly, at comparable doses, these values outperformed the action of the standard antioxidant curcumin. At 6.25, 12.5 and 25 µg/ml, the aqueous extract showed 6.12%, 16.41% and 21.00% RSA respectively. The aforementioned findings offer empirical support for the customary therapeutic use of *G. febrifugum* and underscore its capacity as a natural antioxidant source.

Keywords: *Gymnostachyum febrifugum*, GC-MS, phytochemical screening, antioxidant, DPPH assay**.**

INTRODUCTION

A plant is considered as medicinal, if its stem, roots, leaves, seeds, bark, or any other part of it is utilized for therapeutic, tonic, purgative, or other health-promoting purposes. Traditionally and ethnomedically, medicinal plants have been used since time immemorial to heal their illness based on instinct, taste and experience. They have major source of pharmacological compounds. Present research activities in the area of ethnomedicines have increased tremendously. Indian medicine has long been employed *Gymnostachyum febrifugum* as an indigenous plant, to treat fever, mouth ulcers, cough, stomatitis, and menorrhagia and digestive disorders by local people of Dakshina Kannada district of Karnataka

(Vijayalakshmi et al. 2023). This plant has febrifugal properties and is also used as an antidote for viper bites (Mathew et al. 2024). *G. febrifugum* belonging to family Acanthaceae is an endemic species found in the Western Ghats commonly called *agrada gida* or *nelamuchala*, is a small, nearly stemless herb. The plant usually grows near the banks of small running water streams. The present work was carried out to identify the phytochemical constituents through GC-MS analysis and radical scavenging activity (RSA) in different root extracts of *G. febrifugum*.

MATERIAL AND METHODS

Root samples of *G. febrifugum* were collected from Sonandoor village, Dakshina Kannada district. Collected samples were washed thoroughly with tap water, shade dried and powdered using blender and stored in air tight container. 20 g root powder was added to 200 ml chloroform and kept in water bath for 4 h at 50^0 C. The extract was then filtered using Whatman No. 1 filter paper and stored in cuvette. Qualitative phytochemical screening tests of the root extract were performed according to the standard protocols for detecting the presence of various phytochemicals (Harborne 1998, Khandelwal 2008).

G. febrifugum chloroform root extract was subjected to gas chromatography-mass spectrometry (GC-MS). Clarus 680 GC was used in the analysis, employed a fused silica column, packed with Elite-5MS (5% biphenyl 95% dimethylpolysiloxane, 30 m × 0.25 mm ID × 250 µm df) and the components were separated using helium as carrier gas at a constant flow of 1 ml/min. The injector temperature was set at 260° C during the chromatographic run. 1µL of extract sample was injected into the instrument. The oven temperature was as follows: 60° C (2 min) followed by 300° C at the rate of 10° C min^{-1} and 300° C for 6 min. The mass detector conditions were, transfer line temperature 230° C, ion source temperature 230° C, and ionization mode electron impact at 70 eV, a scan time 0.2 sec and scan interval of 0.1 sec and the fragments from 40 to 600 Da. The spectra of the components were compared with the database of spectra of known components stored in the GC-MS NIST (2008) library.

OBSERVATIONS

Qualitative phytochemical analysis of the root extract with 3 different solvents, polar (dimethyl sulfoxide) (DMSO), mid-polar (water) and non-polar (chloroform) solvents revealed the presence of various phytoconstituents. All are secondary metabolites except carbohydrates (Table 1).

The chloroform root extract of *G. febrifugum* was subjected to GC-MS analysis. A total of 66 possible hits were found, of which 6 hits with 5 major phytocompounds were seen namely, lupeol, octadecanoic acid ethyl ester, hexacosanol, 1-decanol, 2-hexyl- and pentafluoropropionic acid octadecyl ester (Table 2, Fig. 1). The identification of phytochemical compounds were confirmed on the basis of retention time,

TABLE 1: Qualitative phytochemical screening of *G. febrifugum* root extract.

Phytochemical constituent	Chloroform	Water	DMSO
Carbohydrates	++	+	+
Alkoloid	+	+	-
Flavonoids	-	++	++
Phenolics	+	++	++
Saponins	-	+	+
Tannins	-	-	-
Volatile oils	-	-	+
Nonvolatile oils	-	+	+
Gums and mucilage	-	+	-

Presence (+) or absence (-) in different extracts.

peak area, molecular weight and formula. Phytoconstituents obtained through GC-MS was interpreted by using the database of GC-MS NIST (2008) library. GC-MS chromatogram of the chloroform root extract along with retention time is shown in Fig. 1.

DPPH free RSA was determined spectrophotometrically following the protocol of Blois (1958) and as reported by Kumar et al. (2014). 0.2 mM of DPPH solution was mixed with various concentrations of the sample (1–5 mg/ml), vortexed and incubated for 30 min at 37° C in dark. Curcumin is used as a standard absorbance and was read by using a double beam UV-visible spectrophotometer. Methanol with no samples was taken as control. RSA percentage was calculated using the following formula:

$$\% \text{ RSA} = (A_{control} - A_{sample} / A_{control}) \times 100$$

Where A = absorbance at 517 am.

DPPH is a fast, reliable, simple and most preferred method employed for evaluating the antioxidant potential of a wide array of test samples. DPPH is a stable free radical since the delocalisation of spare electrons occurs over the entire molecule. Proton donation by the antioxidants present in test samples to the DPPH radicals reduces deep violet colour to colourless in the reaction mixture. (Irshad et al. 2012, Zhou & Yu 2004). Tables 3–5 highlight the percentage of RSA exhibited by standard curcumin, aqueous root extract and DMSO root extract of *G. febrifugum* at various concentrations of extracts.

In vitro antioxidant assay was carried out on the root extract of *G. febrifugum* using DPPH. The percentage of RSA obtained by curcumin, aqueous and DMSO root extracts of *G. febrifugum* is given in Tables 3–5. The RSA of standard curcumin, DMSO root extract and aqueous root extract of *G. febrifugum* is represented graphically in Figs 2–4.

TABLE 3: Percentage of RSA exhibited by standard curcumin.

Concentration (µg/ml)	Blank	Absorbance	RSA (%)	IC-50
3.12	0.71	0.70	2.50	3.36
6.25	0.71	0.69	3.33	7.58
12.5	0.71	0.63	12.10	16.04
25	0.71	0.56	20.86	32.95
50	0.71	0.46	35.60	66.78
100	0.71	0.18	74.96	134.43

TABLE 2: 2D structures of 5 major compounds from *G. febrifugum* chloroform root extract.

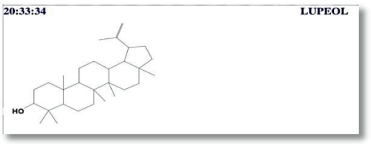
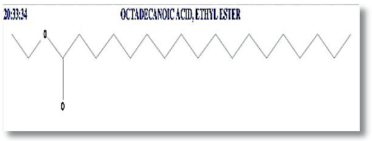
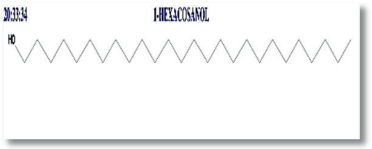
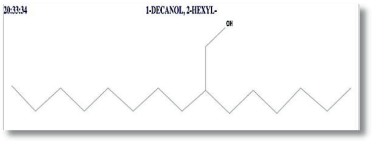
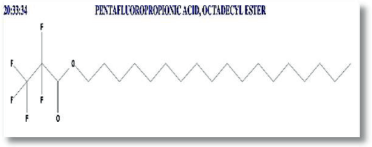
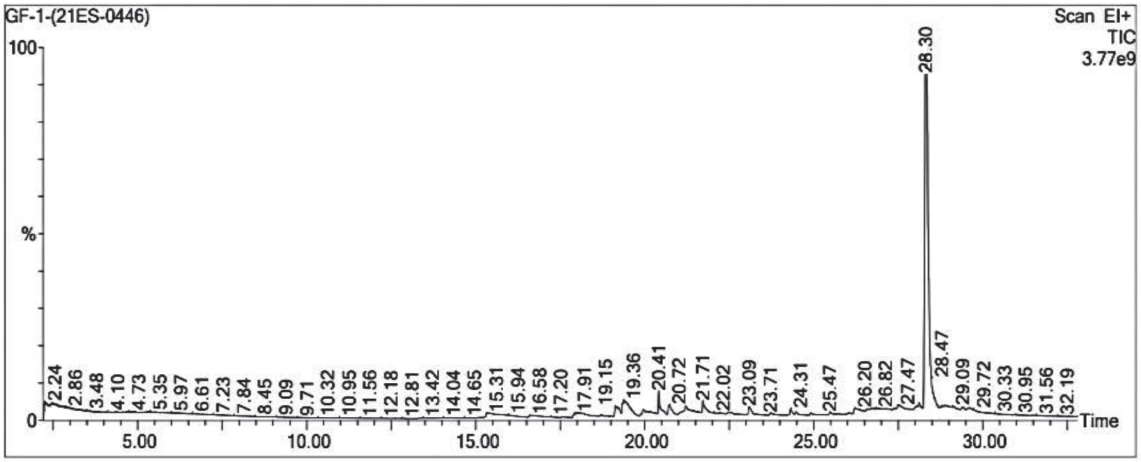
Compound	Retention time	Molecular weight	Molecular formula	Structure
LUPEOL	28.31	426	$C_{30}H_{50}O$	
OCTADECANOIC ACID, ETHYL ESTER	19.38	312	$C_{20}H_{40}O_2$	
1-HEXACOSANOL	19.12	382	$C_{26}H_{54}O$	
1-DECANOL, 2-HEXYL-	21.71	242	$C_{16}H_{34}O$	
PENTAFLUOROPROPIONIC ACID, OCTADECYL ESTER	20.41	416	$C_{21}H_{37}O_2F$	

TABLE 4: Percentage of RSA exhibited by aqueous root extract of *G. febrifugum.*

Concentration (µg/ml)	Blank	Absorbance	RSA (%)	IC-50
3.12	0.71	0.71	1.11	0.12
6.25	0.71	0.67	6.11	5.08
12.5	0.71	0.60	16.41	14.99
25	0.71	0.56	21.00	34.81
50	0.71	0.49	30.87	74.46
100	0.71	0.23	66.89	153.75

TABLE 5: Percentage of RSA exhibited by DMSO root extract of *G. febrifugum.*

Concentration (µg/ml)	Blank	Absorbance	RSA (%)	IC-50
3.12	0.71	0.71	0.27	0.05
6.25	0.71	0.66	6.95	6.13
12.5	0.71	0.61	14.88	18.29
25	0.71	0.58	19.33	42.62
50	0.71	0.56	21.69	91.28
100	0.71	0.31	56.60	188.59



#	RT	Scan	Height	Area	Area %	Norm %
1	19.125	3384	105,136,944	15,368,335.0	3.122	3.81
2	19.385	3436	155,543,008	38,803,104.0	7.882	9.63
3	20.411	3641	234,857,312	13,063,831.0	2.654	3.24
4	20.706	3700	92,900,744	11,496,792.0	2.335	2.85
5	21.711	3901	125,631,264	10,740,311.0	2.182	2.67
6	28.314	5221	3,567,260,160	402,850,208.0	81.826	100.00

Fig. 1: GC-MS chromatogram details of chloroform extract of *G. febrifugum.*

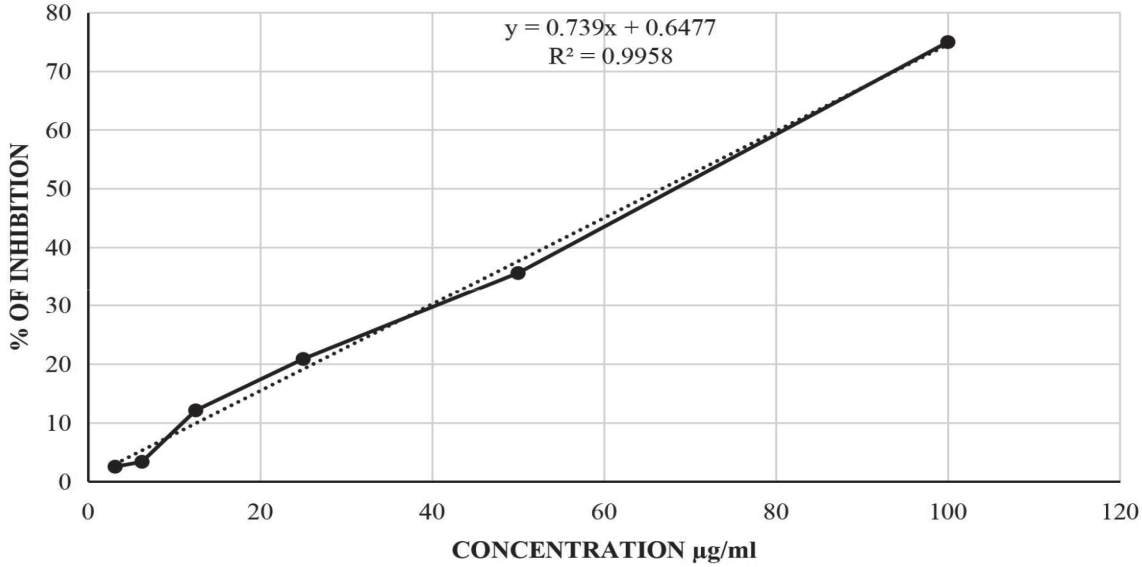


Fig. 2: *G. febrifugum*. Graph showing standard curcumin radical scavenging activity.

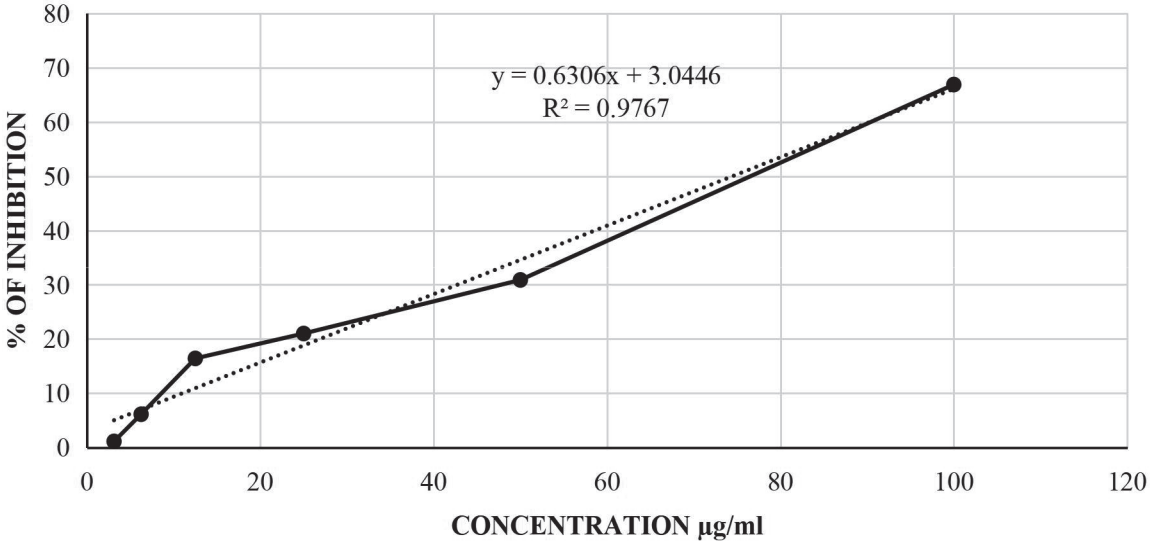


Fig. 3: *G. febrifugum*. Graph showing aqueous extract radical scavenging activity.

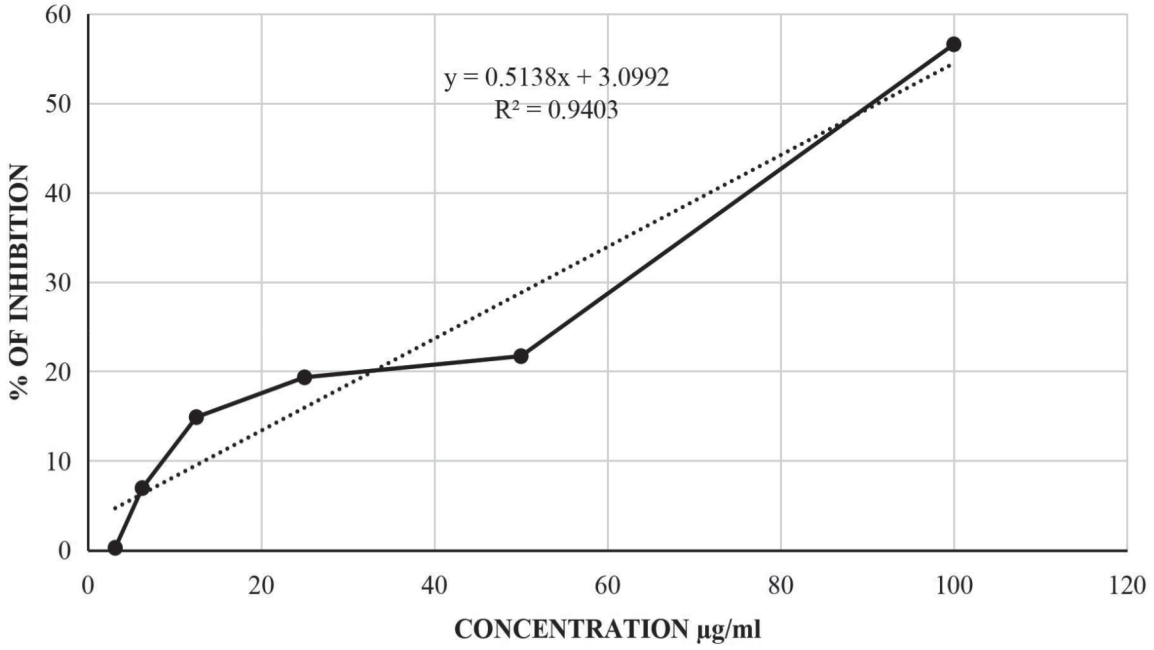


Fig. 4: *G. febrifugum*. Graph showing DMSO extract radical scavenging activity.

DISCUSSION

Arunachalam & Parimelazhagan (2011) conducted various antioxidant assays using methanolic root extract of *G. febrifugum.* They compared scavenging ability of the root extract on hydroxyl with ascorbic acid and the results revealed that the extract exhibited more pronounced hydroxyl RSA as compared to ascorbic acid in a dose dependent manner. While the scavenging ability of methanolic extract of *G. febrifugum* of root on DPPH radical compared with standard vitamin E. The IC-50 value of the extract and vitamin was 206.74 and 295.15 µg/ml respectively.

During the present investigation, the better effect of root extract on DPPH radical scavenging is thought due to better hydrogen donating ability indicating *G. febrifugum* exhibiting a powerful antioxidant activity against in vitro oxidative systems. The obtained results might be considered sufficient to further studies for isolation and identification of active principles. Nair et al. (2016) carried out free RSA of methanolic leaf extract of *G. warrieranum*. Their findings revealed that there is a significant increase in the antioxidant activity due to scavenging ability of extracts. The IC-50 values were found to be 60 µg/ml and 80 µg/ml for ascorbic acid and *G.warrieranum* respectively.

The current study on *G. febrifugum* has provided new information on the phytochemical makeup and antioxidant capacity of this plant. A wide range of bioactive chemicals were identified by GC-MS analysis of the chloroform root extract, with lupeol, octa-decanoic acid ethyl ester, hexacosanol, 1-decanol, 2-hexyl- and pentafluoropropionic acid octadecyl ester being the main components. These results are especially significant since they give *G. febrifugum's* traditional medical usage for treating fever, oral ulcers, and digestive disorders, a solid scientific foundation.

Due to its purported antiinflammatory, antimicrobial, and anticancer characteristics, the presence of lupeol, a pentacyclic triterpenoid, is particularly interesting (Gallo & Sarachine 2009). This substance may have a major role in the plant's therapeutic properties, lending credence to the colloquial term "Agrada gida" or fever plant. Likewise, the discovery of octadecanoic acid ethyl ester, which is recognised for its antibacterial properties, is consistent with the plant's customary application in the management of oral ulcers (Agoramoorthy et al. 2007).

Both the aqueous and DMSO extracts of *G. febrifugum* roots showed encouraging free RSA in our antioxidant tests that used the DPPH technique. Notably, both extracts showed stronger radical scavenging efficacy than the conventional curcumin at lower doses of 6.25 µg/ml and 12.5 µg/ml (Tables 3–5). The extract's strong antioxidant potential may be due to the combined actions of several phytochemicals, such as flavonoids and phenolics, as shown by our first phytochemical screening.

Its potential as a natural antioxidant source is highlighted by the fact that *G. febrifugum*

extracts have been shown to have antioxidant activity that is comparable with that of several other medicinal plants. This characteristic not only supports its long-standing usage but also creates opportunities for its use in contemporary nutraceuticals and therapies. It is noteworthy that our results on *G. febrifugum's* antioxidant capacity complement those of Arunachalam & Parimelazhagan's (2011). Although they found strong antioxidant activity in methanolic extracts, the present study shows that aqueous and DMSO extracts are equally effective in scavenging free radicals. This implies that *G. febrifugum's* bioactive components that provide antioxidant activity may be extracted using a variety of solvents, boosting its potential for diverse applications.

This study provides comprehensive evidence supporting the traditional medicinal uses *G. febrifugum*. The identification of bioactive compounds through GC-MS analysis and the demonstration of potent antioxidant activity underscore the therapeutic potential of this endemic plant. Our findings not only validate the ethnomedicinal knowledge associated with *G. febrifugum* but also pave the way for its potential use in modern medicine and the nutraceutical industry.

The superior antioxidant activity of *G. febrifugum* extracts, even surpassing that of curcumin at certain concentrations, warrants further investigation into its mechanisms of action and potential applications. Future research should focus on isolating and characterizing the individual bioactive compounds responsible for these properties and evaluating their specific pharmacological activities. Moreover, given the endemic nature of *G. febrifugum* and its significant medicinal properties, there is an urgent need for conservation efforts to ensure the sustainable utilization of this valuable plant resource. This study thus not only contributes to the scientific understanding of *G. febrifugum* but also emphasizes the importance of preserving the rich biodiversity of the Western Ghats, a treasure trove of medicinal plants.

In the light of these findings, we recommend further in vivo studies to fully elucidate the therapeutic potential of *G. febrifugum* and explore its possible integration into modern healthcare systems. This research serves as a stepping stone towards the development of novel, plant-based therapeutics, aligning traditional knowledge with modern scientific validation.

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Declarations

Consent to participate

We hereby certify that we have participated sufficiently in the analysis and interpretation of the data as well as the writing of the manuscript, to take public responsibility for it and have agreed to have our name listed as contributors.

Authors' contribution

PKS and NSS were involved in the collection of material, experimentation and data collection. Overall supervision of the study as well as drafting and correction of the manuscript was done by LR.

Conflict of interest

The authors declare no conflict of interest.

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