ABSTRACT
Alpinia galanga (L.) Willd. (Zingiberaceae), or galangal, has been previously reported as active against Mycobacterium tuberculosis (TB) in vitro. The present study assessed a novel antitubercular mechanism of galangal through M. tuberculosis shikimate kinase (MtSK) inhibitory assays. Sequential extractions of nonpolar solvents hexane and dichloromethane (DCM) were performed on galangal and screened in MtSK inhibitory assays to identify potential activity. Samples were then subjected to high resolution (HR) LC–MS chemical fingerprinting and analysis. Additionally, a novel approach was undertaken for galangal using methods such as mass professional profiler (MPP) and global natural products social (GNPS) molecular networking for structure elucidation.

1. Introduction
Mycobacterium tuberculosis (Mt) is one of the top 10 causes of death worldwide and is the leading cause of death from a single infectious agent (WHO, 2020). In 2018...
alone, a total of 10 million people contracted TB, with 1.5 million people dying from the disease. Drug resistant strains, labelled as multi- (MDR) and extensively-drug resistant (XDR), have a low successful treatment rate, with only 56% being successfully treated (WHO, 2020).

The emergence of drug resistant strains demonstrates the need for further research on new mechanisms of action that can be utilised to combat TB. *Alpinia galanga*, (Zingiberaceae) or galangal, has been reported to have antitubercular activity and has been used in traditional Ayurvedic, Unani, Chinese and Thai folk medicine (Chouni & Paul, 2018). This study utilised galangal extracts to assess a novel mechanism of inhibition of the fifth step in the MtSK pathway; a step in which shikimate 3-phosphate (S3P) is produced.

A sequential extraction on galangal rhizomes was performed with nonpolar compounds hexane and DCM, which each afforded a partition. In addition, a known galangal antitubercular compound, 1β-s-1′acetoxychavicol acetate (ACA) (Warit et al. 2017), and a known inhibitor, rottlerin, were utilised for comparison. Nonpolar galangal extracts have not previously been assessed for inhibition of Mtb via this mechanism. Thus, a novel LC–MS based inhibitory assay was designed to test the inhibition of S3P in the presence of each aforementioned test sample. To identify putative bioactive compounds within the molecular network, the partitions were subjected to targeted LC–MS–MS, mass professional profiler (MPP) and GNPS analysis. MPP analysis was utilised for deducing the relative abundance of major compounds in the partitions. Following MPP, GNPS molecular networking was integrated for timely structure elucidation and identification. GNPS is an interactive, small molecule-focused tandem mass spectrometry (MS/MS) data curation and analysis infrastructure. Use of MPP and GNPS in this study represents a new approach for galangal rhizomes that was beneficial by both reducing time for chemical fingerprinting and profiling and increasing accuracy of compound identification.

Compounds not previously reported in galangal were identified through these methods. Acacetin (6) and Cirsimaritin (7) have not been previously reported in the literature as being present in the Zingiberaceae family, Alpinia genus or galanga species. Naringenin (4) and 1,7-diphenyl-4-hepten-3-one (5) have only been found in the Alpinia genus and not the galanga species. Three compounds previously identified in galangal were also found, with those compounds being genistein (1), pinocembrin (2) and kaempferide (3).

2. Results and discussion

2.1. *Mycobacterium tuberculosis shikimate kinase inhibitory activity*

The hexane extract was classified as active and displayed an MtSK inhibition of 47%. The other samples were considered as inactive; with rottlerin having an inhibitory rate of 36%, followed by DCM with 27% and ACA with 10%. The activity of the hexane extract can potentially be attributed to compounds working synergistically within the novel MtSK pathway inhibition mechanism that was assessed, as ACA alone was inactive.
2.2. **Auto MS–MS and MPP analysis**

Auto MS–MS was used to identify fragmentation of compounds within the samples. The data from these experiments were transferred to MassProfiler to be converted into a form suitable for MPP analysis, which was performed to compare the partitions and identify any similarity in composition. Next, batch recursive feature extraction and data alignment of filtering by occurrence was utilised to create a Venn Diagram shown in the Supplementary Material as Figure S1. The hexane partition underwent compound identification due to its activity. Compounds selected for further elucidation efforts via targeted MS–MS were pulled from the MPP analysis.

2.3. **Targeted LC–MS–MS and GNPS analysis**

Targeted MS–MS was utilised to confirm compound presence. Compounds were identified through retention times, exact masses, spectral data and ensuing literature searches. To identify entities from targeted MS–MS the MassBank of North America (MoNA) was used, which serves as a centralised database of metabolite mass spectra, metadata and associated compounds. GNPS analysis was utilised to identify additional compounds as it performs library searches in a manner that is faster than doing so by hand. Prior to GNPS utilisation, only compound 5 has been identified (MoNA, 2020b). GNPS was able to quickly and effectively discern compounds 1–4, 6 and 7 in the samples (Shang et al. 2017; Ristivojevic et al. 2015; Fabre et al. 2001; MoNA, 2020a). It did so in an efficient time-frame by comparing compounds against their public spectral database. Identified compounds were then further visualised in a molecular network to compare chemical similarities.

Compounds 1–3 have been previously reported in galangal while compounds 4 and 5 were reported in the *Alpinia* genus. In Table S1, shown in the Supplementary Material, the identified compounds are listed along with the confidence level of the identification measured by the standards set in the publication by Johnson and Carlson (2015). These seven compounds are present in both partitions and could act as potential MtSK inhibitors. Through various literature searches, it has been identified that genistein, naringenin (Chen et al. 2010), pinocembrin (Chou et al. 2011), cirsimartitin (Mujovo et al. 2008) and acacetin (Suksamrarn et al. 2004), have shown antimycobacterial activity against *Mycobacterium tuberculosis* H$_{37}$R$_{v}$ *in vitro* with MIC values of 35, 2.8, 3.5, 200 μg/mL and 704.2 μM, respectively. The respective chemical structures of the seven compounds are illustrated in Figure 1.

3. **Experimental**

The experimental information is supplied in the Supplementary Materials.

4. **Conclusions**

This study assessed a novel MtSK inhibitory mechanism exerted by nonpolar *A. galanga* extracts. The hexane extract of galangal displayed newly identified inhibitory
activity of the pathway and is reported for the first time here. By using MPP and GNPS, the study additionally utilised original approaches in the investigation of galangal constituents for timely and effective structure elucidation. Seven compounds were identified, with four compounds (4–7) not previously being reported in galangal.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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