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TERPENOID-RICH METABOLITES FROM *NEONOTHOPANUS NAMBI* AS POTENTIAL TOPICAL ANTIBACTERIAL AGENT AGAINST MRSA: BIOMARKER QUANTIFICATION AND TOXICITY EVALUATION

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The bioluminescent basidiomycetes *Neonothopanus nambi* FRIM550, produces secondary metabolites with potential antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA), which causes serious acute and chronic skin infections. A dimeric sesquiterpenoid known as aurisin A was identified as the anti-MRSA marker compound. Aiming at exploring terpenoid-rich metabolites from *N. nambi* as a novel topical antibacterial agent, this study describes biomarker compound quantification for quality control and toxicity evaluation of the active ingredient for therapeutic applications. Culture filtrate of *N. nambi* FRIM550 from stirred-tank bioreactor fermentation was extracted and subjected to column chromatography to isolate the terpenoid-rich active ingredients (TRAI), which exhibited strong inhibition against reference MRSA and clinical FRMRSA (fusidic acid-resistant MRSA) strains. TRAI showed a minimum inhibitory concentration (MIC) of 3.91 and 7.81 μg mL^{-1} against MRSA and FRMRSA, respectively. A method for quantitative analysis of aurisin A was developed using reversed-phase HPLC coupled with a diode array detector (HPLC-DAD). *In vitro* toxicity evaluation showed that TRAI did not adversely affect the viability of BALB/3T3 clone A31 (fibroblast) cells at the anti-MRSA MIC concentrations, showing more than 90% cell viability. *In vivo* acute dermal toxicity study indicated that topical administration of a formulation containing 2% (w/w) TRAI was non-irritant with no signs of toxicity or mortality and no gross abnormalities in any of the organs in test animals during the 14-day period, showing a median lethal dermal dose (LD_{50}) of > 2000 mg kg^{-1} body weight. Moreover, *in vivo* acute oral toxicity study showed that oral administration of TRAI at a dose of 2000 mg kg^{-1} body weight did not result in adverse behavioral changes or death in the animals, and no gross abnormalities were observed in any of the organs. Therefore, TRAI did not cause any treatment-related adverse effects in the rats at 2000 mg kg^{-1} dose. Our results are significant as they highlighted the potential use of the terpenoid-rich active ingredients from *N. nambi* FRIM550 to develop alternative treatments for MRSA skin infection.

Keywords: Methicillin-resistant S*taphylococcus aureus*, MRSA skin infections, basidiomycetes, secondary metabolites, antibacterial

INTRODUCTION

The emergence of antimicrobial-resistance in pathogenic bacteria is one of the major threats to public health worldwide. Methicillin-resistant *Staphylococcus aureus* (MRSA) is an example, where community-acquired and nosocomial or hospital-acquired MRSA strains exhibit resistance to multiple classes of antibiotics, causing life-threatening infections in humans (Clericuzio et al. 2021). Being an opportunistic pathogen that can infect open wounds and surgery sites, MRSA causes serious acute and chronic skin and soft tissue infections, leading to patient morbidity and mortality. The use of topical antibiotics in treatment has advantages over systemic treatments, which include delivery of high concentrations of antibacterial agents at the required site and reduction in systemic toxicity (Stevens et al. 2014). Formulations containing mupirocin, retapamulin and fusidic acid have been used as topical antibacterial agents. However, the resistance of *S. aureus* to these antibiotics has been repeatedly reported (Principi et al. 2020). Thus, continuous research is urgently needed to find new, safer alternative treatments to tackle serious MRSA topical infections (Terreni et al. 2021)

Basidiomycetes fungi are largely untapped sources of valuable antimicrobial natural products with the potential to be developed as mycopharmaceuticals or basidiomycetes-derived drugs (Vimalah et al. 2020, Al-Obaidi et al. 2021). Terpenoids are among the major classes of antibacterial metabolites produced by basidiomycetes (Dasgupta & Acharya 2019). *Neonothopanus nambi* (Speg.) R.H. Petersen & Krisai is a bioluminescent basidiomycete species that has been studied extensively in recent years as a rich source of secondary metabolites with a diverse range of pharmacological properties (Kanokmedhakul et al. 2012, Boueroy et al. 2021, Getha 2024). Previously, Getha et al. (2009) observed strong antibacterial activity in submerged mycelial culture extract of *N. nambi* strain FRIM550, which was isolated from root rot disease-infected *Acacia mangium* roots. Terpenoid-rich metabolites fractionated from the basidiomycete extract were responsible for the strong antibacterial activity (Getha et al. 2021). Aurisin A, a dimeric sesquiterpenoid, was identified as the key anti-MRSA compound in the enriched extract (Getha et al. 2023). Thus, aurisin A could be considered a biomarker for quality standardisation of the terpenoid-rich antibacterial extract from *N. nambi*.

High-performance liquid chromatography (HPLC) analysis for generating chemical fingerprints and quantifying the marker compound present in natural product-based active pharmaceutical ingredients, is one of the key strategies in quality control (Sunthudlakhar et al. 2022). Therefore, this study aims to first compare the terpenoid-rich active ingredients (TRAI) from *N. nambi* with standard antistaphylococcal antibiotics for their anti-MRSA efficacy, and then to develop a HPLC method for quantification of the key biomarker (aurisin A) in TRAI. *In vitro* and *in vivo* toxicity studies were also conducted to assess the toxicity effects of the API, given its potential application as an antibacterial and anti-MRSA agent for managing topical infections.

MATERIALS AND METHODS

Fermentation of *N. nambi* FRIM550 in a stirred-tank bioreactor

Mycelial cultures of strain FRIM550 were grown on Potato Dextrose Agar (PDA) at 28 oC. After 8 days of growth, 5-mm-diameter mycelial plugs were cut from the edge of the PDA cultures using a sterile cork-borer and transferred to 1 L Erlenmeyer flasks containing Y5MG10 fermentation medium [5 g L^{-1} yeast extract; 5 g L^{-1} malt extract; 10 g L^{-1} D(+)glucose anhydrous; 3 g L^{-1} KH_2PO_4; 1 g L^{-1} $MgSO_4.7H_2O$; pH 5.5]. The flasks were incubated on a rotary shaker at 200 rpm and 28 ± 2 °C for 4 days. The culture (5%, v/v) was then inoculated into 3 L fermentation medium in a 5 L stirred-tank bioreactor (Minifors-Infors HT). Batch fermentation was carried out for 9 days at 26 ± 2 °C and initial pH 5.5, with an agitation speed of 150–300 rpm, dissolved oxygen concentration of 25 ± 5% and aeration rate of 0.5–1.5 vvm (Getha et al. 2021).

Preparation of terpenoid-rich active ingredient (TRAI)

Cell-free culture filtrate harvested from the bioreactor after 9 days, was extracted with butyl acetate (1:1.5, v/v). The BuOAc extract of fungal secondary metabolites was then dissolved in methanol (MeOH) and fractionated using column chromatography (CC) in lipopilic Sephadex LH-20 (Sigma-Aldrich USA) with MeOH as the mobile phase and a flow rate of 0.5–0.7 mL min-1. Terpenoid-rich fractions eluted between 17–25 hours were combined and dried under a vacuum to obtain the enriched active ingredient coded as TRAI (Muhammad-Syamil et al. 2018).

Isolation of aurisin A compound

Aurisin A was isolated according to the methods described by Getha et al. (2023). The BuOAc extract of strain FRIM550 was first subjected to Sephadex LH-20 CC (elution with 100% MeOH). Then fractions containing aurisin A were further purified by preparative HPLC using Zorbax XDB-C18 column (21.2 × 150 mm; Agilent Technologies USA) in an Agilent 1260 Infinity II system equipped with diode array detector (DAD), with a gradient elution of 5 mM ammonium formate (pH 3.5) and acetonitrile as the mobile phases. Based on retention time (16 min) and UV spectrum (Getha et al. 2023), aurisin A was eluted as a

yellow amorphous solid. Working solutions of aurisin A were then prepared for the HPLC quantification analysis.

HPLC method for quantitative analysis of aurisin A

The HPLC method for analysis of aurisin A content was developed based on methods described by Dorrani et al. (2014). Triplicate samples of aurisin A were dissolved in MeOH (1 mg mL^{-1}), and subsequent dilutions were made to a concentration range of 10–160 µg mL^{-1}. Working solutions were filtered using a 0.45 µm syringe filter before the analysis. Sample quantification and calibration curves were performed using a Zorbax XDB-C18 column (4.6 × 100 mm, 3.5 µm; Agilent Technologies USA) on an HPLC Agilent 1260 Infinity series equipped with DAD set at 320 nm for detection of aurisin A under analytical conditions described above. The relative standard deviation (RSD) of replicates at each concentration was calculated, and a calibration curve was obtained by plotting the average HPLC peak area and compound concentration. Data were evaluated for slope, intercept values and correlation coefficient (R). The linearity of the calibration curves was determined ($R^2 > 0.998$), and the limit of detection (LOD) and limit of quantification (LOQ) were determined based on the standard response deviation and slope of the regression line to perform the sensitivity analysis (Rosing et al. 2000).

Quantification of aurisin A in TRAI samples

For quantification of the biomarker in TRAI, samples were prepared from BuOAc extracts produced in three different fermentation batches of *N. nambi* FRIM550 (23-263-TRAI, 23-264-TRAI, 23-266-TRAI). The samples were dissolved in MeOH at a concentration of 2 mg mL^{-1} and vortexed to allow efficient dissolution. Sample solutions were then analysed in triplicate HPLC analysis using the method and analytical conditions described above to quantify aurisin A. Content of the biomarker compound was calculated based on a linear equation obtained from the calibration curve, and given as g per 100 g of TRAI (%, w/w).

Antibacterial activity by minimum inhibitory concentration (MIC) assay

Antibiotics oxacillin, vancomycin and fusidic acid were purchased from Sigma-Aldrich. Methicillin-resistant *Staphylococcus aureus* (MRSA) reference strain ATCC 33591 was purchased from the American Type Culture Collection (ATCC USA), and the MRSA clinical strain BD 16876 was obtained from the Faculty of Health Sciences, Universiti Kebangsaan Malaysia. Test bacteria were cultured overnight at 37 oC in Mueller-Hinton broth, and the culture suspension was adjusted to an OD reading of 0.08–0.13 at λ_{625} nm using a spectrophotometer, which corresponds to a cell density of 10^6 colony forming units (CFU) mL^{-1}. MIC values of the test samples against MRSA were determined by broth microdilution assay according to the methods of Clinical and Laboratory Standards Institute guidelines (CLSI 2018). The MIC is the lowest concentration of the sample at which no visually detectable bacterial growth was observed.

In vitro toxicity

Fibroblast cell line BALB/3T3 clone A31 was obtained from the American Type Culture Collection (ATCC). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated newborn calf serum and 1% penicillin/streptomycin, and were maintained at 37 °C and 5% CO_2/95% air. Exponentially growing cells were seeded in a 96-well plate at 1×10^4 cells per well density in complete culture medium (100 µL per well). After an overnight incubation, the culture medium was removed, and the cells were washed with phosphate buffered saline (PBS). The cells were then treated with various concentrations of TRAI sample (0–320 µg mL^{-1} in serum-free medium) for 24 hours. The concentration of dimethyl sulphoxide used to dissolve the test sample was maintained at 0.5% in the test system. Sodium lauryl sulphate (0–60 µg mL^{-1}) was used as the positive control. After the treatment incubation period, cell viability was assessed by the MTT (3-[4,5-dimethylthiazolyl-2]-2,5-diphenyl tetrazolium bromide) assay based on Mosmann (1983) with slight modifications. A dose-response curve was plotted and the 50%

inhibitory concentration (IC_{50}) was determined from the dose-response curve by non-linear regression using GraphPad Prism 8.4.3 software. Each data is represented by mean ± standard error of the mean (S.E.M.) of at least three independent experiments.

In vivo acute dermal toxicity

TRAI was dissolved in a suitable vehicle as described by Getha et al. (2021) at a concentration of 2% (w/w) and evaluated for acute dermal toxicity on 8–9 weeks old female *Sprague Dawley* rats. The test method was in accordance with the Fixed dose procedure OECD TG 402 guidelines of the Organisation for Economic Cooperation and Development (OECD 2017), and performed by an independent testing laboratory (SIRIM QAS International Sdn Bhd). A test sample was first administered topically (dermal) in a single application on the animals' test site, and detailed clinical observations were done immediately after patching. Following a 24-hour exposure, the test sample was removed and clinical observations were done on the rats based on the Draize criteria as in OECD TG 402.

In vivo acute oral toxicity

A 14-day acute toxicity study on TRAI sample in healthy female (7–8 weeks old) *Sprague Dawley* rats was conducted to assess its safety in case of accidental ingestion of products containing the active ingredient. The study was conducted in accordance with the OECD TG 420 guidelines (OECD 2002), and the experimental procedures were approved by the Institutional Animal Care and Use Committee, FRIM (IACUC No. IACUC-FRIM/04/1-2020). Rats were housed under standard environmental conditions of 12-hour light/12-hour darkness, given *ad libitum* access to a standard pellet diet and water and acclimatized to the new environment for 7 days. The sighting dose of TRAI was started at 300 mg kg^{-1} body weight based on the recommendation of the OECD guideline on one animal, and the single dose was orally gavaged in the animals. Based on the sighting dose results, the main dose study was conducted at a 2000 mg kg^{-1} dosage with another four animals added to the first tested animal. The animals were observed for behavioral changes, signs of toxicity and mortality at the first, second, fourth and sixth hour, and once daily for 14 consecutive days. The control group were not given TRAI during the study. Daily observation for mortality and abnormal clinical manifestations such as piloerection, salivation and lacrimation were done on the five rats and weight was recorded on days 1, 7 and 14. All animals were sacrificed by carbon dioxide inhalation euthanasia at the end of the study (15th day). Vital organs (liver, kidney, spleen, heart, lung, ovary and stomach) were harvested, weighed and a gross macroscopic physical examination was performed. All data were presented as mean ± standard deviation and analysed using the Statistical Package for the Social Sciences (SPSS) software. Statistical differences between the means for each measured variable were determined by Analysis of Variance (ANOVA), and results were considered statistically significant when $p < 0.05$.

RESULTS

Quantitative analysis of aurisin A content in *N. nambi* FRIM550 active ingredient

An HPLC method based on analytical conditions described by Getha et al. (2023), was developed for the quantitative analysis of the dimeric sesquiterpenoid aurisin A. Table 1 shows the mean HPLC peak area data for each concentration of the compound. The method's accuracy was determined after three injections of known concentration of aurisin A were done on three different days (inter-day analysis). A calibration curve was plotted using the mean values for each set as shown in Figure 1. Linearity of the calibration curve was determined (R^2 value of 0.9998), confirming the analytical method's linearity. The LOD and LOQ values were 3.44 µg mL^{-1} and 10.43 µg mL^{-1}, respectively, determined from the generated calibration curve (Figure 1).

The developed HPLC method was then used for performing quantitative analysis of aurisin A present in triplicate samples of the terpenoid-rich active ingredient (TRAI) produced from different fermentation batches of *N. nambi* FRIM550. Figure 2 shows that the HPLC fingerprint of the TRAI samples exhibited

Table 1 HPLC peak area (mean ± standard deviation) of triplicate aurisin A samples from inter-day analysis (RSD, relative standard deviation)

Aurisin A	Inter-day analysis*	
(μg mL^{-1})	Peak area (mAU.s)	%RSD
10	169.447 ± 3.524	2.08
20	255.658 ± 6.194	1.54
40	513.922 ± 11.667	1.33
80	1062.499 ± 172.365	8.63
160	2408.282 ± 449.672	10.21

*Each concentration was injected three times and all aurisin A samples analysed on three different days

specific chromatographic characteristics. The chromatograms of each sample showed good separation of the peak corresponding to aurisin A, from other peaks in the active ingredient under the analytical conditions. Moreover, the compound peak in each sample was similar to the UV spectra and peak retention time of pure aurisin A compound (16 min), as reported previously (Getha et al. 2023). Quantification using the plotted calibration curve showed that the biomarker compound was present in the concentration range of 33–60% w/w in TRAI (Table 2).

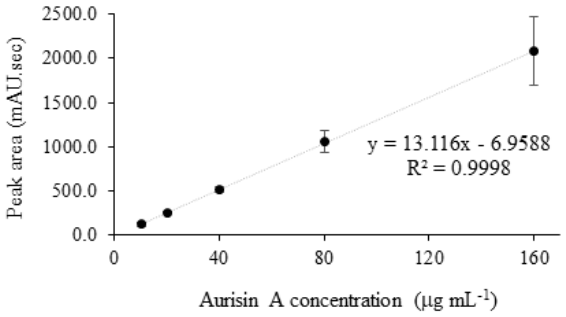

Figure 1 Calibration curve of aurisin A (at 320 nm) plotted using average HPLC peak area values of each concentration

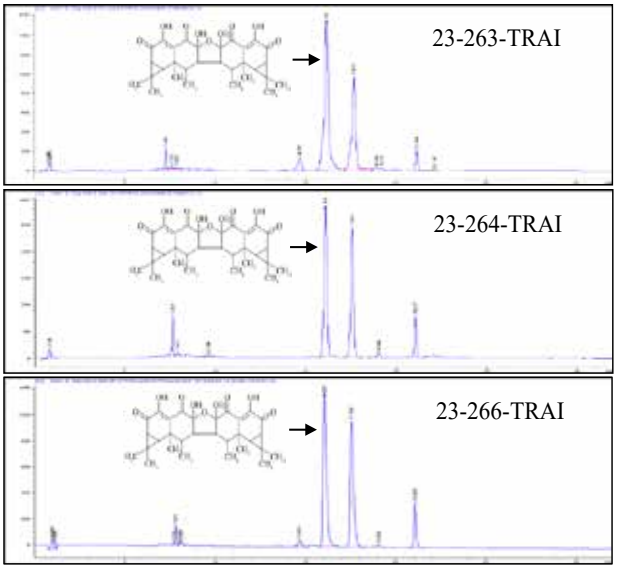

Figure 2 HPLC-DAD chromatograms (320 nm detection wavelength) of triplicate TRAI

Table 2 HPLC analysis for quantification of aurisin A in terpenoid-rich active ingredient (TRAI) samples obtained from three different fermentation batches (results from triplicate analysis of each sample)

TRAI sample codes		23-263-TRAI	23-264-TRAI	23-266-TRAI
Sample concentration (μg mL^{-1})		2000	2000	2000
Sample dilution factor		8	6	8
Peak area (mAU.s)	Analysis 1	1989.132	2271.287	1060.733
	Analysis 2	1998.100	2264.760	1095.641
	Analysis 3	1938.704	1894.743	1053.779
Aurisin A (μg mL^{-1}; mean ± s.d.)		151.13 ± 2.44	163.96 ± 16.43	82.11 ± 1.71
Aurisin A (% w/w; mean ± s.d.)		60.45 ± 0.98	49.19 ± 4.93	32.85 ± 0.68

TRAI and aurisin A are active against fusidic acid-resistant MRSA

In vitro antibacterial activity of the terpenoid-rich active ingredient and biomarker aurisin A of *N. nambi* FRIM550 against MRSA, was analysed and compared to that of some antistaphylococcal antibiotics (oxacillin, vancomycin, fusidic acid). One reference MRSA (ATCC 33591) and another clinical strain (MRSA BD 16876) were used in these studies, the results of which were summarised in Table 3. Both TRAI and aurisin A exhibited potent anti-MRSA activity (MIC 4–8 μg mL^{-1}). A notable finding was the high potency observed against the clinical MRSA BD 16876 strain, which is also highly resistant to fusidic acid (MIC 78.1 μg mL^{-1}). Fusidic acid has been used as a topical treatment option for *S. aureus* and MRSA. In addition, fusidic acid is an important and valuable alternative to vancomycin to combat resistant *S. aureus* (Foster 2017). However, high resistance to this antibiotic in MRSA has been evident in recent years due to its extensive and prolonged use (Hajikhani et al. 2021). The active ingredient from *N. nambi* FRIM550, however, retained its activity against the MRSA BD 16876 strain. Thus, TRAI and aurisin A remain active against MRSA, even in cases where fusidic acid fails due to resistance. As expected, both MRSA strains were resistant to the β-lactam antibiotic oxacillin, but highly susceptible to the last-line antibiotic vancomycin (Table 3).

In vitro cytotoxicity

We have demonstrated that the antibacterial activity of TRAI is strongly inhibitory against reference MRSA and clinical FRMRSA strains with MIC of 3.91 and 7.81 μg mL^{-1}, respectively. These results motivated us to evaluate the cytotoxicity effect of the active ingredient by measuring the degree of the substance's destructive action on BALB/3T3 clone A31 fibroblast cells originating from mouse embryos. Cytotoxicity was assessed by the MTT assay. This is a colorimetric assay based on mitochondrial succinate dehydrogenase potential to reduce MTT. Since reduction of MTT can only occur in metabolically active cells, the activity level indicates cell viability (Mosmann 1983). It was observed that the MIC concentrations of TRAI against MRSA and FRMRSA strains (4–8 μg mL^{-1}), were much lower compared to the concentration causing significant cytotoxic effects. Results in Figure 3 showed that the percent viability of fibroblast cells was more than 80% after 24 hours of treatment with TRAI at the MIC concentration. Thus, the *N. nambi* active ingredient showed that there was no possibility of viability reductive damages in fibroblast cells at the anti-MRSA effective dose.

In vivo dermal toxicity

In the in vivo acute dermal toxicity test, a formulation containing 2% (w/w) TRAI was administered topically on animal test sites, and clinical observations were done after 24 hours of exposure. Under the test conditions, 2% TRAI did not show animal mortality and did not demonstrate any abnormal behavior during the observation period. Necropsy observations showed no gross abnormalities in all organs and the animals did not show any skin damage, including erythema and eschar or edema formation. The in vivo dermal toxicity test indicated that TRAI showed positive biocompatibility results at the test concentration. The active ingredient is non-irritant to animal

Table 3 Minimum inhibitory concentrations (MICs) of TRAI, aurisin A and standard antibiotics (FA, fusidic acid; OXA, oxacillin; VAN, vancomycin) against methicillin-resistant *Staphylococcus aureus* (MRSA)

MRSA strains	MIC value (μg mL^{-1})				
	TRAI	Aurisin A	FA	OXA	VAN
MRSA ATCC 33591	3.91	7.81	1.563	250.0	3.125
MRSA BD 16876 (FRMRSA)*	7.81	7.81	7.81	125.0	3.125

*FRMRSA, fusidic acid-resistant MRSA

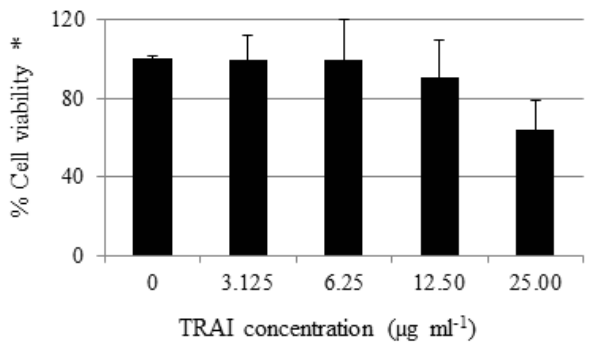

Figure 3 The survival of BALB/3T3 clone A31 (fibroblast) cell lines after 24 hours of treatment *each data is represented by mean ± standard error of mean (S.E.M.) of at least three independent experiments

skin with a median lethal dermal dose (LD_{50}) of more than 2000 mg kg^{-1} body weight and was classified as category 5 (unclassified) according to the Globally Harmonised System (GHS).

In vivo acute oral toxicity

An acute oral toxicity test was carried out in rats according to OECD TG 420-Fixed Dose Procedure guidelines to determine the level of toxicity in case of accidental ingestion of TRAI in topical formulations. Results did not show any adverse effects on the behavioral responses in the TRAI-fed animals up to 14 days of observation. The daily food and water intake of each animal measured in the 14-day toxicity test showed that all animals in the control and TRAI-treated groups exhibited consistent and normal intake values. Body weight change was an important index or indicator for assessing toxicity (Vahalia et al. 2011). All treatment and control group animals showed increased body weight (Table 4). There were no significant ($p > 0.05$) differences between the control rats and those fed with TRAI in their body weight, indicating that the test sample did not affect normal metabolism in the treated rats.

Gross macroscopic examination of vital organs such as liver, kidney, spleen, heart, lung, ovary and stomach of the treated animals revealed no abnormalities in the colour or texture when compared with the organs of the control group at the end of the study period. There were also no significant differences ($p > 0.05$) in the relative organ weight observed between the control and TRAI-treated rats. Any drastic changes in relative organ weight between the normal (control) and treated animals could serve as a useful indicator of toxicity as organ weight changes will be expressed in the suppression or increment of body weight (Satyapal et al. 2008). The results showed that TRAI did not interfere with the treated animals' normal development and growth of the internal organs. No mortality was observed after 14 days among the TRAI-fed rats at the highest test dose of 2000 mg TRAI kg^{-1} body weight. Thus, TRAI is categorised as non-toxic (GHS Category 5 or unclassified) with a LD_{50} value of > 2000 mg kg^{-1} body weight.

DISCUSSION

HPLC fingerprinting has been widely accepted for performing quality control on natural products-based active ingredients. Previously, Sunthudlakhar et al. (2022) developed a reliable HPLC method for quantitative analysis of protocatechuic acid for quality assessment of extracts from *Phellinus* mushrooms (basidiomycetes). Protocatechuic acid found in the extracts of most Phellinus spp. has a wide range of pharmacological effects including antioxidant, anti-inflammatory, neuroprotective, antibacterial, antiviral and anticancer activities (Chen et al. 2016). Thus, the compound was used as a marker and quantified using HPLC methods for quality control of the raw materials and pharmaceutical products containing the extracts. Optimisation of the conditions used in HPLC analysis is an important step for good sensitivity, feasibility, and reproducibility of data (Sunthudlakhar et al. 2022).

Neonothopanus nambi is a bioluminescent basidiomycete species available in Malaysia, as well as other tropical regions such as Thailand, Singapore and Vietnam (Chew et al. 2015). This species has been reported for its ability to produce a diverse group of bioactive secondary metabolites with nematicidal, anticancer and antibacterial activities (Tsarkova et al. 2016, Sangsopha et al. 2020, Boueroy et al. 2021, Wisetsai et al. 2021, Getha et al. 2023). However, there are not many studies on the quality and safety assessments of the bioactive natural products from this species. To date, there have been no reports on the quantification of the

antibacterial marker compound aurisin A using quantitative HPLC analysis, particularly for quality control purposes. The HPLC method used in this study for quantifying the aurisin A content in terpenoid-rich active ingredient (TRAI) of *N. nambi* FRIM550, was validated in terms of linearity, limit of detection (LOD) and limit of quantitation (LOQ). The calibration curve of aurisin A was linear in the concentration range of 10–160 µg mL^{-1} (Figure 1). The correlation coefficients (R) of the equations were higher than 0.99 indicating good linearity. Therefore, the results obtained from this experiment indicate that the developed HPLC method has good specificity and is suitable for the quality control of antibacterial (and anti-MRSA) formulations containing TRAI as the active ingredient.

The HPLC fingerprint of all three samples of TRAI produced from different batches of fermentation, exhibited quite similar chromatographic patterns and showed the peak corresponding to aurisin A at the retention time of 16 min (Figure 2). The active ingredient samples were produced by submerged fermentation of *N. nambi* FRIM550 in a stirred-tank bioreactor. Submerged fermentation is a promising alternative method for efficient production of mycelium and metabolites from basidiomycetes, compared to the usual method of solid-substrate cultivation from fruiting body. Besides reducing the cultivation time from several months to days, submerged fermentation in bioreactor enables precise measurement and strict control of critical process parameters to ensure consistent quality of the final product or active ingredients from basidiomycetes (Chaverra-Munoz & Huttel 2022).

Development of resistance to two of the most commonly used topical antibiotics, fusidic acid and mupirocin, has led to the urgent need for new topical agents for managing the treatment of bacterial skin infections (Chen et al. 2020). The antibacterial assay results demonstrated that TRAI and aurisin A are active against MRSA with a MIC of < 8µg mL^{-1} (Table 3). In contrast to fusidic acid which is used in the treatment of skin infections caused by MRSA, both TRAI and aurisin A retained the high level of activity against a fusidic acid-resistant MRSA strain. These results further supported the selection of aurisin A as a suitable antibacterial biomarker for the quality control of TRAI. The HPLC analysis developed in this study showed that the active ingredient samples contained a relatively high content of aurisin A in a range of 33–60% w/w (Table 2), suggesting TRAI as a good source for developing antibacterial formulations in future.

Over the years, there has been an increasing trend in studies on the potential use of basidiomycetes as a wound healing agent. The antibacterial, antioxidant and anti-inflammatory activities exhibited by many basidiomycetes such as *Lignosus rhinoceros,* the highly valued medicinal mushroom, play an important role in enhancing wound healing (Yap et al. 2023). Bacterial infection caused by *S. aureus* and MRSA causes prolonged inflammation which leads to delayed wound healing. Thus, an effective antibacterial agent is necessary to reduce bacterial colonisation and infection, which in turn will reduce the duration of inflammation and improve healing process (Negut et al. 2018). Previous studies by Getha et al. (2009) and Getha et al. (2023) have shown that the extracts and aurisin A of *N. nambi* FRIM550 exhibit strong activity against Gram positive bacteria, but not against Gram negative bacteria. Narrow spectrum antibacterial agents are increasingly recognised as an important advancement in bacterial infection treatment. These agents potentially provide a substantial advantage in protecting the natural microbiome by not killing the beneficial commensal bacteria. They are also preferred due to the low ecological impact, especially to control the emergence of even broader antimicrobial resistance (Diamantis et al. 2022).

When tested for cytotoxicity on BALB/c mouse embryo fibroblast cells, TRAI showed no toxicity effects in the concentration range of its anti-MRSA MIC values. Furthermore, the results from *in vivo* acute dermal and acute oral toxicity tests demonstrated that TRAI is biocompatible to animal skin and did not show any adverse effects on growth or caused mortality, respectively. Both tests also showed that when the active ingredient was administered topically or orally, no gross abnormalities were observed in the vital organs of test animals at the highest test dose of 2000 mg kg^{-1} body weight. These collective properties make TRAI an appealing active ingredient candidate for

use against MRSA skin infections, particularly in cases of fusidic acid resistance. An important next step will be to develop a suitable topical formulation incorporated with TRAI, such as cream, ointment or hydrogel, and evaluate the formulation with regards to both the efficacy against MRSA and also toxicity.

CONCLUSION

The current study developed a HPLC method suitable for quantitative analysis of the content of aurisin A, an antibacterial marker compound, as well as quality assessment of the terpenoid-rich active ingredients (TRAI) produced by mycelial culture of *N. nambi* FRIM550. Our findings showed that TRAI and biomarker aurisin A exhibit strong inhibition against methicillin-resistant *S. aureus* (MRSA) and a clinical MRSA strain with acquired fusidic acid (FRMRSA) resistance. The complex and intricate process of wound healing has a severe impact on patient's life as well as causing economic burdens in healthcare institutions. People with poor healing abilities like the elderly, chronically ill, bedridden or non-ambulatory are more susceptible to wound infection because of their declining immune systems (Yousefian et al. 2023). *Staphylococcus aureus,* a leading cause of skin infections, is the causative agent in up to 75% of primary pyoderma. An increased prevalence of infections in hospitals and community settings caused by MRSA, has contributed to an increase in the number of skin infections reported in recent years (Molne & Tarkowski 2000). Control of infection in wounds is mainly achieved by bactericides and/or antibiotics. Terpenoid-rich active ingredients from strain FRIM550 may function to provide an efficacious treatment option for extensive topical infections caused by drug-resistant *S. aureus*, which are not resolved with bactericides within appropriate timeframe. Methods of delivering the active ingredients to infected wound sites include formulations such as ointments, creams or other suitable wound dressing products. Thus, we conducted toxicity analysis on TRAI to support its practical application in such products. Our findings revealed favorable *in vitro* and *in vivo* toxicity profiles for TRAI, whereby the cytotoxicity study showed no possibility of viability reductive damages in fibroblast cells at the anti-MRSA effective dose. The active ingredient also provided a good level of skin biocompatibility in the acute dermal toxicity study. In addition, oral administration of TRAI in rats did not cause acute toxicity at the highest dose of 2000 mg kg^{-1} body weight. Further investigations on TRAI using topical wound infection animal models will be important to show the efficacy of *N. nambi* FRIM550 active ingredients in wound healing.

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