Indian Journal of Traditional Knowledge
Vol 24(2), February 2025, pp 146-157
DOI: 10.56042/ijtk.v24i2.6368

Phytochemical characterization and evaluation of the antibacterial activity of *Amruthotharam kwath*, a traditional polyherbal formulation on multidrug resistant clinical isolates from the respiratory tract

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Received 03 January 2024; revised 27 December 2024; accepted 03 February 2025

Antibiotic resistance is a multifaceted medical issue affecting the global healthcare sector. Despite continued research and development to combat antibiotic resistance, the development of new antibiotics lags behind the rates of drug resistance. Among the various research strategies under development, traditional polyherbal formulations have greater potential to resolve infectious conditions caused by antibiotic resistant pathogens. Unlike monotherapy antibiotics, polyherbal formulations contain a variety of secondary antibacterial metabolites and their enhanced synergistic action renders them potent therapeutic agents. Multitarget action of phytocompounds at low concentrations poses very low selection pressure that prevents bacteria from developing new resistance mechanism against polyherbals. The study was focused on exploring the antibacterial efficacy of a traditional polyherbal formulation *Amruthotharam kwath* which had been used to treat respiratory tract infections, one of the leading infectious conditions prevalent in our country. Respiratory tract infection caused by multidrug resistant gram-negative pathogens leads to ineffective empirical treatments and worsens the situation. The study involves preliminary phytochemical analysis, quantification of phytoconstituents, metabolite profiling of the formulation and its antibacterial effect on multidrug resistant clinical isolates from the respiratory tract. The phytochemical analysis revealed the presence of various classes of phytocompounds with antibacterial activity. Antimicrobial assays reported promising antibacterial effect. MIC values were found to be in the range of 6.25 mg/mL-12.5 mg/mL and its MBC ranges from 6.25 mg/mL-128 mg/mL. The formulation exhibited bactericidal effect on majority of the isolates.LC-MS/QToF revealed the presence of 76 secondary metabolites, majority of which possessed antibacterial activity. The SEM image analysis confirmed complete destruction of cell wall as well as cell membrane of the pathogens. This study suggests the promising herbal therapy of *Amruthotharam kwath* which can be proposed as an alternative for treating infections caused by multidrug resistant pathogens.

Keywords: Antibiotic resistance, Metabolite profiling, Synergistic action, Traditional polyherbal formulation

IPC Code: Int Cl.[25]: A61K 36/00, A61K 9/00

Medical professionals, governments, and international organizations are increasingly reporting on antimicrobial resistance (AMR), as one of the most important public health issues. According to World Health Organization (WHO), AMR is among the top 10 global public health hazards to humanity and poses a threat to human well-being globally[1]. The Lord Jim O'Neill report stated that by 2050, AMR might kill 10 million people annually[2]. The world will eventually run out of antibiotics, according to a 2017 World Health Organization report[3], as the current drugs in clinical use were created by altering the existing classes and have demonstrated minimal impact cycles. Additionally, according to a prior prediction by the US Center for Disease Control (CDC, 2019 AR Threat Report), there will be roughly 23000 deaths in US each year due to microbes that are resistant to antibiotics[4]. Drug resistance index (DRI) has been developed as an important parameter to analyze the effectiveness of antibiotic treatment regimes. DRI of India is relatively high indicating lowest relative effectiveness of antibiotic therapy[5]. Antibiotic consumption increased from 3.2 to 6.5 billion (103%) defined daily doses (DDDs) in India during 2000 to 2015[6]. As per WHO report, based on AWaRe (Access, Watch and Reserve) categorization of drugs it was found that there is a high degree of consumption of third generation cephalosporin in India. This report can be correlated to high degree of cephalosporin resistance patterns reported[7]. A recently published report revealed a fatal outbreak of colistin resistant, carbapenem resistant

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Klebsiella pneumoniae in a tertiary care hospital in Delhi which was later contained by effective infection control interventions[8].

The development of resistance was found to be linked with the antibiotic abuse or oversuse[9]. According to CDC, Multidrug Resistant Organisms (MDROs) are those pathogens showing non susceptibility to at least one antibiotic in three or more than three categories of antibiotics[10]. Respiratory tract infection is one among the most prominent category of infectious disease reported to involve multidrug resistant pathogens. Even though respiratory tract infections are caused by bacteria as well as viruses, 60% of the viral respiratory tract infections are followed by secondary bacterial infection[11] due to antibiotic resistant pathogens that makes the situation even worse. In the upcoming years, the existence of MDR-GNB (Gram Negative Bacteria) and the absence of novel antibiotic development pose a serious danger to respiratory medicine[12]. This can lead to an increased mortality rate and a greater economic burden to the nation. This study makes an attempt to explore the effectiveness of a polyherbal classical preparation prescribed generally by Ayurveda Physicians for treating infectious conditions especially respiratory tract infections. The National Action Plan (NAP) and The Kerala State Antimicrobial Resistance Strategic Action Plan (KARSAP) emphasize on developing herbal remedies as an alternative therapy to address this problem[13].

The Indian traditional medical system has contributed a good number of traditional herbal formulations as drugs which millions of people are using today for various ailments. The herbal drugs often contain multiple compounds that can interact with multiple targets in the body. This is often referred to as the "polypharmacology" of herbal medicines. Unlike allopathic drugs, which typically focus on single-molecule therapy, herbal drugs utilize a combination of compounds that can have synergistic effects[14]. In allopathic medicine, the use of single-molecule drugs can create a strong selective pressure on pathogens, leading to the development of resistance. However, with herbal drugs, the complex mixture of compounds makes it challenging for pathogens to develop resistance mechanisms that can effectively counteract the multiple targets and actions of the drug. Furthermore, herbal drugs often work through various mechanisms of action simultaneously, making it more difficult for pathogens to adapt and develop resistance. The synergistic effects of multiple compounds in herbal drugs can enhance their therapeutic efficacy while reducing the likelihood of resistance. This unique feature sets polyherbal apart from other research interventions, such as quorum sensing inhibitors, phage therapy, antimicrobial peptides, nanoparticles, and vaccines, which typically focus on a single target or a specific MDR strain. Systemic monotherapy techniques may not be sufficient in a future where drug resistance outpaces novel therapeutic discovery. The presence of multiple mechanisms of resistance within the bacterial population makes it difficult to eliminate the infection using standard antibiotic treatments. It may be unable to tackle the trends in resistance by the development of new drugs or adjustments to the available options. Instead, novel combinations of biologically inspired agents may be more effective in combating antibiotic resistance

This interdisciplinary study intends to provide light on the antibacterial activity of *Amruthotharam kwath* (AK), a therapeutically established composition, with a particular emphasis on its efficacy against multidrug-resistant clinical isolates from the respiratory tract. Ayurveda physicians have traditionally employed AK, a popular polyherbal preparation, to treat infectious diseases especially respiratory tract infection and long-term fevers. It has a 3:2:1 combination of the herbs *Tinospora cordifolia* (Amruta), *Terminalia chebula* (Haritaki) and *Zingiber officinale* (Sunthi). This combination of herbs offers unique therapeutic properties that contribute to the overall efficacy of AK. The aqueous stem extract of *Tinospora cordifolia* reported promising broad spectrum antibacterial activity[15]. Antibacterial studies on *Terminalia chebula* fruit extract showed appreciable effect on gram positive and gram-negative pathogens[16]. AK was one of the drug recommended by AYUSH (Ayurveda. Yoga and Naturopathy, Unani, Siddha and Homeopathy) for post Covid conditions for improving the immunity and to tackle secondary bacterial infection[17]. The study utilizes HPTLC for confirming the presence of biomarker in all the batches, since it is the most recommended and routinely used analytical technique for handling multiple samples for fingerprinting, standardization and quantification of polyherbals[18].

Materials and Methods

Preparation and characterization of the formulation

Collection and authentication of raw drugs

The raw materials required for the preparation of the drug: the stem of *Tinospora cordifolia*, fruit pericarp of *Terminalia chebula* and rhizome of *Zingiber officinale*

were collected from the local market and were authenticated at the Raw Material Divison, Care Keralam Koratty. The herbarium of sample specimens were deposited (*Acc No: 18845 Terminalia chebula; Acc No: 18846 Zingiber officinale*; *Acc No: 19371 Tinospora cordifolia*) at Kerala Forest Research Institute (KFRI) Peechi for future reference.

Preparation of the polyherbal formulation

The AK was prepared as per the procedure described in Sahasrayogam[19]. The AK was prepared by combining the crushed stem of *Tinospora cordifolia*, crushed fruit pericarp of *Terminalia chebula* and crushed dried rhizome of *Zingiber officinale* in a 3:2:1 ratio. The herbal mix of 48 g was taken and added to 16 times water (768 mL) and boiled under low flame until the volume reduced to one by eighth of the initial volume. 6 batches were prepared. One market sample was also purchased (Mfg: Dec. 2020 and Exp: Nov. 2023) from one of the most trusted brand in Kerala for further HPTLC analysis and comparison.

Organoleptic evaluation

The freshly prepared three batches of AK was checked for its organoleptic parameters like color, odor, taste and state of matter using sensory organs, based on the protocol[20] as follows. The freshly prepared *kwath* samples were given to a panel of ten members. Organoleptic parameters were assessed based on their feedback.

Physicochemical parameters

AK was checked for consistency in various physicochemical parameters like specific gravity using specific gravity hydrometer (Tel-Tru, Model no: 100A, Range: 1.000-1.2000, Delhi), brix using hand refractometer (Shiv Sceintific, Model No:511, Range 0-32%, Delhi), total dissolved solids using digital TDS meter (Esico International, Model No: 651, Haryana), pH using digital pH meter (Infra Instruments Pvt Ltd, Chennai; Model no: IR 50/A) and reading were recorded.

Phytochemical screening

The formulation was screened for analyzing the presence of various groups of phytoconstituents. The following qualitative tests were performed: Dragendorff's test for alkaloids, Shinoda test for flavonoids, Ferric chloride test for tannins, Froth formation test for saponins, Picric acid test for glycosides and Benedict's reagent test for carbohydrates[21].

Quantification of major phytocompounds

The formulation was quantitatively estimated for major phytocompounds like alkaloids, tannins and phenols. The phenolic content was expressed in terms of milligram equivalents of gallic acid per gram of sample[21,22], the alkaloids were quantified by sequential extraction using chloroform[20] and the tannins were quantified using titrimetric method[23].

Microbial load

The freshly prepared formulation was tested for the presence of bacteria and fungi by plate count method[21].

Heavy metal analysis

HNO_3 and $HCLO_4$ in the ratio 5:2 were used to digest the formulation. The digested sample was then diluted to 100 mL using deionized water and analysed using an ICP-AES system (Thermo Electron, IRIS INTREPID II XSP DUO, Munich, Germany). The detection limits were Cd (0.01 ppm), Pb (0.05 ppm), Hg (0.1 ppm), and As (0.04 ppm).

High Performance Thin Layer Chromatography (HPTLC)

Three batches (AK 1, AK 2, AK 3) of freshly prepared AK, lyophilized AK (AK 4) sample and a market sample (AKM) were subjected to HPTLC (CAMAG, Switzerland) analysis along with another batch (AK 5) prepared at high temperature(>100ºC). The samples were run along with gallic acid as the biomarker compound. The samples were dried in a water bath at a fixed temperature and were extracted with methanol. 8 µL of the samples were spotted on HPTLC silica gel 60F 254 (Merck) plate as bands of length 8 mm at a distance of 10 mm. The mobile phase giving the best separation of components in all the 7 samples was optimized as Chloroform: Ethyl Acetate: Formic Acid (5:4:0.1). The plates were kept for development in the CAMAG twin-trough glass chamber, which was saturated with the solvent for 30 min. After development, the plates were dried in an oven at 60ºC and scanned in absorbance mode with a CAMAG TLC Scanner. The data was processed using the win CATS planar chromatography manager programme. At 254 and 366 nm, the compounds were scanned.

Antimicrobial studies

Collection of MDR isolates

The study received Institutional Ethics Committee approval (IEC.No.15/20/IEC/JMMC&RI) to collect MDR bacteria from Department of Microbiology, Jubilee Mission Medical College and Research

Institute (JMMC&RI), Thrissur. The isolates were obtained from sputum sample collected from patients with respiratory tract infection using standard microbiological techniques. The antibiotic susceptibility test (AST) was done using automated method (Vitek-2) and interpreted using CLSI 2020 guidelines (Table 1). These MDR isolates were collected and were sub cultured and stored in agar slants, at 4ºC.

Agar well diffusion assay

Preliminary evaluation of the antibacterial activity was carried out using agar well diffusion assay. Overnight cultures were kept for 24 h at 36ºC±1ºC, after incubation, bacterial suspension (inoculum) was diluted with sterile saline and standardized to 0.5 MacF. The media was given time to solidify. Using a sterile cup-borer, wells measuring 6 mm in diameter were cut into the solidified agar medium. 100 µL (100 mg/mL, 50 mg/mL, 25 mg/mL) of the *kwath* was added into sample wells. Standard antibiotic Amikacin (HiMedia) was used as positive control. To verify that all results were consistent, the experiment was run in triplicate under very rigorous aseptic conditions. The antibacterial activity was expressed in terms of the mean of diameter of zone of inhibition ± standard deviation at the end of incubation period

Microtiter plate dilution assay

Microtiter plate assay was carried out as per Clinical and Laboratory Standards Institute (CLSI) guidelines[24]. According to CLSI, the Minimum Inhibitory Concentration (MIC) is the antimicrobial agent concentration at which the organism's growth in the microdilution wells is totally inhibited, as seen with the naked eye[25]. Minimum Bactericidal Concentration (MBC) is defined as the lowest concentration of the antibiotic that could kill 99.9% of the inoculum. The experimental conditions were standardized using *E. Coli* (ATCC 25922) using Amikacin as standard antibiotic. MIC (4 µg/mL) value found to be in compliance with CLSI guidelines[24]. 24 h grown culture is inoculated into fresh media and its turbidity was adjusted to 0.5 McFarland. 100 µL of AK (100 mg/mL) was added to first wells and two fold diluton was carried. The culture was diluted to 1:20 to yield $5*10^6$ CFU/mL. 0.01 mL of the above suspension was inoculated into the microtiter well to yield the final test concentration of bacteria was approximately $5*10^5$ CFU/well. For each culture a sterile control was maintained, a growth control was maintained where no AK was added. and a positive control where standard antibiotic was kept. After 20 h of incubation at 35ºC, 30 µL visualising agent, Resazurin (15% w/v, HiMedia) was added to all wells and MIC was determined after two hours of incubation with the dye[25]. Metabolically active bacteria reduced the blue/purple dye to pink/red color. MBC was found by inoculating a loopful of culture from each well with concentration at MIC and concentration greater than MIC and checked for the presence of colonies.

Statistical analysis

MIC values of the *kwath* was analysed by independent t test using SPSS software. Significance of statistical test was predetermined at $p<0.05$.

Time kill assay

The best technique for figuring out the bactericidal or fungicidal effect is the time-kill test. A time-dependent or concentration-dependent antibacterial

Table 1 — Antibiotic resistance profile of the bacterial isolates

Test Bacteria	ID	A	AK	C3	C4	CFS	CIP	COL	COT	G	M	TG
A. baumanii	ISL-1	R	S	R	R	S	R	I	R	I	S	R
K. pneumoniae	ISL-2	R	S	R	R	S	R	I	R	R	S	S
P. aeruginosa	ISL-3	R	S	I	S	S	I	I	I	R	R	R
K. pneumoniae	ISL-4	R	S	R	R	S	S	R	S	s	R	R
K. pneumoniae	ISL-5	R	S	S	R	S	S	S	R	S	R	S
K. pneumoniae	ISL-6	R	S	R	R	S	R	S	R	R	R	R
K. pneumoniae	ISL-7	R	S	S	S	S	S	R	R	S	R	R
K. pneumoniae	ISL-8	R	S	R	R	S	R	S	R	R	R	R
K. pneumoniae	ISL-9	R	S	R	R	S	R	S	R	R	R	R
P. aeruginosa	ISL10	R	S	I	S	S	S	S	R	S	R	R

A-Ampicillin; AK-Amikacin; C3- Ceftriaxone; C4- Cefipimine; CFS-Cefoperazone Sulbactam; CIP-Ciprofloxacin; COL-Colistin; COT-Co-trimoxazole; G-Gentamycin; M-Minocyclin; TG-Tigycycline
R-Resistant; S-SensitiveI-Intermediate

action is revealed by the time-kill test. The assay was carried out as per the CLSI guidelines[26]. The standardized suspension (1 McFarland standard) was diluted to 1:5 by adding 1 ml of suspension to 4 mL of MHB yielding 6 X 10^7 CFU/mL. 0.1 mL of inoculum containing 6 X 10^7 CFU/mL was added to each tube containing 10 mL MHB containing 2 MIC, MIC, standard antibiotic (MIC) and one tube as growth control. Immediately after inoculation, 0.1 mL was removed from each reaction tube to calculate bacterial plate counts at time zero. Incubate tubes at 35±2°C immediately after sampling. Colony count on each bacterial count plate was taken at time intervals of 2,4,6,8 and 24 h. Log CFU/mL (y axis) versus time (x axis) was plotted to analyse the mode of action.

SEM analysis

To investigate the morphological alterations occurred in isolates, 2 MDR isolates (treated and non-treated) were taken for SEM analysis using (Make: TESCAN; Model VEGA 3, Czech Republic). The isolates were exposed to treatment at their MBC, followed by overnight incubation with 2.5% glutarldehyde[27]. The samples were centrifuged and the glutaraldehyde layer was removed. The isolates were then subjected to ethanol treatment at varying concentration (25% 50% 75% and 100%) at varying exposure time. The samples were freeze dried (Operon FDU 7003, ¯55ºC- ¯70ºC) and underwent sputter coating with gold and viewed under SEM.

Fig. 1 — Freshly prepared AK

Metabolite profiling by Liquid Chromatography-quadrupole Time-of-flight Mass Spectrometry (LC-MS/MS/QToF)

The metabolite profiling of AK was done by LC-MS/MS/QToF (Model- 6545, Agilent USA). The instrument was equipped with electrospray ionization source with a mass to charge range 100-10,000. Suitable amount of the supernatant of the sample after centrifugation is pipetted out and washed with Petroleum ether to remove organic layer and mixed with Ethanol to precipitate the protein. Centrifuged the remaining solution at 10000 rpm for 20 min, the supernatant was then transferred to a centrifuge tube and vacuum concentrated at 48ºC. 10 mg of the residue was mixed with Methanol and syringe filtered, injected to LC-MS chromatography set up.

Results

Organoleptic evaluation

The freshly prepared *kwath* (Fig. 1) was a colloidal suspension and one could observe fine particle settled at the bottom after 30 min of preparation. Table 2 shows the evaluation of organoleptic parameters.

Physicochemical evaluation

The specific gravity of the *kwath* was found to be in the range of 1.024-1.033 for all the batches. The pH was found to be in the range of 4 - 4.5. and brix value was in the range of 8-11.

Preliminary phytochemical screening and quantification

Table 3 shows the phytochemical screening of the *kwath* and its ingredients The screening confirmed the

Table 2 Organoleptic evaluation of the three batches of *Amruthotharam kwath*

Sample ID	Color	Odor	Taste
AK 1	Brown Color	Characteristic odor.	Pungent
AK 2	Brown Color	Characteristic odor.	Pungent
AK 3	Brown Color	Characteristic odor.	Pungent

Table 3 — Preliminary phytochemical screening of the formulation and its ingredients

Phytochemical test	Phytoconstituent	*AK*	*T. cordifolia*	*T. chebula*	*Z. officinale*
Dragendorff' reagent method	Alkaloids	++	++	++	Nil
Shinoda test	Flavanoids	Nil	Nil	Nil	Nil
Picric acid test	Glycoside	Nil	Nil	Nil	Nil
Folin Ciocalteu reagent test	Phenol	++	++	++	++
Foam test	Saponins	++	Nil	Nil	++
Ferric Chloride test	Tannins	++	++	++	Nil
Benedict reagent test	Carbohydrate	++	++	++	++

++ indicates presence of constituents

presence of alkaloids, phenols, tannins, saponins and carbohydrate in the *kwath*. The prominent groups of phytocompounds were quantified and was found to be as follows: alkaloids: 0.21%; phenols: 2.42%; tannins: 3.46%.

Microbial load

Microbial analysis revealed that the AK was free from both aerobic bacterial and fungal contaminants.

Heavy metal analysis

The heavy metal analysis revealed the absence of cadmium, lead and mercury but arsenic was present in trace amounts. The concentration was much below than the specified allowed limit.\

HPTLC

(Fig. 2 & Fig. 3) depicts the HPTLC pattern of 6 *kwath* samples along with gallic acid as the marker compound.

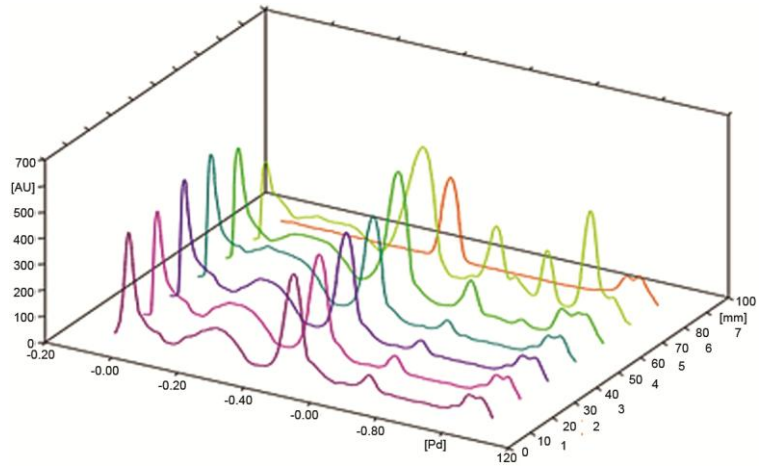

Fig. 2 — HPTLC Densitogram- Track 1: AK 1, Track 2: AK 2. Track 3: AK 3; Track 4: AK 4, Track 5: AK 5, Track 6: AKM; Track 7: Gallic acid (Marker compound)

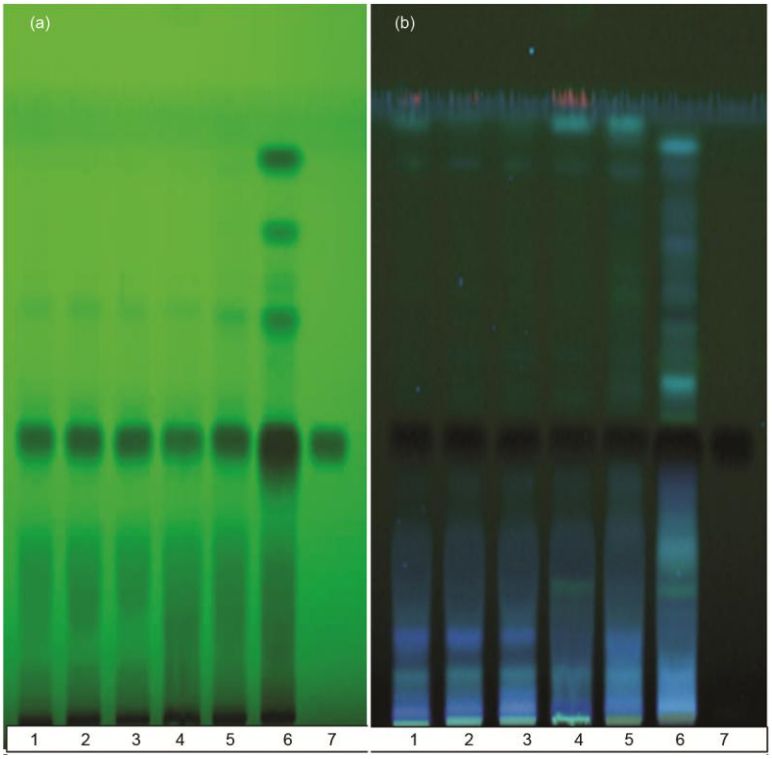

Fig. 3 (a) — HPTLC plate under 254 nm. (b) HPTLC plate under 366 nm): Track 1: AK 1, Track 2: AK 2; Track 3: AK 3; Track 4: AK 4, Track 5: AK 5; Track 6: AKM; Track7: Gallic acid

Antimicrobial studies

Table 4 & Table 5 shows the results of agar well diffusion assay and microtiter plate assay respectively. The results showed promising antibacterial effect of the *kwath* on MDR isolates. (Fig. 4) shows the mode of action of the *kwath* on the microbial cells.

Metabolite profiling

Metabolite profiling identified the presence of 76 secondary metabolites (supplementary data) in the kwath. (Fig. 5a) depicts the LC-MS profile of the *kwath* followed by mass spectrum of quercetin and the prominent marker compound gallic acid (Fig. 5b & Fig. 5c) respectively.

SEM analysis

The intact rod shaped *bacilli* were clearly visible in SEM images (Fig. 6a & Fig. 6c) and the treated cells lost their membrane integrity and clumps of cells were visible in the SEM images (Fig. 6b & Fig. 6d).

Discussions

Organoleptic evaluation aids in preliminary quality assessment of crude drug based on the perception by the sense organs. The organoleptic parameters of

Table 4 — Well diffusion assay

Isolate ID	Zone of Inhibition of AK/Antibiotic			
	25 mg/mL	50 mg/mL	100 mg/mL	Standard antibiotic
ISL-1	20.7±0.15	21.3±0.16	21.3±0.15	14.0±0.2
ISL-2	14.9±0.93	18.7±0.11	23.3±0.06	10.9±0.83
ISL-3	16.7±0.11	12.7±0.15	12.7±0.15	20.7±0.15
ISL-4	14.0±0.2	20.0±0.2	22.7±0.06	18.0±0.46
ISL-5	20.7±0.15	21.3±0.16	21.3±0.15	14.0±0.2
ISL-6	13.3±0.31	16.0±0.2	16.7±0.31	17.3±0.61
ISL-7	9.3±0.15	17.3±0.15	20.0±0	16.0±0.2
ISL-8	16.7±0.15	18.0±0.29	20.0±0.6	14.7±0.31
ISL-9	7.3±0.15	17.3±0.15	18.0±0.6	16.7±0.06
ISL-10	16±0.83	19.5±0.1	22.5±0.1	16.0±0.63

Table 5 — Microtiter plate assay

Isolate ID	*Amruthotharam kwath*		Standard antibiotic	
	MIC (mg/mL)	MBC (mg/mL)	MIC (µg/mL)	MBC (µg/mL)
ISL-1	6.25	50	16	128
ISL-2	12.5	25	4	16
ISL-3	6.25	6.25	4	8
ISL-4	6.25	12.5	4	8
ISL-5	6.25	12.5	4	4
ISL-6	6.25	25	2	4
ISL-7	6.25	12.5	4	32
ISL-8	6.25	25	32	128
ISL-9	6.25	25	32	32

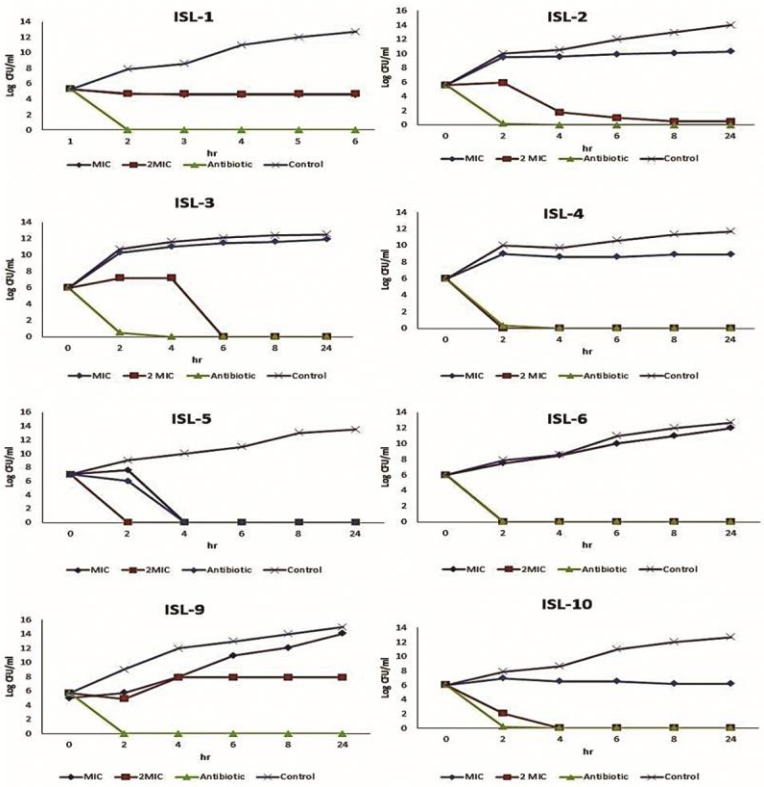

Fig. 4 — Time kill assay kinetics (ISL-1-ISL-10)

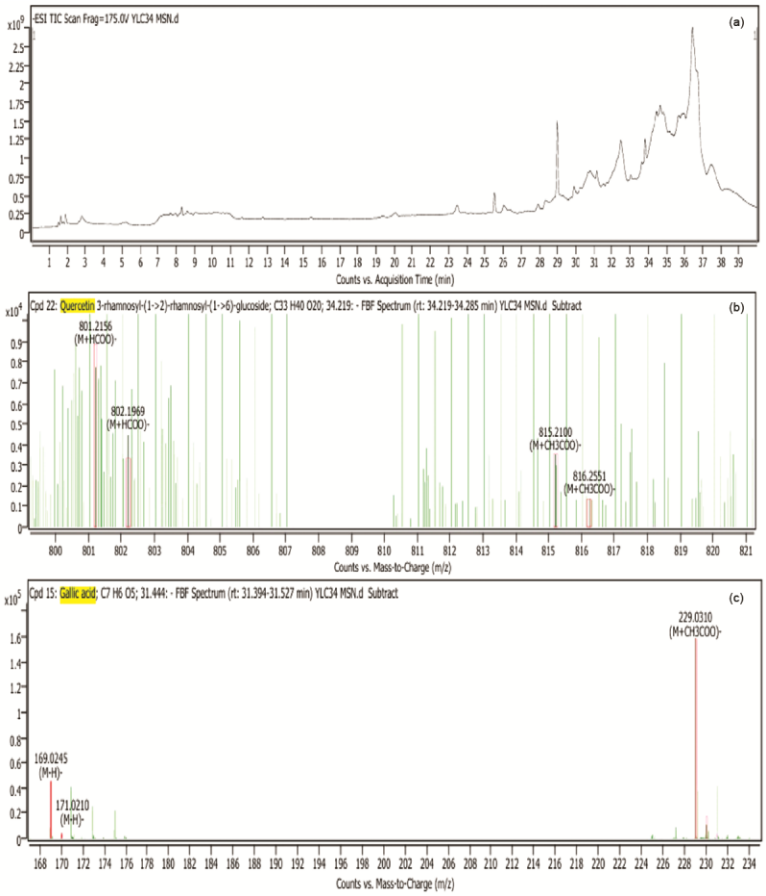

Fig. 5 — Metabolite profiling of AK by LC-MS/QToF. (a) LC-MS/QToF spectrum. (b) The mass spectra of the prominent marker Gallic acid. (c) Mass spectrum of quercetin in AK

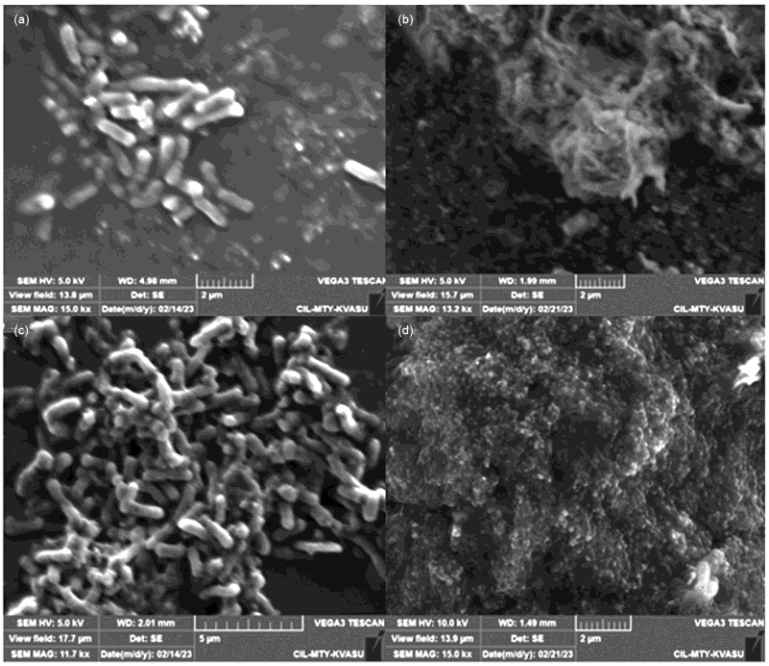

Fig. 6 — SEM Imaging (a) Before treatment with AK (1SL-2). (b) After treatment with AK (1SL-2). (c) Before treatment with AK (ISL-3). (d) After treatment with AK (ISL-3)

6 batches of *Amruthotharam kwath* such as color, appearance, and sensation by tongue were checked and were identified as brown in color, colloidal in appearance, and bitter in taste. Phytochemical examination could unlock the diverse group of chemical compounds in plants. The preliminary phytochemical analysis revealed the presence of alkaloids, phenols, saponins, and tannins in the *kwath.* The literature survey reported different antibacterial mechanisms for alkaloids which includes inhibition of DNA and protein synthesis, causing damage to cell wall and cell membrane, inhibition of efflux pumps and inhibition of bacterial metabolism[28]. Phenolic compounds were also found to associate with destruction of bacterial cell membrane[29]. Khan *et al*. (2018) reported that the antibacterial action of saponins is caused by damaging cell wall[30]. Hence the presence of these classes of phytocompounds could contribute to the therapeutic efficacy of the formulation. Evaluation of organoleptic properties, physicochemical properties, heavy metals analysis, and the absence of microbial contaminants indicates quality and the authenticity of prepared lab samples.

In order to ensure batch to batch consistency of the formulation, 6 batches of the *kwath* were subjected to HPTLC analysis, which produced a fingerprint pattern with similarity in the number, retention factor, intensity, and colour, indicating that the active constituents present in all batches were similar aiding in the establishment of batch-to-batch consistency of the formulation. Lyophilization could be suggested for long term storage of the sample and for future reference since there are not many variations observed in AK 4 fingerprint compared to other batches. Additional secondary peaks were observed in the market sample (AKM) that could be due to the presence of preservative added or due to the formation of secondary products by the cross reaction of active components with preservative. HPTLC confirmed the predominance of the marker compound (gallic acid) in all the 6 samples of the *kwath*.

Agar well diffusion assay is the preliminary evaluation of antibacterial effect and the results showed clear zone of inhibition by the *kwath* on all isolates. The tested isolates showed appreciable zone of inhibition in the assay with values ranging from 16.67±1 mm to 22.67±5 mm at 100 mg/mL of the *kwath*. MIC values were found to be in the range of 6.25 mg/mL-12.5 mg/mL and its MBC ranged from 6.25 mg/mL-128 mg/mL. Statistical analysis revealed higher values for average MIC values of the *kwath* when compared to that of standard antibiotic. The higher MIC and MBC values observed for the polyherbal formulation could be attributed due to the complex composition of the formulation, the presence of various bioactive compounds at lower concentrations and the potential influence of extraction method. These factors should be taken into account when assessing the efficacy of polyherbal formulations against microorganisms. This justifies the prescription of higher doses of the herbal extract by physicians to ensure an adequate amount of the active compounds reach the target site in the body to exert a therapeutic effect. Time kill assays are commonly used to evaluate the effectiveness of antimicrobial agents against bacteria. These assays measure the reduction in bacterial population over a specified time period when exposed to a drug. The kill kinetic profiles displayed varied degrees of bactericidal (ISL-2, ISL-3, ISL-4, ISL-5, ISL-6, ISL-10) and bacteriostatic (1SL-1, ISL-7, ISL-8, ISL-9) activity. The killing rate of the *kwath* was slower in the case of ISL-2, ISL-3, ISL-5, ISL-10 where the bactericidal activity was seen only after 4 h of incubation at 2X MIC. The variations in the time kill pattern of a drug can indeed be influenced by the resistance profile of drug-resistant bacteria and also the tested strain. The antibacterial activity may be due to the concerted effect of tannins, alkaloids and various phenolic compounds present in the formulations as evidenced in the qualitative phytochemical analysis. The effect of standard antibiotic was found to be bactericidal in action on all the ten isolates.

The LC-MS/QToF identified 76 (Supplementary data) metabolites in the AK formulation. Metabolites like quinic acid, protocatechuic acid, gallic acid, chebulic acid were identified in the AK in a study conducted by Sulaiman *et al*[31]. Among the 76 metabolites identified, the following metabolites *i.e.*, 6 -gingerol[32] 10- gingerol[33], 6- shogoal[34], apigetrin[35], betulinic acid[36], catechin[37], ellagic acid[38], ferulic acid[39] methyl gallate[40], gentisic acid[41] salicylic acid[42], vanillic acid[43] were reported to demonstrate antibacterial activity.

The SEM images showed drastic morphological alterations that the intact cells underwent after being exposed to AK treatment. Borges *et al*. reported that the presence of gallic acid, and ferullic acid can lead to membrane permeabilization with consequent

leakage vital cellular constituents[39]. Vanillic acid is capable of destroying carbapenem-resistant *Enterobacter cloacae* by destabilizing bacterial cell membrane[43]. There are various studies that confirms the affinity of phenolic compounds towards bacterial membrane lipids[44,45]. This justifies the higher susceptibility of gram-negative bacteria for having greater interaction with phenolic antimicrobial metabolites[43]. The presence of these phytocompounds in the formulation might have led to membrane protein solubilization, pore development and alterations in membrane structure/function that ultimately caused the death of the treated isolates as evidenced in SEM images.

Conclusion

Antibiotic resistance continues to be a global threat to our healthcare system. In this scenario, polyherbal drugs could contribute a significant role in combating antibiotic resistance. At present this is the first published exploratory study on AK that revealed promising antibacterial activity against multidrug resistant clinical isolates taken from the human respiratory tract. The *in vitro* scientific evidence justifies its clinical use as a treatment option during the patient's infectious condition. The phytochemical characterization shows that the formulation is enriched with a vast array of bioactive antibacterial metabolites. The findings show that the formulation can function extremely well as an anti-infective agent irrespective of the resistance profile of the isolates. Thus, *Amruthotharam kwath* can be proposed as a potential complementary strategy for treating respiratory tract infections caused by gram-negative antibiotic-resistant pathogens. The findings based on the *in vitro* study, need to be further investigated through *in vivo* studies and clinical trials, to confirm the efficacy and safety of the *kwath* for treating antibiotic-resistant infections in humans. Even though the formulation is partially standardized, the obstacles that affect the therapeutic efficacy of the formulation in providing consistent antimicrobial activity, the absence of complete standardization of the formulation and the reproducibility of quality herbal drugs need to be crossed for total allopathic medical acceptance.

Supplementary Data

Supplementary data associated with this article is available in the electronic form at https://nopr.niscpr.res.in/jinfo/ijtk/IJTK_24(2)(2025)146-157_SupplData.pdf

Acknowledgements

The authors acknowledge Jubilee Mission Medical College and Research Institute, Thrissur; Confederation for Ayurvedic Renaissance-Keralam Limited (CARe Keralam), Koratty; Kerala Forest Research Institute (KFRI), Peechi; Kerala Agricultural Veterinary and Animal Science (KVASU), Mannuthy for extending their support for the smooth conduction of the research work.

Conflict of Interest

The authors of the present work affirm that there was no conflict of interest.

Author Contributions

MC, NJ, ME, SH: Conceptualization, Methodology, Investigation, Writing - original draft. LI & CV supervision, review & editing

Ethics Approval

The study received Institutional Ethics Committee approval (IEC.No.15/20/IEC/JMMC&RI) to collect MDR bacteria from Department of Microbiology, Jubilee Mission Medical College and Research Institute (JMMC&RI), Thrissur.

Informed Consent

There is no direct involvement of human volunteers in this study. So informed consent is not required in this context.

Data Availability

The authors confirm that the data supporting the findings of this study are available within the article and also in the supplementary material. Data may be obtained from the corresponding author upon reasonable request.

References

1 Anonymous, Antimicrobial resistance, *Bull World Health Organ*, 93 (6) (2015) 363-364.
2 Jim O' Neill, Antimicrobial resistance: Tackling a crisis for the health and wealth of nations, Wellcome Trust, London (2014) 4-9.
3 Aljeldah M M, Antimicrobial resistance and its spread is a global threat, *Antibiotics*, 11 (8) (2022) 1082. doi 10.3390/antibiotics11081082.
4 Anonymous, Antibiotic resistance threats in the united states 2019, *CDC*, Centers for disease prevention and control, USA, 10 (1) (2019) 100-140.
5 Klein E Y, Tseng K K, Pant S & Laxminarayan R, Tracking global trends in the effectiveness of antibiotic therapy using the drug resistance index, *BMJ Glob Health*, 4 (2) (2019) e001315.
6 Klein E Y, Van Boeckel T P, Martinez E M, Pant S, Gandra S, *et al*., Global increase and geographic convergence in

antibiotic consumption between 2000 and 2015, *Proc Natl Acad Sci U S A*, 115 (15) (2018) E3463-E3470.

7 Anonymous, WHO report on surveillance of antibiotic consumption, World health organization, Switzerland, (2018) 51-55

8 Sharma S, Banerjee T, Kumar A, Yadav G & Basu S, Extensive outbreak of colistin resistant, Klebsiella pneumoniae in a large tertiary care hospital, India, *Antimicrob Resist Infect Control*, 11 (1) (2022) 1-9.

9 Srivastava N, Sheetal V, Singh M & Kumar A, A short-term study on statistical numeration of multidrug resistant *Escherichia coli* isolates among the patients with urinary tract infection, *Indian J Exp Biol*, 62 (2) (2024) 119-125.

10 Magiorakos A P, Srinivasan A, Carey R B, Carmeli Y, Falagas M E, *et al*., Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: An international expert proposal for interim standard definitions for acquired resistance, *Clin Microbiol Infect* 18 (3) (2012) 268-281.

11 Manohar P, Loh B, Nachimuthu R & Hua X, Secondary bacterial infections in patients with viral pneumonia, *Front Med*, 7 (2020) 420.

12 Rodrigo-Troyano A & Sibila O, The respiratory threat posed by multidrug resistant Gram-negative bacteria, *Respirology*, 22 (7) (2017) 1288-1299.

13 Anonymous, Kerala State Antimicrobial Resistance Strategic Action Plan, (2018) 59.

14 Parasuraman S, Thing G S & Dhanaraj S A, Polyherbal formulation: Concept of ayurveda, *Pharmacogn Rev*, 8 (16) (2014) 73-80.

15 Jeyachandran R, Xavier T F & Anand S P, Antibacterial activity of stem extracts of *Tinospora cordifolia* (willd) hook. f & thomson, *Anc Sci Life*, 23 (1) (2003) 40-43.

16 Kannan P, Ramadevi S R & Hopper W, Antibacterial activity of *Terminalia chebula* fruit extract, *Afr J Microbiol Res*, 3 (4) (2009) 180-184.

17 Anonymous, Ayurveda for management of COVID-19, Ministry of AYUSH, Govt. of India, (2019) 14-15

18 Patel K C, Patel K G, Aboli G, Ganesh S, Parmar R R, *et al*., Optimization of HPTLC densitometric method for curcuminoids and polyphenolics in an ayurvedic Emblica officinalis and Curcuma longa based Nishamalaki formulation by Box-Behnken design, *Indian J Tradit Know*, 23 (8) (2024) 719-728.

19 Anonymous, Sahasrayogam, Sujanapriya Commentary, Vidhyarambham publishers Alappuzha, Jwarakashaya, Sloka, 12 (1998) 29

20 Gopal S & Ittiachen L, Analysis of quality parameters and preservative concentrations in Sahacharadi Kwatha: A comparative study of three commercial brands, *J Ayurveda Integr Med*, 15 (5) (2024) 101029. doi 10.1016/ j.jaim.2024.101029.

21 Lohar D R, *Protocol for Testing: Ayurvedic, Siddha & Unani Medicines*, (Government of India Department of AYUSH, Ministry of Health & Family Welfare, Pharmacopoeial Laboratory For Indian Medicines, Ghaziabad), (2011) 127.

22 Sulaiman C T & Balachandran I, Chemical profiling of an Indian herbal formula using liquid chromatography coupled with electro spray ionization mass spectrometry, *Spectrosc Lett*, 48 (3) (2014) 222-226.

23 Gupta S, Acharya R, Gamit R V & Shukla V J, Quantitative analysis of tannins, alkaloids, phenols, and flavonoids in *Ficus semicordata* leaf, stem, stem bark, root, and fruit powder, *J Indian Syst Med*, 9 (3) (2021) 171-174.

24 Anonymous, CLSI M100-ED29: 2019 Performance Standards for Antimicrobial Susceptibility Testing, 29th Edition, Clinical laboratory standards institute, USA, (2019) 210-252.

25 Elshikh M, Ahmed S, Funston S, Dunlop P, McGaw M, *et al*., Resazurin-based 96-well plate microdilution method for the determination of minimum inhibitory concentration of biosurfactants, *Biotechnol Lett*, 38 (6) (2016) 1015-1019.

26 Army L Leber, *Clinical Microbiology Procedures Handbook*, (American Society for Microbiology) 5th Chapter, (2016) 818-830.

27 Ali R, El-Boubbou K & Boudjelal M, An easy, fast and inexpensive method of preparing a biological specimen for scanning electron microscopy (SEM), *Methods X*, 8 (2021) 101521. doi 10.1016/j.mex.2021.101521.

28 Yan Y, Li X, Zhang C, Lv L, Gao B, *et al*., Research progress on antibacterial activities and mechanisms of natural alkaloids: A review, *Antibiotics*, 10 (3) (2021) 318. doi 10.3390/ antibiotics10030318.

29 Rempe C S, Burris K P, Lenaghan S C & Stewart C N Jr, The potential of systems biology to discover antibacterial mechanisms of plant phenolics, *Front Microbiol*, 8 (2017) 422.

30 Khan M I, Ahhmed A, Shin J H, Baek J S, Kim M Y, *et al*., Green tea seed isolated saponins exerts antibacterial effects against various strains of gram positive and gram-negative bacteria, A comprehensive study *in vitro* and *in vivo*, *Evid Based Complement Altern Med*, 2018 (2018) 3486106. doi 10.1155/2018/3486106.

31 Sulaiman C T, Ramesh P R, Mahesh K, Madhu K M, Anandan E M, *et al*., Chemical profiling of a polyherbal formulation by tandem mass spectroscopic analysis with multiple ionization techniques, *Future J Pharm Sci*, 6 (1) (2020) 40.

32 Ahmed S H, Gonda T & Hunyadi A, Medicinal chemistry inspired by ginger: exploring the chemical space around 6-gingerol, *RSC Adv*, 11 (43) (2021) 26687-26699.

33 Nagoshi C, Shiota S, Kuroda T, Hatano T, Yoshida T, *et al*., Synergistic effect of [10]-gingerol and aminoglycosides against vancomycin-resistant enterococci (VRE), *Biol Pharm Bull*, 29 (3) (2006) 443-447.

34 Rigane G, Mnif S & Ben Salem R, One step purification of 6-shogaol from *Zingiber officinale* rosco, a phenolic compound having a high effectiveness against bacterial strains, *Revue Roumaine de Chimie*, 63 (1) (2018) 5-10.

35 Hadrich F & Sayadi S, Apigetrin inhibits adipogenesis in 3T3-L1 cells by downregulating PPARγ and CEBP-α, *Lipids Health Dis*, 17 (1) (2018) 95.

36 Bildziukevich U, Rarova L, Janovska L, Saman D & Wimmer Z, Enhancing effect of cystamine in its amides with betulinic acid as antimicrobial and antitumor agent *in vitro*, *Steroids*, 148 (2019) 91-98.

37 Kajiya K, Hojo H, Suzuki M, Nanjo F, Kumazawa S, *et al*., Relationship between antibacterial activity of (+)-catechin derivatives and their interaction with a model membrane, *J Agric Food Chem*, 52 (6) (2004) 1514-1519.

38 De R, Sarkar A, Ghosh P, Ganguly M, Karmakar B C, *et al*., Antimicrobial activity of ellagic acid against Helicobacter pylori isolates from India and during infections in mice, *J Antimicrob Chemother*, 73 (6) (2018) 1595-1603.

39 Borges A, Ferreira C, Saavedra M J & Simões M, Antibacterial activity and mode of action of ferulic and gallic acids against pathogenic bacteria, *Microb Drug Resist*, 19 (4) (2013) 256-265.

40 Choi J-G, Kang O-H, Lee Y-S, Oh Y-C, Chae H-S, *et al*., Antibacterial activity of methyl gallate isolated from galla rhois or carvacrol combined with nalidixic acid against nalidixic acid resistant bacteria, *Molecules*, 14 (5) (2009) 1773-1780.

41 Li H, Pan Y, Li C, Yang Z, Rao J, *et al*., Design, synthesis and characterization of lysozyme-gentisic acid dual-functional conjugates with antibacterial/antioxidant activities, *Food Chem*, 370 (2022) 131032. doi 10.1016/*J.Foodchem*.2021.131032

42 Song X, Li R, Zhang Q, He S & Wang Y, Antibacterial effect and possible mechanism of salicylic acid microcapsules against *Escherichia coli* and *Staphylococcus aureus*, *Int J Environ Res Public Health*, 19 (19) (2022) 12761. doi 10.3390/ijerph191912761.

43 Qian W, Fu Y, Liu M, Wang T, Zhang J, *et al*., In vitro antibacterial activity and mechanism of vanillic acid against carbapenem-resistant *Enterobacter cloacae*, *Antibiotics*, 8 (4) (2019) 220. doi 10.3390/antibiotics8040220.

44 Karonen M, Insights into Polyphenol-Lipid Interactions: Chemical methods, molecular aspects and their effects on membrane structures, *Plants*, 11 (14) (2022) 1809. doi 10.3390/plants11141809.

45 Yu X, Chu S, Hagerman A E & Lorigan G A, Probing the interaction of polyphenols with lipid bilayers by solid-state NMR spectroscopy, *J Agric Food Chem*, 59 (12) (2011) 6783-6789.