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Insights into the antibacterial and anti-inflammatory potential of *Marchantia polymorpha* L.

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Marchantia polymorpha, a liverwort of the Marchantiaceae family, has been used in traditional medicine to treat inflammation, fever, pustules, diuresis and skin-related disorders. However, the scientific evaluation and validation of these traditional practices are needed to improve human health care. Among different solvent extracts employed for the analysis of antioxidant, antibacterial and anti-inflammatory potential of *M. polymorpha*, methanol extract exhibited the highest ferric reducing antioxidant potential, i.e. 122.35±0.57 (µM $FeSO_4$ E) and antibacterial potential as the growth of both gram-positive (*S. aureus*, *B. subtilis* and *L monocytogenes*) and gram-negative (*E. coli*, *K. pneumoniae* and *P. mirabilis*) bacterial cultures was inhibited. More specifically, methanol extract inhibited pro-inflammatory mediators like nitric oxide (NO) in a dose-dependent manner and inflammatory cytokines, i.e. Tumour necrosis factor (TNF-α) and interleukins IL-6 and IL-1β in LPS-induced RAW 264.7 macrophage cell line. Both LC-MS and GC-MS analysis of methanolic extract revealed the presence of numerous phytochemicals responsible for its antibacterial and anti-inflammatory potential, such as Pentadecanal, 3-β-Hydroxy-5-cholen-24-oicacid, Stigmasterol, Phytol, n-Hexadecanoic acid, Ergosterol, Caryophyllene, some important bis-bibenzyls like Marchantin A, Marchantin M, Riccardin D, Plagiochin E and some flavonoids like Rutin, Kaempferol 3-O-glucosyl-rhamnosyl-glucoside and Luteolin 7-O-diglucuronide etc while FTIR characterisation revealed the presence of functional groups like alkenes, alkynes, alcohols, phenols and aromatic compounds etc. Therefore, this study recommends further exploration and development of *M. polymorpha*-based products in the pharmaceutical and healthcare industries.

Keywords: Antibacterial, Anti-inflammatory, GC-MS, LC-MS, *Marchantia polymorpha*

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Introduction

The ethnomedical utilisation of various plants and herbs for managing different illness problems has developed into an excellent endeavour for people. In India, tribal people and folklore traditions employ a variety of herbs, plant-based decoctions, or pastes to cure cuts and wounds. A plant's natural phytochemical components considerably contribute to its value in treating various diseases. About 7000-7500 plant species are employed in India's traditional medical practices[1].

Inflammation is an immune system response mechanism that may be caused by pathogenic microorganisms like bacteria and viruses, as well as particulates, toxic substances, or cells that are injured. Inflammation control is linked to immune function and is stimulated by inflammatory mediators such as nitric oxide (NO) and cyclooxygenase-2 (COX-2)[2]. Furthermore, active macrophages produce pro-inflammatory mediators such as interleukin-6 (IL-6), interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α), which augment the inflammation. Induced nitric oxide synthase (iNOS) gene expression in murine macrophages triggered by lipopolysaccharide (LPS) results in enormous nitric oxide synthesis using L-arginine and molecular oxygen as substrates and NADPH[3].

Since inflammation is recognised as having a significant role in various illnesses, many synthetic medications have demonstrated significant anti-inflammatory efficacy. However, due to adverse effects like gastrointestinal bleeding and ulceration, the interests of scientists have shifted towards developing safer natural products for treating inflammation-mediated disorders. Moreover, a rise in

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research has been observed on the anti-inflammatory potential of phytochemical compounds employed in traditional medicine in the last few years[4].

The genus *Marchantia* L. includes the small, non-vascular plants known as liverworts that belong to the division Marchantiophyta. Liverworts are found worldwide in damp habitats, such as on moist soils, rocks, and the edges of bogs. Many indigenous tribes worldwide employ *Marchantia*, which has demonstrated great promise in medicine as an antimicrobial, anticancerous, antioxidant, anti-inflammatory, hepatoprotective, muscle relaxant and skin care agent[5]. Investigations on the crude extract and isolated compounds like Marchantin A, Marchantin B and Marchantin H isolated from *Marchantia polymorpha* L. revealed significant cytotoxic, anticancerous and anti-inflammatory activities[6]. Marchantins (A, B, D and E), Paleatin B, Isoriccardin C, Riccardin C, Perrottetin D, Radulanin H and Indomethacin were found to inhibit the production of pro-inflammatory substances, such as nitric oxide and prostaglandins[7]. The present study was designed to assess the phytochemical content and antibacterial and anti-inflammatory potential of *M. polymorpha*.

Materials and Methods

Chemicals

Analytical grade solvents i.e. methanol, acetone, chloroform, diethyl ether and dimethyl sulfoxide (DMSO) (99.5%), Linalool, Tannic acid, Rutin, Gallic acid, Sulphuric acid (H_2SO_4), Aluminium Chloride ($AlCl_3$), Sodium Carbonate (Na_2CO_3), Sodium Acetate (CH_3COONa), Ferric Chloride ($FeCl_3$), Ferrous Sulphate ($FeSO_4$), Folin & Ciocalteus Phenol (FCP) Reagent and 2,4,6-Tripyridyl-S-triazine (TPTZ) reagent was procured from Sisco Research laboratories (SRL, Gurugram, India). Mueller Hinton (MH) broth and MH agar were procured from HI media (Mumbai, India). The murine (mouse) RAW 264.7 macrophage cell line was procured from the National Centre for Cell Science (NCCS), Pune, India. Dulbecco's modified Eagle's medium (DMEM) and Penicillin/streptomycin (P/S) were obtained from Gibco (Thermofisher Scientific, United States). Lipopolysaccharide (LPS) was procured from Cusabio Biotech (China). Fetal Bovine Serum (FBS) from MP biomedicals andTNF-α, IL-6, and IL-1β ELISA kits have been procured from Elabscience, USA. Phosphate buffered saline (PBS) was purchased from Lonza Bioscience, Switzerland.

Plant collection

M. polymorpha (Marchantiaceae) was collected from Glen Forest of Shimla, Himachal Pradesh, India, in September 2021 and was authenticated by morphological characters using keys[8]. The voucher specimen was deposited in the Herbarium (PAN 6437), Department of Botany, Panjab University, Chandigarh, India. The plant was thoroughly washed, dried, ground to fine powder and stored in an airtight container under aseptic conditions.

Extraction procedure and sample preparation

Five grams of the dried plant was extracted in 50 mL of each solvent, like methanol, acetone, chloroform, diethyl ether and aqueous, using a Soxhlet apparatus. The solvents were heated to their boiling temperature, i.e., methanol (64.7°C), acetone (56°C), chloroform (61°C), diethyl ether (34.6°C) and aqueous (100°C) and the plant material was subjected to extraction for 6-8 h at 2-4 cycles h^{-1}. The obtained extracts were filtered, and the filtrate was dried through a rotary evaporator. The dried extracts were quantified, and stock solutions were prepared by their reconstitution to a concentration of 10 mg/mL using 5% DMSO, as it is a versatile solvent that can solubilise both polar and non-polar compounds. Extracts were stored at 4ºC for future use.

Quantitative estimation of phytochemical content

Estimation of Total Phenolic Content

To 1 mL of diluted plant extract, 0.5 mL of Folin-Ciocalteu reagent was added. After 10 minutes, 3 mL of 20% sodium carbonate (Na_2CO_3) was added and kept in the dark for 30 min. The absorbance of the finally obtained blue-coloured mixture (molybdenum–tungsten complex) was measured at 765 nm. The standard curve was prepared using different concentrations of gallic acid, and the results were expressed as mg gallic acid equivalents (GAE) per gram of the sample in dry weight (mg/g)[9].

Estimation of Total Flavonoid Content

Briefly, to 100 µL of plant extract diluted with 900 µL of methanol, 1 mL of 10% aluminium chloride ($AlCl_3$) solution was added. After 5 min, 1 mL of 1 M sodium acetate (CH_3COONa) solution was added and kept for 45 min at room temperature with intermittent shaking. Absorbance was measured at 415 nm against a blank. Rutin was used to make the standard calibration curve. Results were expressed as mg Rutin Equivalents (RE) per gram of sample in dry weight (mg/g)[9].

Estimation of Total Tannin Content

Briefly, 100 µL plant extract was diluted with 7.5 mL of distilled water, 0.5 mL of Folin-Ciocalteu reagent and 1 mL of 35% sodium carbonate (Na_2CO_3) were added. The mixture was shaken thoroughly and kept at room temperature for 30 minutes. Absorbance was taken at 700 nm. Tannic acid was used to make the standard calibration curve. Results were expressed in terms of mg tannic acid per gram of sample in dry weight (mg/g) in the sample[10].

Estimation of Total Terpenoid Content

Briefly, to 200 µL of plant extract, 1.5 mL of chloroform was added, vortexed thoroughly and kept at 37°C for 3 minutes. It was followed by the addition of 100 µL of conc. H_2SO_4, and incubating at 37°C for 1.5-2.0 h in dark conditions, a reddish-brown precipitate was formed. The supernatant of the reaction mixture was decanted carefully, and 1.5 mL of methanol was added to the precipitate and mixed thoroughly until the precipitate was dissolved. The optical density was measured at 538 nm. The total terpenoid content was calculated by a calibration curve of linalool, and the results were expressed as mg linalool per gram of sample in dry weight (mg/g)[11].

Estimation of total antioxidant capacity

Briefly, to 3 mL of working FRAP reagent [300 mM acetate buffer, 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3$ at a ratio of 10:1:1 (v/v/v)], 100 µL of plant extract was added, kept at room temperature for 30 min and absorbance was measured at 593 nm. Different concentrations of $FeSO_4$ were used to make the Standard Curve. Results were expressed in terms of µM $FeSO_4$ E/g dw[9].

Screening of antibacterial activity

Bacterial cultures

Six bacterial cultures namely *Staphylococcus aureus* (MTCC-3160), *Escherichia coli* (MTCC-585), *Bacillus subtilis* (MTCC-441), *Listeria monocytogenes* (MTCC-839), *Klebsiella pneumoniae* (MTCC-109) and *Proteus mirabilis* (MTCC- 425) were procured from MTCC-IMTECH, Chandigarh, India and were grown on Mueller-Hinton agar plates by incubating at 37°C for 24 h. Bacterial growth and colony-forming units (CFU/mL) of each bacterial culture were assessed to determine the bacterial cell counts by combining serial dilution, spread plating technique and optical-density measurement at 600 nm.

Agar Well Diffusion assay

The crude extracts of *M. polymorpha* were analysed for their antibacterial activity by agar well diffusion method[12]. Briefly, 100 µL of bacterial culture (10^6 CFU/mL) was spread plated on Mueller Hinton agar plates. Subsequently, wells of 8 mm diameter were punched into the agar medium and filled with 100 µL of the crude extract (10 mg/mL) of *M. polymorpha*. The same volume of DMSO (5%) was taken as a negative control. Ciprofloxacin (5 µg/mL) was used as a positive control because it is a broad-spectrum antibiotic that inhibits gram-positive and gram-negative bacteria. The plates were incubated at 37°C for 24 h and monitored for zones of inhibition (mm).

Determination of minimum inhibitory concentration

Minimum inhibitory concentration (MIC) of each extract was calculated using the broth micro-dilution method with minor modifications to the Clinical and Laboratory Standards Institute's criteria[12]. The tested concentration of *M. polymorpha* crude extracts ranged from 0.0097 to 20 mg/mL. The lowest concentration of plant extract that inhibited the visible growth of bacterial cultures was selected as MIC. The lowest concentration of plant extract that prevented bacterial growth on Mueller Hinton agar plates was selected as minimum bactericidal concentration (MBC).

Anti-inflammatory activity

Cell culture

The murine RAW 264.7 macrophage cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) media supplemented with 10% Fetal Bovine Serum (FBS) and 1% Antibiotic (penicillin/ streptomycin in 0.9% saline) and incubated at 37°C in 5% CO_2 humidified incubator and were regularly sub-cultured every two days.

Cell viability assay

MTT assay was performed to assess the effect of methanolic extract of *M. polymorpha* on the viability of RAW 264.7 cells[13]. Briefly, Raw 264.7 cells ($1x10^5$ cells/well) were seeded in 96-well culture plates, treated with various concentrations (1, 2, 4, and 8 mg/mL) of methanolic extract and incubated in a humidified incubator under 5% CO_2 at 37°C. Untreated Raw 264.7 cells were used as a negative control. After 24 h, the supernatants were discarded, and 100 µL/well of 2,5-diphenyltetrazolium bromide (MTT) solution (0.5 mg/mL) was added, followed by incubation at 37°C in the dark for 4 h. Finally, after

removing the supernatant, the formazan crystals were dissolved in 100 µL of DMSO. The optical density was measured at 570 nm using a microplate reader (M200 PRO, Tecan Life Science).

Nitric oxide (NO) inhibition assay

In order to assess the anti-inflammatory potential of crude methanolic extract of *M. polymorpha*, the extract was tested for its ability to reduce LPS-induced nitric oxide (NO) generation in the murine macrophage RAW 264.7 cell line[13]. Briefly, RAW 264.7 cells seeded in a 48-well microplate were treated with *M. polymorpha* extract with or without LPS. LPS alone (1 µg/mL) was taken as the positive control. After incubation at 37°C in 5% CO_2 humidified incubator for 16 h, 100 µL of supernatant was treated with 150 µL of Griess reagent (1% sulfanilamide and 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride in 5% phosphoric acid) for 15 min followed by measurement of optical density at 540 nm. Results were expressed in terms of per cent NO production.

Cytokines assessment (TNF-α, IL-6 & IL-1β)

Briefly, RAW 264.7 cells were stimulated with 1 µg/mL of LPS in a 96-well microtiter plate for 1 h, treated with *M. polymorpha* extract and incubated at 37°C for 24 h. In the supernatants, Sandwich immunoassay quantification of TNF-α, IL-6, and IL-1β production was determined using enzyme-linked immunosorbent assay (ELISA) kits by the manufacturer's instructions (Elabscience, USA). The absorbance was measured at 450 nm.

LC-MS Characterisation

Methanolic extract of *M. polymorpha* was characterised by LC-MS analysis to identify various bioactive compounds using LC-MS Waters, Synapt XS HDMS with UPLC Acquity H class series system. The LC column was C18 Waters, Acquity BEH (2.1 x 100 mm, 1.7 µm thickness). Briefly, mobile phases used for the analysis were (A) 0.1% formic acid in water solvent and (B) 0.1% formic acid in acetonitrile (ACN) solvent. For MS spectra, N_2 with a supply pressure of 6-7 bar and Argon with 5-6 bar was used, and the desolvation temperature was set to 450ºC, and the source temperature was 120ºC. The gas flow rate was 850 L/h of desolvation gas and 50 L/h of cone gas. The injection volume used was 5 µL with a flow rate of 0.15 mL/min and a gradient starting at starting at 0% B at t=0 and kept for 1.0 min, then to 100% B at 15 min and kept for 0.5 min, then back to 0% B over 0.1 min and maintained for 4.4 min[14].

GC-MS analysis

GC-MS was employed to identify various volatile phytocompounds in the methanolic extract *M. polymorpha* using Shimadzu triple-quad GCMS-TQ8050 NX equipped with a standard capillary column. Briefly, Helium was used as a carrier gas with a flow rate of 1 mL/min, with column oven temperature set at 50°C for 2 min and then increased to 300°C for 10 minutes at a rate of 5°C/min. The volume injected was 2 µL, Split ratio was 10:1, solvent cut time was 4.50 min, ion source temperature was 200°C and interface temperature was 260°C. The phytocompounds were identified based on retention time and retention indices, and the spectra were matched with the NIST17R mass spectral library database[15].

FTIR analysis

The Fourier Transform Infrared Spectrophotometer (FTIR) was employed to determine the types of chemical bonds/functional groups present in the methanolic extract of *M. polymorpha*[16]. Briefly, 2 mg dried powdered methanolic extract was mixed with a Potassium bromide (KBr) pellet of 13 mm diameter and loaded in the FTIR spectroscope. The chemical bonds of a compound can be estimated by reading the infrared absorption spectra with a measurement range of 400 to 4000 cm^{-1}.

Statistical analysis

All the experiments were carried out in triplicate with three independent measurements. Results were analysed statistically, and data were expressed as Mean±SD. Statistical significance was calculated using One-way ANOVA followed by the Tukey's test for multiple comparisons. $P \leq 0.05$ was considered significant. GraphPad Prism (ver. 8.0.1) software was used for all analysis.

Results

Quantitative estimation of phytochemical content

Quantitative analysis of phytochemicals in different solvent extracts revealed that methanol extract showed the presence of distinct phytochemical compounds, i.e. phenols, flavonoids, terpenoids and tannins, compared to other solvents. In contrast, aqueous extract had the least phytochemicals. Total phenolic content of the assessed methanolic extracts

of *M. polymorpha* ranged from 44.89±0.20 to 137.65±0.56 mg GAE/g dw, while total flavonoid (25.48±1.02 to 109.34±0.73 mg RE/ g dw), total tannin (21.99±0.45 to 100.17±0.39 mg tannic acid/g dw) and total terpenoid (3.98±0.01 to 40.62±0.14 mg linalool/ g dw), respectively (Table 1).

Antioxidant potential

Antioxidant potential of different extracts of *M. polymorpha* revealed that antioxidant potential increased gradually in a dose-dependent manner (20 µg/mL to 100 µg/mL) among all the solvent extracts and ranged from 1.23±0.01 to 122.35±0.57 µM $FeSO_4$ E/g dw, respectively (Fig. 1). Interestingly, methanolic extract (100 µg/mL) showed the strongest antioxidant potential, followed by acetone, chloroform and diethyl ether extracts (Fig. 1).

Antibacterial activity

It was interesting to observe that most of the different organic extracts of *M. polymorpha* showed antibacterial potential against both gram-positive and gram-negative bacteria, except *Proteus mirabilis.* More specifically, methanolic extract of *M. polymorpha* exhibited maximum antibacterial potential with gram-positive (*S. aureus, B. subtilis* and *L monocytogenes*) and gram-negative (*E. coli* and *K. pneumoniae*) bacterial strains. Hence, the maximum zone of inhibition (20.0±0.45 mm) was observed with 0.625 mg/mL (MIC) and 1.25 mg/mL (MBC) against *L. monocytogenes,* while lowest MIC (0.3125 mg/mL) and MBC (0.625 mg/mL) was found with chloroform extract against *B. subtilis* with 12.9±0.30 mm zone of inhibition (Fig. 2; Table 2).

Anti-inflammatory activity

Cell viability assay

The methanolic extract of *M. polymorpha* showed no cytotoxicity at doses up to 4 mg/mL, as 90% of the cells remained alive compared with 8 mg/mL extract having maximum toxicity (Fig. 3a). Therefore, 4 mg/mL methanolic extract was employed for further investigations.

Nitric oxide (NO) inhibition assay

It was observed that the tested concentrations of methanolic extract (1, 2 and 4 mg/mL) reduced the NO production in LPS-stimulated RAW 264.7 cells to 77.54, 65.29, and 49.95%, respectively, compared with untreated LPS-stimulated macrophages having 100% NO production. It is interesting to note that these methanolic extracts did not cause NO production in non-LPS stimulated RAW 264.7 cells (Fig. 3b). Since 4 mg/mL methanolic extract had maximum NO inhibition and was selected for the assessment of cytokines (TNF-α, IL-6, and IL-1β).

Cytokines assessment

It was observed that the supernatant derived from the RAW 264.7 cells co-treated with 4 mg/mL

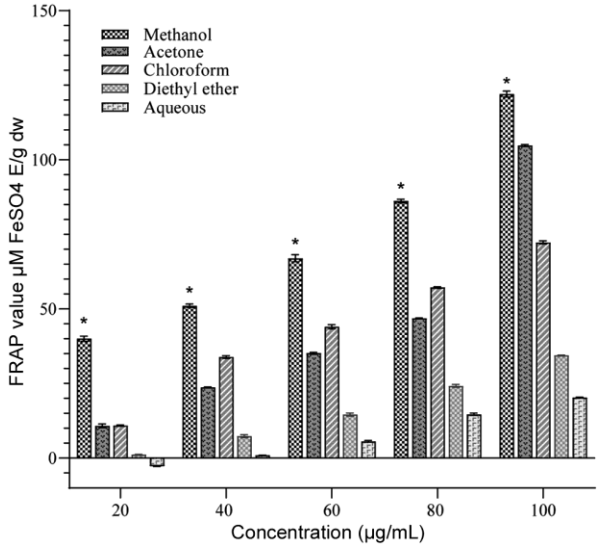

Fig. 1 — Antioxidant activity of varied concentrations of different extracts of *M. polymorpha*. Values are Mean ± SD. *represent significant difference within a distinct concentration (20,40, 60 etc.) at $p \leq 0.05$.

Table 1 — Total phenolic content, flavonoid content, tannin content and terpenoid content of *M. polymorpha* in different solvent extracts

Plant extract	Total Phenolic Content (TPC) (mg GAE/g dw)	Total Flavonoid Content (TFC) (mg RE/ g dw)	Total Tannin Content (TTC) (mg tannic acid /g dw)	Total Terpenoid Content (TTRC) (mg linalool / g dw)
Methanol	137.65±0.56[a]	109.34±0.73[b]	100.17±0.39[c]	40.62±0.14[d]
Acetone	102.33±0.26	64.37±0.53	96±0.39	23.16±0.12
Chloroform	85.40±0.60	41.22±0.53	43.43±0.30	24.53±0.14
Diethyl ether	131.43±0.63	81.97±0.62	21.99±0.45	31.71±0.09
Aqueous	44.89±0.2	25.48±1.02	98.28±0.34	3.98±0.01

Values are Mean ± SD. [a]p <0.001 compared to TPC of other solvent extracts. [b]p <0.001 v/s TFC of other solvent extracts. [c]p <0.001 v/s TTC of other solvent extracts. [d]p <0.001 v/s TTRC of other solvent extracts. GAE: Gallic Acid Equivalent; RE: Rutin Equivalent

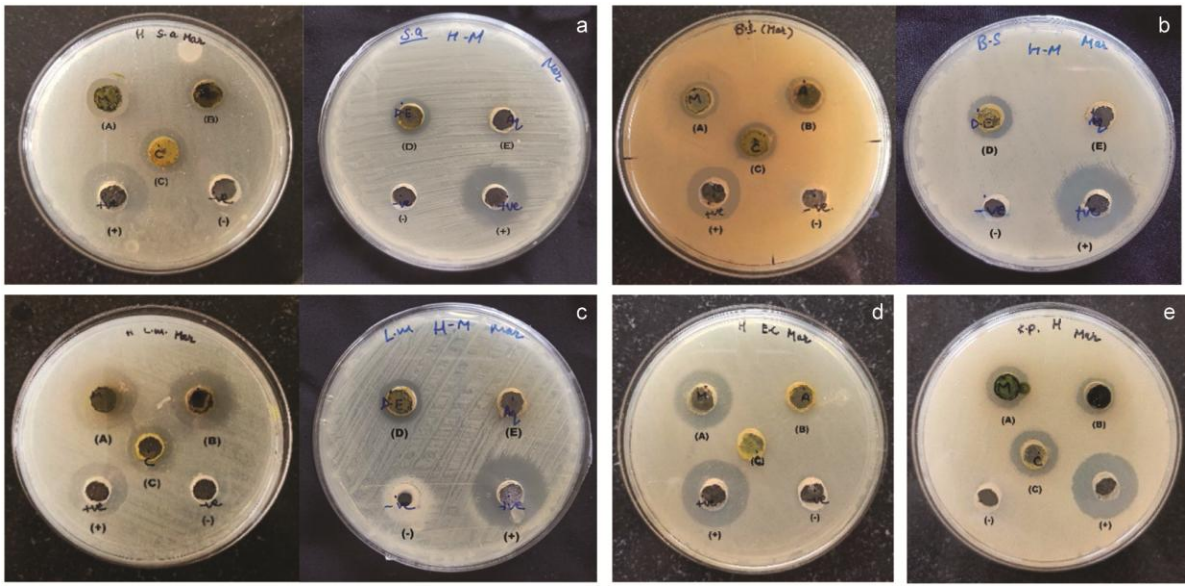

Fig. 2 — Antibacterial activity of extracts of *M. polymorpha* (A) Methanol, (B) Acetone, (C) Chloroform, (D) Diethyl ether and (E) Aqueous against a) *Staphylococcus aureus*, b) *Bacillus subtilis*, c) *Listeria monocytogenes*, d) *Escherichia coli* and e) *Klebsiella pneumoniae*. (-) represents negative control DMSO and (+) represents positive control Ciprofloxacin.

Table 2 — Antibacterial assay of different organic extracts of *M. polymorpha* against various gram-positive and gram-negative bacteria

Bacteria	Plant extract	Zone of Inhibition (mm)*	Minimum Inhibitory Concentration (mg/mL)	Minimum Bactericidal Concentration (mg/mL)
S. aureus	Methanol	15.0^{a}±0.25	0.625	1.25
	Acetone	13.2^{b}±0.46	1.25	2.5
	Chloroform	14.9^{a}±0.25	0.625	1.25
	Diethyl ether	12.0^{b}±0.45	1.25	2.5
	Aqueous	-	-	-
E. coli	Methanol	17.1^{c}±0.36	5	10
	Acetone	-	-	-
	Chloroform	-	-	-
	Diethyl ether	-	-	-
	Aqueous	-	-	-
B. subtilis	Methanol	13.1^{b}±0.40	1.25	2.5
	Acetone	12.2^{b}±0.43	1.25	2.5
	Chloroform	12.9^{b}±0.30	0.3125	0.625
	Diethyl ether	12.9^{b}±0.40	0.625	1.25
	Aqueous	-	-	-
L. monocytogenes	Methanol	20.0^{d}±0.45	0.625	1.25
	Acetone	18.0^{c}±0.47	1.25	2.5
	Chloroform	15.0^{a}±0.20	0.625	1.25
	Diethyl ether	11.9^{b}±0.38	1.25	2.5
	Aqueous	-	-	-
K. pneumoniae	Methanol	13.0^{b}±0.41	1.25	2.5
	Acetone	11.9^{b}±0.50	1.25	2.5
	Chloroform	15.2^{a}±0.58	2.5	5
	Diethyl ether	-	-	-
	Aqueous	-	-	-

(*Contd*.)

Table 2 — Antibacterial assay of different organic extracts of *M. polymorpha* against various gram-positive and gram-negative bacteria (*Contd*.)

	Methanol	-	-	-
P. mirabilis	Acetone	-	-	-
	Chloroform	-	-	-
	Diethyl ether	-	-	-
	Aqueous	-	-	-

*Values are Mean ± SD. The zone of inhibition results is compared, each different letter (a, b, c, etc.) corresponds to each mean showing a statistical difference with p <0.05.(-) represents no activity

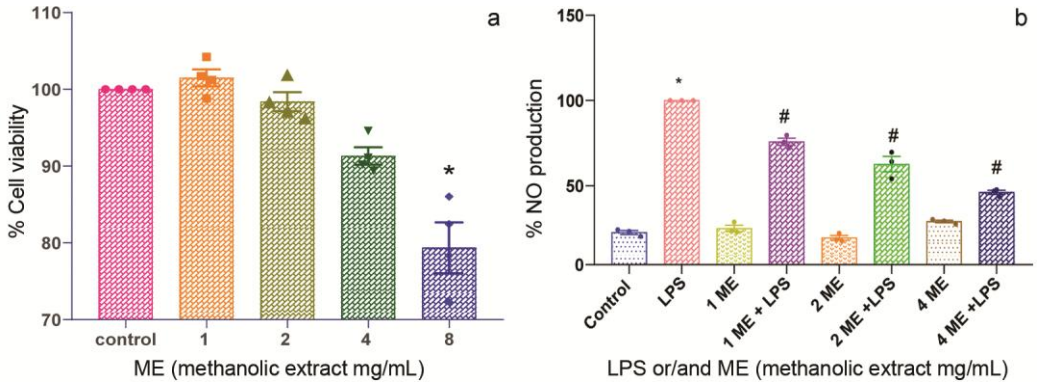

Fig. 3 — Effect of *M. polymorpha* methanolic extracts on a) Cell viability of RAW 264.7 cells, and b) NO production in methanolic extract. Values are Mean ± SEM. *p <.0001 v/s control and $^{\#}p$ <.0001 v/s LPS (Lipopolysaccharide).

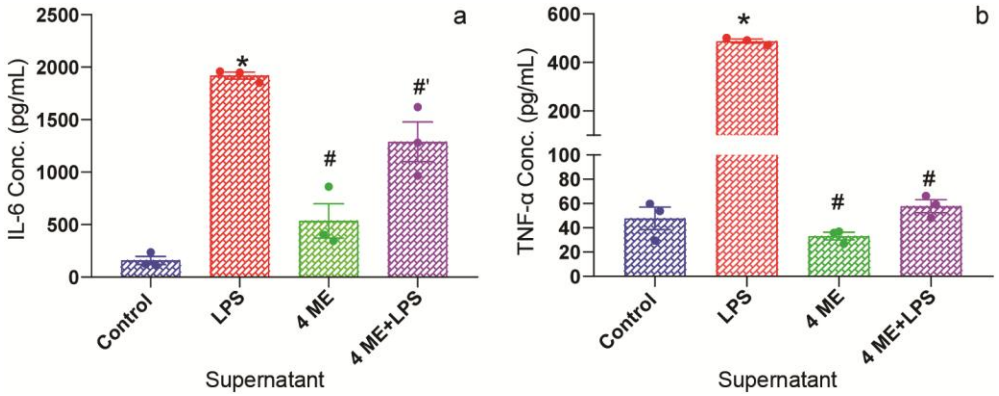

Fig. 4 — Effect of *M. polymorpha* methanolic extracts on cytokines level: a) IL-6, and b) TNF-α measured in supernatants of LPS-stimulated RAW 264.7 cells. Values are Mean ± SEM. *p <.0001 v/s control, $^{\#}p$ <.0001 v/s LPS and $^{\#'}p$ <.001 v/s LPS. LPS: Lipopolysaccharide; ME: Methanolic Extract.

methanolic extract and LPS significantly reduced the level of IL-6 and TNF-α production in the cell culture and showed no effect on IL-1β production (Fig. 4).

LC-MS analysis

LC-MS with TOF-MS ES+ and positive ionisation mode was performed for identification of phytocompounds in methanolic extract of *M. polymorpha* and 15 prominent phytocompounds were identified based on the degree of similarity between their structure and molecular mass. The majority of compounds detected were flavonoids, phenolic acids, terpenoids and bisbibenzyl ethers. In this analysis, the four most important bisbibenzyl ethers, i.e. Marchantin A, Marchantin B, Dehydromarchantin A and Plagiochin E, and the seven flavonoids, i.e. 6"-O-Malonylglycitin, Apigenin 7-O-diglucuronide, Kaempferol 3-O-glucosyl-rhamnosyl-galactoside, Chrysin 5,7-dimethyl ether, Isopeonidin 3-rutinoside, Kaempferol 3-O-rutinoside, Luteolin 7-O-diglucuronide, were identified (Table 3). In addition to these compounds, one terpenoid, Absinthin and one phenolic acid, 1,2-Disinapoylgentiobios, were also identified. The structures of bioactive compounds detected using LC-MS analysis are presented in Fig. 5.

Table 3 — LC-MS profile of methanolic extract of *M. polymorpha* showing various phytochemical constituents

S. No.	RT (in min.)	m/z	Proposed Compound	Compound class	Molecular formula
1	20.740	455.1530	Marchantin M	MacrocyclicBisbibenzyl	$C_{29}H_{26}O_5$
2	25.115	439.1559	Riccardin D	Macrocyclic Bisbibenzyl	$C_{28}H_{24}O_4$
3	25.701	441.1685	Marchantin A	MacrocyclicBisbibenzyl	$C_{28}H_{24}O_5$
4	27.959	496.3391	Absinthin	Terpenoid	$C_{30}H_{40}O_6$
5	27.959	425.1749	Plagiochin E	Macrocyclic Bisbibenzyl	$C_{28}H_{24}O_4$
6	29.576	474.3783	Palaetin A	Acyclic bisbibenzyl	$C_{29}H_{28}O_6$
7	29.576	611.2836	Rutin	Flavonoid	$C_{27}H_{30}O_{16}$
8	32.609	623.2526	Apigenin 7-O-diglucuronide	Flavonoid	$C_{27}H_{26}O_{17}$
9	32.609	756.5557	Kaempferol 3-O-glucosyl-rhamnosyl-glucoside	Flavonoid	$C_{33}H_{40}O_{20}$
10	34.726	282.2817	Chrysin 5,7-dimethyl ether	Flavonoid	$C_{17}H_{14}O_4$
11	37.570	639.2761	Luteolin 7-O-diglucuronide	Flavonoid	$C_{27}H_{26}O_{18}$

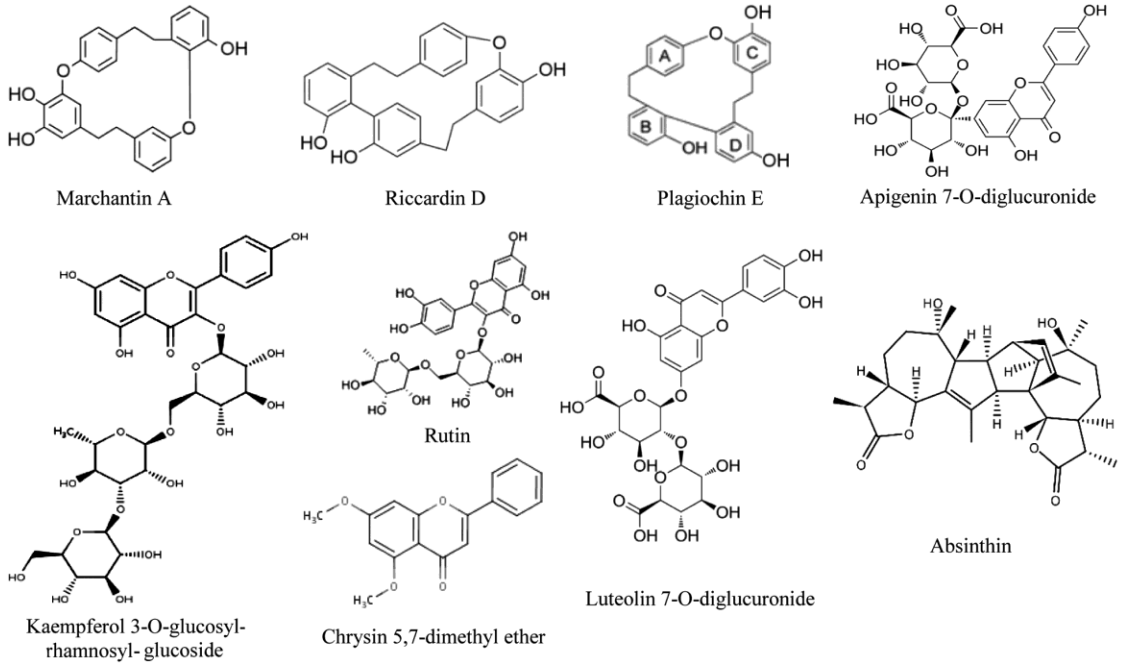

Fig. 5 — Structures of bioactive compounds detected in the methanolic extract of *M. polymorpha* using LC-MS analysis.

GC-MS analysis

A total of nine pharmacologically important volatile phytocompounds, i.e. Pentadecanal, 3-β-Hydroxy-5-cholen-24-oic acid, Stigmasterol, Phytol, n-Hexadecanoic acid (Palmitic acid), methyl ester, Corticosterone, Ergosterol, Caryophyllene and 1H-Cyclopropa[a]naphthalene, were identified from the GC-MS analysis of methanolic extract of *M. polymorpha* (Table 4). The structures of bioactive compounds detected using GC-MS analysis presented in Fig. 6.

FTIR analysis

The FTIR spectrum of methanolic extract of *M. polymorpha* showed numerous peaks revealing its complexity (Fig. 7). The region of 3367.98 cm^{-1} denotes the H-bonded stretching of polymeric hydroxyl groups (O-H), which is typical of polyphenolic compounds. It can be both symmetric and asymmetric. The $-CH_2$ and -CH stretching vibrations that originate from the sugars and carbohydrates are visible in the area of 2918.85 and 2851.13 cm^{-1}. The peak at 1735.03 cm^{-1} is highly related to six-membered ring lactone and aromatic combination bands. Proteins were also observed at the peak 1570.09 cm^{-1}on the basis of amide II bond (>N-H bend). The stretching of C=C–C aromatic bond was observed at 1508.97 and 1459.42 cm^{-1}. The peak at 1398.78 cm^{-1} was attributed to phenol or tertiary alcohol with O-H bend. Furthermore, the peaks at 1216.97, 1167.82, 1043.13 cm^{-1} could be attributed to

Table 4 — GC-MS analysis of methanolic extract of *M. polymorpha* showing the presence of bioactive phytocompounds on the basis of retention time and retention index with their biological activity

S. No.	Peak Area (%)	Retention Index	RT (min)	Compound Name	Nature of compound	Molecular Weight	Molecular Formula
1	28.44	1701	30.639	Pentadecanal	Fatty-acid	226	$C_{15}H_{30}O$
2	26.82	2719	53.904	3-β- Hydroxy-5-cholen-24-oic acid (Lithocholic acid)	Bile-acid	374	$C_{24}H_{38}O_3$
3	13.92	3170	54.284	Stigmasterol	Triterpene	412	$C_{29}H_{48}O$
4	4.45	2122	36.273	Phytol	Diterpenoid	296	$C_{20}H_{40}O$
5	3.84	1960	32.590	n- Hexadecanoic acid (Palmitic acid), methyl ester	Fatty-acid	270	$C_{17}H_{34}O_2$
6	1.95	2531	50.134	Corticosterone bis(pentafluoropropionate)	-	638	$C_{27}H_{28}F_{10}O_6$
7	1.21	2650	50.236	Ergosterol	Triterpene	396	$C_{28}H_{44}O$
8	1.08	1415	22.462	Caryophyllene	Sesquiterpene	204	$C_{15}H_{24}$
9	0.96	1398	22.657	1H-Cyclopropa[a]naphthalene	Ethylene	204	$C_{15}H_{24}$

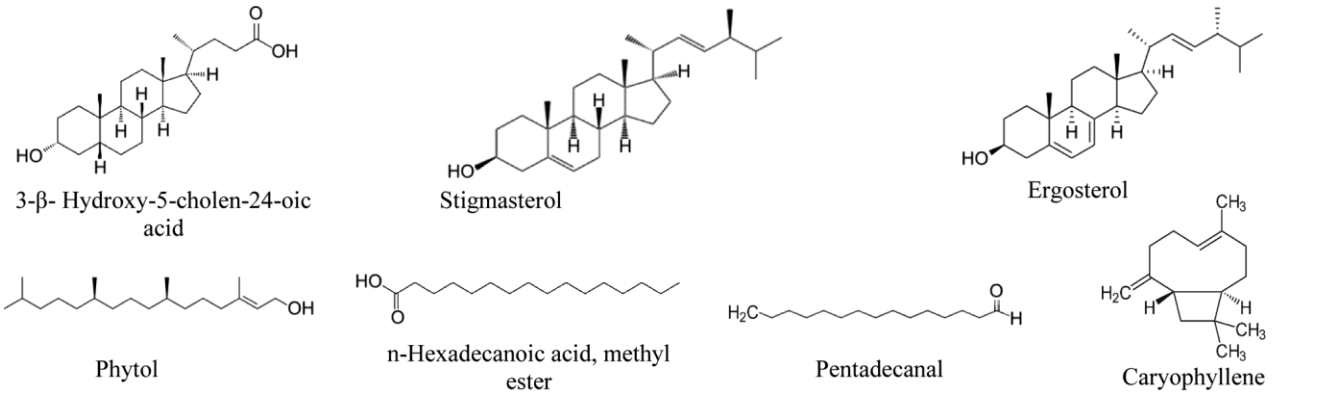

Fig. 6 — Structures of bioactive compounds detected in the methanolic extract of *M. polymorpha* using GC-MS analysis.

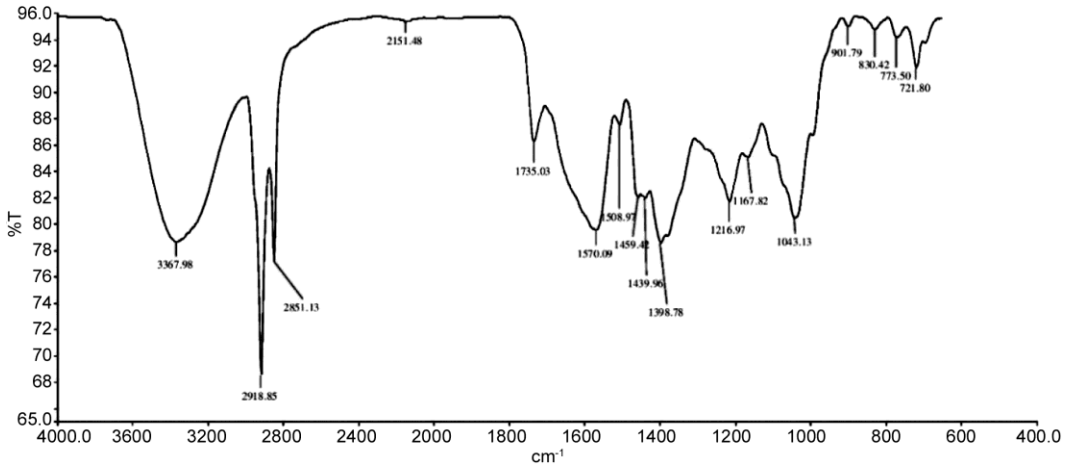

Fig. 7 — FTIR spectrum of methanolic extract of *M. polymorpha.*

Aromatic C-H in-plane bending and peaks at 830.42, 773.50, 721.80 cm^{-1} are attributed to aromatic C-H out-of-plane bending, and these peaks are also responsible for skeletal C-C vibrations.

Discussion

Antimicrobial agents are recommended normally to combat infections. However, they are also associated with adverse effects like diarrhoea, allergic reactions

or even elevated reactive oxygen species (ROS), posing a serious threat to human beings. Besides these, the occurrence of drug resistance by microbes is becoming a major problem, so to overcome these adverse effects, plant-based phytochemicals are preferred as antimicrobial agents to ameliorate the various human infections[17]. Therefore, in the present study, an attempt has been made to enlighten the antioxidant, antibacterial and anti-inflammatory potential of methanolic extract of *M. polymorpha*. Although *M. polymorpha* has been used for ages in folk medicine to treat inflammation, fever, skin diseases, and other conditions, this research fills the gap between traditional use and current scientific proof.

It was found that among multiple organic solvents employed, methanolic extract of *M. polymorpha* exhibited maximum phytocompounds, which may be due to their enhanced solubility in polar solvent, as the polarity indices of the organic solvents affect the percentage yield[18]. Further, it was found that *M. polymorpha* showed maximum total phenols (137.65±0.56 mg GAE/g dw), flavonoids (109.34±0.73 mg RE/g dw), terpenoids (40.62±0.14 mg linalool/g dw) and tannins (100.17±0.39 mg tannic acid/g dw) in its methanolic extract and is in concordance with earlier studies. Studies on different plants showed an ample amount of total phenols in their methanolic extracts, i.e., 225.45±1.48 mg GAE/g in *M. polymorpha*[19], 19.3 mg GAE/g in *Plagiochila beddomei*[20], 12.42±0.47 mg GAE/g in *Lepidozia boreensis*[21]. Similarly, a remarkable amount of total flavonoids has been reported in the ethanolic extract of mosses of Tianmu Mountain (ranged from 1.8 to 22.3 mg/g)[22], in methanolic extract of *Riccia billardieri* (51.04±0.36 mg/g QE)[23] and in methanolic extract of a liverwort *Dumortiera hirsuta* (91.14±0.71 mg RE/g dw)[24]. These phytochemicals have been found to exhibit the plant's antimicrobial efficacy mainly due to their ability to alter the cell membrane permeability, resulting in the leakage of various cell constituents such as proteins, nucleic acids, and inorganic ions vis-à-vis inhibiting energy metabolism of the bacterial cell.

Methanolic extract of *M. polymorpha* also showed high antioxidant potential (122.35±0.57 μM $FeSO_4$ E/g dw) in agreement with earlier studies[19]. Antioxidant potential of a moss *Rhodobryum roseum* (34.73±0.44 μM FeSO4 E/g dw)[25] was also reported high in the methanolic extracts, which may be due to the presence of active phytocompounds that act as exogenous antioxidants, which get oxidised directly by radicals to form less reactive species, therefore responsible for antioxidant potential.

It was interesting to observe that though all the organic extracts of *M. polymorpha* exhibited antibacterial potential, the methanolic extract showed maximum antibacterial potential against both gram-positive and gram-negative bacteria. This may be attributed to the presence of various phytochemicals like phenols, flavonoids and terpenoids, having the ability to modulate the cell-membrane permeability or disrupt the bacterial plasma-membrane, leading to leakage of intercellular content and finally cell-death[19]. Some scientists have also found the presence of various phytochemicals exhibiting antimicrobial potential against *S. aureus*, *P. mirabilis*, *E. coli*, *L. monocytogenes*, *B. subtilis* and *X. oryzae*. Antibacterial activity of crude methanolic and flavonoid extracts of *M. polymorpha* was found more effective (IZ: 20.6 mm and 19.6 mm, respectively) against *S. aureus* than *P. mirabilis* and *E. coli*[26]. Investigations on n-hexane, chloroform, ethyl-acetate and ethanol extracts of *M. polymorpha* against *Vibrio parahaemolyticus*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Staphylococcus aureus* and *S. epidermidis* revealed remarkable antimicrobial activity of all the extracts except ethanol extract[27].

However, very little research has been done on the effects of phytocompounds from *M. polymorpha* on their ability to reduce inflammation. The present study demonstrated the anti-inflammatory potential of methanolic extract of *M. polymorpha* as it reduced LPS-induced generation of nitric oxide (NO) and inflammatory mediators like TNF-α and IL-6 in RAW 264.7 macrophage cells. The decreased level of pro-inflammatory cytokines in the present study may be due to the suppression of inflammatory pathways like nuclear factor kB and MAPKs by methanolic extract of *M. polymorpha*[28]. Scientists have also demonstrated that the methanolic extract of *M. polymorpha* markedly decreased LPS-induced NO production in HaCaT cells[29]. Moreover, NO generation in LPS and hPrx1 induced macrophage RAW 264.7 cell line was found to decrease by 32 species of bryophytes (including both liverworts and mosses)[30].

The observed antibacterial and anti-inflammatory potential in the methanolic extract of *M. polymorpha* was further supported by LC-MS, GC-MS and FTIR as these analyses documented the presence of

phytochemicals like flavonoids, phenols, terpenoids, fatty acids, tannins, phytosterols etc. and allowed the identification of a wide array of bioactive compounds, which have been reported for their medicinal properties in previous studies. n-Hexadecanoic acid, methyl ester demonstrated antimicrobial and anti-inflammatory properties[31], while 3-β-Hydroxy-5-cholen-24-oic acid (Lithocholic acid) exhibited antibacterial potential[32]. Rutin was shown to possess antimicrobial, anti-diabetic, and anticancerous properties[33]. Apigenin 7-O-diglucuronide displayed anti-inflammatory potential[34], and Phytol was highlighted as an antioxidant, antimicrobial, and anti-inflammatory agent[35]. Riccardin D was reported to have anticancerous activity, Marchantin A was found to have antioxidant, cytotoxic and antimicrobial properties, while Plagiochin E was specifically noted for its antimicrobial activity[6]. Stigmasterol demonstrated antimicrobial, anti-inflammatory, and anticancerous properties[36]. Among the volatile compounds detected by GC-MS analysis, sesquiterpenoids were displayed as the major compounds in the diethyl-ether extract of *M. polymorpha*[37], and this extract of *M. polymorpha* also revealed many other interesting compounds like 13-Docosenoicacid, 6-Octadecenoicacid, n-Hexadecanoic acid, Stigmasterol and Phytol[38].

Conclusion

The results of this investigation highlighted the potential of the methanolic extract of *M. polymorpha* as a potent antibacterial and anti-inflammatory drug, owing to its rich phytocompound profile. The extract showed strong inhibitory activities against Gram-positive and Gram-negative bacteria, and a dramatic decrease in inflammatory mediator production, including NO, TNF-α, and IL-6, in LPS-stimulated RAW 264.7 cells. These results confirm the traditional use of *M. polymorpha* in treating inflammatory, infectious and oxidative stress-related diseases and provide a scientific rationale for its possible integration into modern healthcare practices. More research in isolating and identifying the responsible compounds for these biological effects, *in vivo* experiments, and clinical trials will be needed to confirm their therapeutic value and determine any possible toxicity.

Conflict of interest

The authors have no competing interests to declare that are relevant to the content of this article.

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