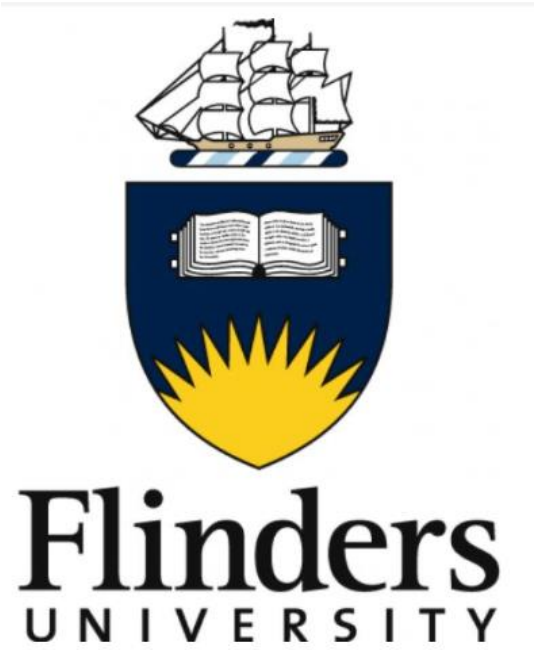

Comparison of green technology extracts and methanol crude extracts of *Panax quinquefolium* and *Nigella sativa* for antimicrobial activity and interactions with antibiotics

By

Hanan Abdul Rahim

15th July 2021

Project facilitated by

College of Medicine and Public Health

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Fig.13. The MIC of synergy exhibited from the combination of Nigella extract (Z) at 40 and 20 mg/ml with Gentamycin (K) at 2 and 1 µg/ml, respectively.

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LIST OF ABBREVIATIONS

AMR- ANTIMICROBIAL RESISTANCE

AAM- ANTIBIOTIC ASSAY MEDIUM

CFU- COLONIES FORMING UNIT

HPLC- HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

LPS- LIPO POLYSACCHARIDE

MAE- MICROWAVE-ASSISSTED EXTRACTION

MBC- MINIMUM BACTERICIDAL CONCENTRATION

MDR- MULTIDRUG RESISTANT

MIC- MINIMUM INHIBITORY CONCENTRATION

MRSA- MULTI DRUG RESISTANT STAPHYLOCOCCUS AUREUS

NS- *Nigella sativa*

OD- OPTICAL DENSITY

PQ- *Panax quinquefolius*

SFE- SUPERCRITICAL FLUID EXTRACTION

SAE- SHAKING-ASSISTED EXTRACTION

TLC- THIN-LAYER CHROMATOGRAPHY

TSA- TRYPTIC SOYA AGAR

TSB- TRYPTIC SOYA BROTH

UAE- ULTRASONIC-ASSISTED EXTRACTION

UV- ULTRAVIOLET

WHO- WORLD HEALTH ORGANIZATION

ABSTRACT

The emergence of multidrug-resistant (MDR) bacteria has become a major public health concern and a serious challenge, resulting in fewer and more expensive treatment options for infectious diseases. Synergistic combinations of several drugs with natural antimicrobials have been identified as additional effective strategies contributing to less drug toxicity and resistance. The present study evaluated to compare the antimicrobial properties of crude extracts of *Panax quinquefolium* and *Nigella sativa* extracted using conventional method and green extraction technologies, such as microwave-assisted extraction (MAE), ultrasonic fluid extraction (UAE), and supercritical fluid extraction (SFE) with efficiency yield at the range of 20-36.5%. The compounds in the plant extracts were detected and analysed using Thin-layer chromatography (TLC) and HPLC-UV to confirm the presence of antimicrobial compounds such as ginsenosides in *Panax quinquefolium* and Thymoquinone and Di thymoquinone in *Nigella sativa*.

A well diffusion assay of methanol extracts of plant demonstrated moderate inhibition of bacterial growth of the Gram-negative organisms- *Escherichia coli* and *Pseudomonas aeruginosa* (9-15mm) and the Gram-positive bacteria *Bacillus pumilis* and *Staphylococcus aureus* (11-14mm), with *E. coli* showing no activity for Nigella extract. Comparatively, the MAE had lesser activity. The bacteriostatic and bactericidal activity of plant extracts in combination with antibiotics against the organisms was further analysed by calculating the minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) using microtiter broth dilution method. The combination of *Nigella sativa* (40mg/ml, 20mg/ml) with Gentamycin (2µg/ml) reduces the MIC of gentamycin alone by 25-50% exhibiting synergy against Gram-positive bacteria- *Staphylococcus aureus*.

Hence, the saponins from the plant extract exhibiting antimicrobial activity alone and synergy with antibiotic at specific dilution has opened better options for new treatment with lower toxicity. Future research would be to analyse the active compounds enhancing the activity in synergism with antibiotics and its cellular functions. And test the cytotoxicity on mammalian tissue cultured cells for potential development for applications in treatment of bacterial infections.

DECLARATION:

I certify that the thesis does not incorporate without acknowledgement any material previously submitted for a degree of diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Hanan Abdul Rahim

23rd June 2021

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CHAPTER-1

> "Then there is the danger that the ignorant man may easily underdose himself and, by exposing his microbes to non-lethal quantities of the drug, make them resistant." Alexander Fleming, 1945 Nobel Prize speech

1.INTRODUCTION

1.1 Antibiotics:

The discovery of penicillin as a life-saving antibiotic is one of the most significant accomplishments in human history. Antibiotics have become a foundation of modern healthcare allowing effective infection control for a long time. These developments started in the Golden Era of Antibiotics. Natural products and multiple versions of these drugs were discovered and developed by successfully exploring the biochemistry of bacterial and fungal secondary metabolism, followed by chemically modifying existing antibiotics and creating semi-synthetic antibiotics (Lewis 2020). Antibiotics plays a significant role in surgeries, transplants, treatment of infection as well as cancer treatments. However, the extensive use of antibiotics to treat infectious diseases in humans, as well as in animal feed as a growth stimulant, has produced exceptional conditions for accumulating resistance components in bacterial populations, making them resistant to antibiotics. Due to the global spread of antibiotic-resistance factors, the prolonged efficacy of these life-saving medications is of concern. The experts predict an estimated 10 million deaths per year due to resistant pathogens by the year 2050 (O'Neill 2020). However, the discovery of new antibiotics has been challenging. The initial screening by dereplication, in which the same molecules are identified repeatedly, is currently impacting natural product innovation.

1.2 Antimicrobial resistance (AMR) dilemma

Many microorganisms have evolved to evade the frequently used antibiotic activity to create antimicrobial resistance (AMR). For a long time, this has been endemic in many regions globally, posing a severe threat to public health. Antibiotic-resistant infections are challenging to treat for various reasons, including high treatment costs, high dose utilisation, complex antibiotic manufacture, and resource scarcity, resulting in severe morbidity and mortality. As a result of the AMR dilemma, the world health organisation (WHO) released a list of priority pathogens stating the levels of the criticality of resistance of each organism responsible for the health problems (Tacconelli et al. 2018).

Resistance is due to two significant factors: frequent prescription for nonbacterial diseases and unrestricted usage in lower concentrations, encouraging resistance to spread rapidly. Antibiotic use varies by country, with regular prescriptions of new antibiotics making them less effective as a result of their widespread use, and the pathogenic bacteria they were designed to target adapting and changing, resulting in drug resistance (Ventola & CL 2015). The resistance is seen mainly in the healthcare industry, referred to as "hospital-acquired infections", mostly in immune-compromised patients. In addition, antibiotics are also utilised as prophylactic agricultural additives to stimulate cattle development and prevent illnesses related to the human food chain, further aggravating the antimicrobial resistance problem (Manyi-Loh et.al 2018).

The antibiotics work predominantly on three main targets within the bacterium, with each drug type possessing a different method of action. Inhibition of cell wall production, protein synthesis, and DNA or RNA synthesis (Fig.1). However, antibiotic resistance has evolved in bacteria through three mechanisms: reduced drug penetration via limited permeability and

efflux pumps, mutation or change of the binding site, and degradation of the drug itself. Conversely, the resistance could be attributable to the overall adaptive response of bacterial cells rather than a single mutation (Kapoor et.al 2017). Thus, depending on the resistant characteristics conferred, pathogens exhibit inherent, acquired, and adaptive resistance.

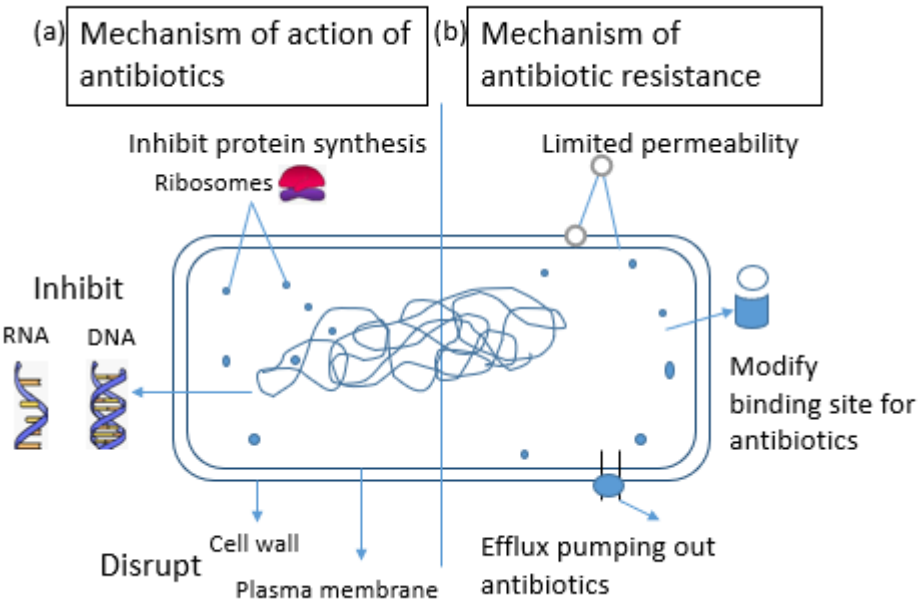


Fig.1 (a) Mechanisms of action of antibiotics in the bacteria (b) Mechanism of antibiotic resistance exhibited by a bacterial cell

The antibiotics work differently on Gram negative and Gram-positive bacteria. Gram-negative bacteria are distinguished by an outer coating called the lipo-polysaccharides (LPS) with hydrophobic lipid and hydrophilic core polysaccharide making a firm outer membrane protecting cell from most antibiotics. While, in gram-positive bacteria the peptidoglycan outer layer allows antibiotics to enter and are easier to kill. Gram-positive bacteria are the focus of many eradication attempts, while Gram-negative bacteria have been gaining deadly resistance and are considered as a more serious concern, and both represent specific hazards to hospitalised patients with compromised immune systems (Christaki, Marcou & Tofarides

2020). So, the new antimicrobials must be aimed at eliminating both type of organism for effective solutions.

With AMR being one of the most serious global health issues, practical solution is urgently required. It is currently being combated by concentrated efforts to enhance diagnosis, antibiotic prescribing methods, and infection prevention techniques. Clinical trials utilising nanocarriers for bioavailability and increased antibiotic availability to bacterial cells are ongoing (Huh et.al 2011). On the other hand, natural bio-actives have effectively interacted with antibiotics due to their pharmacological properties reducing the side effects (Namjoo et.al 2013).

1.3 NATURAL BIO-ACTIVES

Naturally occurring bio-actives from different sources have been explored for their therapeutic potential for beneficial responses towards infections (Pratap et al. 2020). The medicinal plants have been used from ancient times due to their bioactive compounds with many pharmacological benefits. These compounds exert specific defence mechanisms. (Fig.2) Medicinal plants have been utilised as therapeutics for human diseases for ages, and some of these compounds can be employed as antibacterial agents (Buhner 2012). They are the most abundant bio-resources of traditional medical remedies, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceuticals, precursors, and chemicals used to produce synthetic drugs. Bioactive chemicals can be discovered in various plants, fungi, algae, and animals from both marine and terrestrial environments.

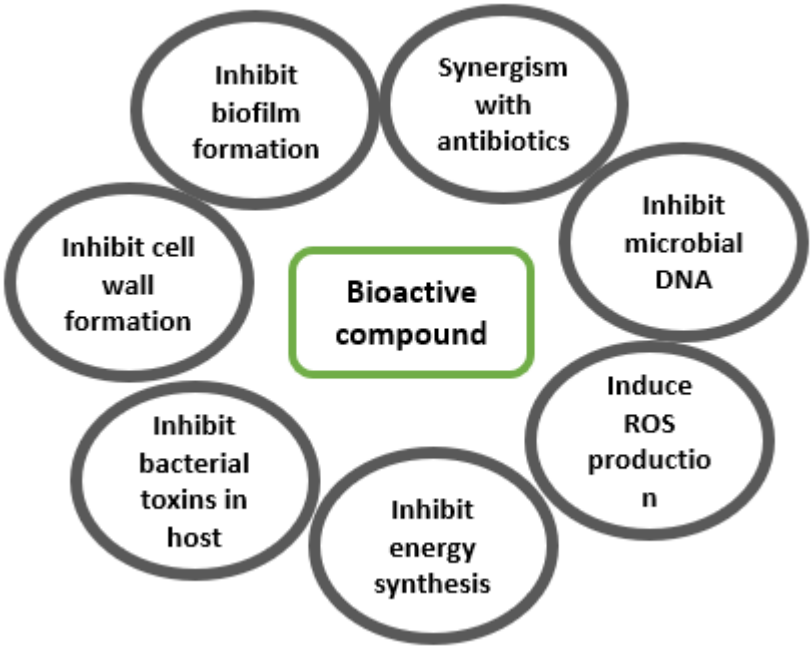


Fig.1. Mechanisms of action exhibited by the natural bio actives with antimicrobial properties.

1.3.1 Animal and other sources

Animals manufacture bio-actives to sustain body functions and defend themselves from external and internal pressures. In certain circumstances, bioactive substances are essential to the animal's existence but may have far more critical activities in unrelated organisms (Zhang, Chen & Wang 2015). Examples of some antimicrobials derived from animal source are shown. (Table.1)

Some produce antimicrobial peptides in polluted environments from their innate immunity for protection from polluted environment (Tonk & Vilcinskas 2017). Similarly, other sources like fish, snakes, and animals feeding on verminous rodents also produce metabolites to protect themselves. More research is required for therapeutical application, considering the disadvantages of toxicity and unpredictability at large-scale production.

Table.1 Antimicrobial from animals and its sources (Boparai et al. 2020)

SCIENTIFIC NAME	ANTIBIOTIC COMPOUND	ACTIVE AGAINST	
Androctonus australis	Androctonin	**Gram-positive** *S. aureus*	**Gram-negative** *E. coli K1 (MRSA)* and *Pseudomonas aeruginosa*
Tachypleus tridentatus (Horseshoe crab)	Tachyplesins		*E. coli*
Crayfish species	Antimicrobial peptides such as astacidin, crustin and antilipolysaccharide factor	*Staphylococcus aureus*	*Shigella flexineri*
Larimichthys crocea	NK-lysin and Collagencin	*S. aureus, Bacillus subtilis*	*E. coli, Vibrio harveyi*

1.3.2 Microorganism

Microorganisms have been an early source of antimicrobial substances. Microorganisms are pharmaceutically important, as they produce secondary metabolites like polyketides, terpenes, shikimates, peptides, and alkaloids categorised based on their metabolic origin (Francis 2017). The discovery of novel antimicrobials from microorganism has been carried out for an extended period. The future aspect would be to find new organism sources that can target new systems while avoiding resistance. Examples of very few antimicrobials from microorganisms are shown (Table.2)

Table.2 Antimicrobial Activity Exhibiting Microorganisms (Debbab et al. 2010)

MICROORGANISM	COMPOUND	MIC	ACTIVE AGAINST	
Ascochyta sp.	*Ascochytatin*		**Gram-positive** . *Bacillus subtilis*	**Gram-negative**
Marinispora strain	*2-alkylidene-5-alkyl-4-oxazolidinones, lipoxazolidinone*	*1.56 to 15.57 µM,* *37.38µM*	Most gram-positive bacteria	*Haemophilus influenzae*
Pseudomonas stutzeri	*Zafrin (4β-methyl-5,6,7,8–tetrahydro-1 (4β-H)–phenanthrenone)*	*235.85–589.62µM*	*Bacillus subtilis*	

1.3.3 Plant-derived bio-actives

Traditional healers have used plants for treating many diseases since ancient times. About 25-50% of antimicrobials are extracted from plants (Calixto 2000). Natural-derived medications are preferred over synthetic due to their possibly less or no post-remedial effects on the human condition. Plants produce these secondary metabolites to protect against predation and, in some case, for pigmentation and flavour. (Table. 3)

Table.3 Plant derived antimicrobials with its activity (Cowan &M.M, 1999)

SCIENTIFIC NAME	**COMPOUND**	**MIC**	**ACTIVE AGAINST**	
Piper nigrum (Black pepper)	Piperine	500–1000µg/mL	Gram-positive *Lactobacillus sp., Micrococcus sp., Enterococcus. faecalis*	Gram-negative *E. coli*
Matricaria chamomilla (Chamomile plant)	Phenolic acid	1.56–3.12 mg/mL	*S. aureus*	*Salmonella typhimurium*
Salix babylonica L. (Weeping willow)	Luteolin, luteolin 7-O-glucoside	1.56-100 mg/mL	*S. aureus,*	
Vernonia adoensis Sch. Bip. ex Walp (African plant).	Chondrillasterol	50 µg/mL	*S. aureus, Listeria monocytogenes*	*E. coli*
Tecoma stans (Yellow elder)	Phenolic compounds	50-600 µg/mL	*S. aureus*	
Caesalpinia coriaria (Libidibia coriaria)	Methyl gallate and gallic acid	1.56–25 mg/mL	*Listeria monocytogenes, S. aureus*	*Salmonella typhi, E. coli,*

				Pseudomonas aeruginosa,
Cassia alata (Senna alata)	4-butylamine, cannabinoid, dronabinol, methyl-6-hydroxy	1.25 1.5 mg/mL	*S. aureus*	*E. coli, P. aeruginosa,*
Cinnamomun Inerme (cinnamon)	5-(1,5-dimethyl-2-4-hexenyl)- methyl phenol)	100–800 g/mL	*S. aureus*	*E. coli*
Artocarpus Communis (Breadfruit)	Atonin E, 2-(3,5-dihydroxy) -(Z)-4-(3 methyl but-1-etnyl	4–512 g/mL	*S. aureus*	*P. aeruginosa, Salmonella typhi, Klebsella pneumoniae*

1.3.3a Effective mechanisms of plant bio-actives

The plant metabolite can alter or suppress protein-protein interactions, making them potent modulators of the immune response like mitosis, apoptosis, and signal transduction. With many studies revealing the incapability of bacteria developing resistance towards multiple plant phytochemicals (Gupta, Daswani & Birdi 2014), while a review by Vadhana et al. have also reported in the rise in resistance towards them. (Vadhana et al. 2015).

Plant secondary metabolites can affect microbial cells in various ways, including altering membrane function and structure, disturbing DNA/RNA synthesis and function, interfering with intermediary metabolism, triggering cytoplasmic constituents to coagulate, and interrupting regular cell communication. However, several components in a crude extract act at different sites, contributing to the extract's total efficacy.

Analysing the desired bio actives in crude extracts of medicinal plants is an essential task and each step is important in the whole process. The efficiency and phytochemical contents of the final extractions are influenced by sample preparation, including grinding, and drying, which influence the final extracts (Azwanida, and N.N. 2015). There is no ideal method for extraction, they are optimised to achieve the final target compound with better quality and high efficiency. Hence, the efficacy of plant extracts may be influenced by extraction efficiency (Kothari et.al 2012). Following research into the antibacterial activity of plant seed extracts presented by Kothari and Vijay 2010, a high positive linear association between extraction efficiency and overall antibacterial activity was observed (Kothari & V 2010).

1.4 Research gap

Microbial resistance is becoming more of an issue, and the future usage of antimicrobial treatments is still dubious. The researchers must take specific steps to mitigate the problem by lowering the use of antibiotics, which can slow down the rise in resistance of pathogens towards the commonly used antibiotics. There has also been a significant reduction in the discovery of new antimicrobials, and as a result the alternative therapies such as traditional plant-based medications, bacteriophage therapies, and combinational therapies are being preferred (Cheesman et al. 2017)

Medicinal plants are gaining popularity as alternative medicines, with one-third of adults in affluent nations using alternative remedies such as herbs. Antibacterial resistance can be

prevented and minimised by various medicinal plant extracts with intrinsic antimicrobial characteristics. A combination approach using plant extracts with antibiotics can raise the possibility of pharmacodynamic and pharmacokinetic interactions with potential therapeutic effect towards resistance (Cheesman et al. 2017).

In contrast, antagonistic interactions can lead to lower efficacy and therapeutic failure. The possibility for herbal medications to interact with allopathic medicines is a significant safety concern, especially for phytochemicals that may result in serious side effects. Synergistic interactions of plant extract with antibiotics have been reported with the intensified antimicrobial impact of antibiotics. An example of such interaction is that American ginseng can interfere with the anticoagulant activity of warfarin drug and for such cases the combination must be validated (Yuan et al., 2004). In some plant-drug interactions, the possibility of reducing antibiotic drug dose by 75% reported with better effect (Ament et al. 2000).

1.4.1 Panax quinquefolius

Panax quinquefolius also known as American ginseng is one of the common herbs used in the US (O'Hara et al. 1998). It is amongst the world's most important herbal remedies used as an adaptogen, which are stress response modifiers and have a wide range of pharmacological properties (Fuzzati 2004).

1.4.1a American ginseng phytochemicals

Ginsenosides are the main bioactive compounds along with polysaccharides, acetylenic alcohols, peptides and fatty acids are accountable for most of the pharmacological properties of ginseng. The ginsenosides and polysaccharides exhibit high antimicrobial activity towards many Gram-positive and Gram-negative organisms (Attele et al. 1999). More than 280 types of ginsenosides have been reported which are chemical compounds of glycosides with

aglycone (non-sugar part) and a sugar chain. According to prior research, the antimicrobial activity of the ginsenosides depends on the glycosyl group (Battinelli et al. 1998). Hence, the bacteriostatic action of these triterpenoid saponin's is also controlled by the arrangement and variety of their side chains. Ginsenosides are bactericidal, preventing bacterial adherence, which is necessary for cell entrance, cytotoxicity, inflammation, and hemagglutination (Kim & Yang 2018). Ginseng defends against bacterial infections by modulating immune responses, damages the cell wall integrity disrupting plasma membrane of the bacteria for maintaining cellular homeostasis, cell division and proliferation (Nguyen & Nguyen 2019).

1.4.2 Nigella sativa

Nigella sativa is another one of the oldest medicinal herbs used in the ancient Islamic system of herbal medicine with multiple pharmacological properties. The black seed was traditionally used as diuretic, anthelmintic and stimulant; it was also used to treat paralysis, jaundice, intermittent fever, dyspepsia, piles and skin diseases and additionally used as spice in many foods as flavourings, preservatives in confectioneries and stabilizing agents (Ramadan 2007).

1.4.2a Phytochemistry of black seeds

Phytochemical analysis has revealed the presence of over 100 compounds in the seeds and oils, including fatty acids, volatile oils, trace elements, alkaloids, and saponins, all of which have pharmacological activities. Other nutritive compounds include carbohydrates, vitamins, minerals, fats, and proteins with eight to nine essential amino acids (Dubey et al. 2016).

Thymoquinone and carvacrol have been shown to be effective in modulating antimicrobial resistance (Mouwakeh et al. 2018). It exhibits bactericidal activity against most of the Gram-positive organisms by preventing cell adhesion to surfaces and inhibiting biofilm formation.

1.5 AIM & OBJECTIVES:

This research aims to study the efficacy of the antimicrobial properties exhibited by *Panax quinquefolius* and *Nigella sativa* towards clinical microorganisms; *Escherichia coli, Staphylococcus aureus, Bacillus pumilis* and *Pseudomonas aeruginosa*. Furthermore, the novelty of possible combinational treatment using plants- *Panax quinquefolius* and *Nigella sativa* with antibiotics are explored for its synergistic effect. Hence the objectives include:

(i) Analyse and confirm the presence of compounds from *Panax quinquefolius* and *Nigella sativa* that exhibit antibacterial activity.

(ii) To Evaluate the antimicrobial efficacy of *Panax quinquefolius* and *Nigella sativa* separately and their interaction with antibiotics.

1.6 SIGNIFICANCE OF STUDY

The spread of infectious diseases and limitations in the treatments due to resistance towards commonly antibiotics has been major issues worldwide. Antimicrobial resistant infections have been causing mortality at faster rate all over. The major setbacks of the antibiotics being neurotoxic, nephrotoxic, ototoxic or hypertensive and some cause severe damage to the liver and affect bone marrow (Chong and Pagano, 1997) and hence pathogens have developed resistance in the long-term use. New strategies are to be addressed to combat the growing crisis of resistance. The antimicrobials from the plants have been giving promising results for long term treatment of wide array of infectious organisms.

Approach of the study was to analyse any synergistic effect of the plants, *Panax quinquefolius* and *Nigella Sativa* exhibiting antimicrobial activity when combined with protein biosynthesis inhibitors, Gentamycin, and Streptomycin against Gram positive and Gram-negative bacteria. In previous research, the Ginseng and Nigella has proven to show protection from the nephrotoxicity of gentamycin with combination treatment on mice *in-vivo* (Qadir et al. 2011)

(Namjoo et al. 2013). Hence, the combination therapy might be considered safe and might provide antimicrobial effect against many organisms which can further be investigated for future applications.

CHAPTER-2

2.1 MATERIALS AND METHODS:

Plant material

American ginseng (*Panax quinquefolius*) which was grown in Wisconsin, W Ginseng Farm, USA was purchased online. The dried roots were cleaned, dried, and blended to powder before extraction. The American Ginseng powder was purchased online via Dubois ginseng products, Victoria, Australia (due to unavailability of previously purchased ginseng roots with covid restriction).

Black seed (*Nigella sativa*) purchased online via HabShifa. These organic black seeds were powdered and sieved for extraction.

Bacterial strains:

Two Gram negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*, and two Gram positive bacteria, *Bacillus pumilis* and *Staphylococcus aureus*, cultivated on blood agar media were received from the SA Health, Flinders Medical Centre. The strains were inoculated onto TSA medium and kept at 4°C.

Chemicals

Chemicals including methanol, ethanol, chloroform, and other reagents used were of analytical grade.

Apparatus:

Microwave assisted extractor (Milestone Innovations), Supercritical fluid extractor (applied separations, SPE-ED SFE, Australia), Freeze dryer (Virtis, NSW), blender (Blendtech Xpress, Australia), Orbital mixer incubator (Adelab scientific, Adelaide), rotary evaporator (Buchi Flawil, Switzerland), Ultrasonic equipment, (Bransonic Ultrasonics), spectrophotometer

(Spectroline, ENF-240C/FE, USA), Hot air oven (Scientific equipment manufacturer SEM), 96 microtiter plates.

METHODS:

2.2 EXTRACTION

2.2.1 Microwave assisted water extraction:

The process was carried out in a StartSYNTH-microwave synthesis labstation, equipped with an industrial magnetron which can deliver up to 1200 Watt. A microwave diffuser which is located above the microwave chamber evenly distributes the microwaves throughout the cavity, preventing localized hot and cold spots. It is operated using a compact terminal, with bright, high resolution, touch screen display in which the parameters such as irradiation power, temperature and time is entered. Exactly 4 grams of the sample was weighed and placed in a 250 ml round bottom flask with a quick-fit wide neck fitting into the condenser tube inside the chamber. All the microwave extractions were performed under a set microwave irradiation power of 150 and 350 W for a specified timing around 15 and 20 min in 80.0 ml of solvent (water). Following the extraction, the extracts were centrifuged at 4500 rpm for 20 minutes to separate the supernatant which was further filtered through a 0.22µm syringe filter for HPLC determination.

2.2.2 Supercritical fluid extraction:

Supercritical fluid extraction uses Carbon dioxide as the solvent for the extraction making it ideal for the extraction of non-polar natural products such as lipid and volatile oil. Sample of 17g powdered nigella seeds was packed into the extractor tube. Nigella seed oil was extracted with supercritical carbon dioxide. Liquid carbon dioxide from a cylinder was passed through a chiller and was then compressed to the operating pressure by a syringe pump. Compressed CO_2 flows into the extractor vessel in a heating chamber that controls the extraction temperature.

The solute extracted was collected into the collection tubes. The amount of oil obtained was measured and the yield was calculated.

2.2.3 Ultrasonic-assisted extraction:

To 200mg of plant powder in a 50ml flask, 15mL of 70% methanol was added and homogenised to obtain uniform solution. The extraction was carried out using 600W pulsed ultrasonic power at frequency of 20KHz, 22% amplitude for 1-2 hours at 25°C. The tubes were placed over ice box during extraction to avoid quick evaporation of solvent. The mixture was then cooled and centrifuged at 5000rpm for 10 minutes. 12.5ml supernatant obtained was then concentrated using a rotary evaporator. The residue was then dissolved in methanol at the required concentration for antimicrobial assay.

2.2.4 Shaking-assisted extraction:

Into 250ml conical flask 10 g of the powdered sample was mixed in 100ml of solvent (methanol, 70% methanol, ethanol). It was kept in shaking incubator with 150 rpm at 25ºC for a period of 24 hours. The methanol sample was then kept in 60ºC water bath for 1 hour. The extracts were filtered using Whatmann filter paper no.2 and centrifuged at 4500 rpm for 10 minutes to get a clear filtrate. The filtrate was then concentrated using a rotary evaporator with the water bath set at suitable temperature for the plant (American ginseng-60ºC and Nigella sativa- 45ºC). The dried residue was transferred into vials and stored at 4°C until further analysis.

2.3 PURIFICATION

2.3.1 Thin-layer chromatography:

Methanol extracts of samples were prepared in 100mg/ml concentration. TLC Silica gel plates 60 F254 aluminium sheets (Merck # 1.05554.0001, Darmstadt, Germany) were cut as required

and start line was marked 1cm from the edges using a pencil. The TLC plates were then kept in 100ºC for 1 hour to be activated. The solvent system which was the lower phase of chloroform: methanol: water (65:35:10) was prepared in a separating funnel inside the fume hood. The TLC plates from the hot air oven were removed and cooled to room temperature. The solvents were poured into closed chromatographic tank and Whatman paper was placed on the sides and left in the closed tank to saturate for 30 minutes. About 10 µl of sample extract was placed on spots marked and the dried plate was placed inside the tank and was left to run until the solvent reaches the top 1cm of the TLC plate. The spots were marked including the maximum solvent flow and read under the UV illuminator at 254nm and 365nm. The Rf values were calculated measuring the distance from the start line to the spots marked using:

$$\text{Rf value} = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

2.4 High performance liquid chromatography (HPLC):

Various extracts of *Panax quinquefolius* and *Nigella sativa* (70% methanol) were prepared in 1:10 dilution with acetonitrile and centrifuged at 15000 rpm for 15 minutes. The 10µl of clear supernatant were loaded into plastic HPLC vials. The protocol for analysis was followed from previous paper using Shimadzu Prominence XR UHPLC system (Yao et al.2011). The system consisted of degasser, 2 x liquid chromatograph units, autosampler, refractive index (RI) detector, UV/V detector, column oven and communications bus module. 10µl sample was injected in 1ml/min flow rate. The wavelength for detection was set at 203nm with room temperature. The mobile phase consisted of water-trifluoro acetic acid (A; 100:0.01, v/v) and acetonitrile-Trifluoro acetic acid (B; 100:0.01, v/v).

The plant bio actives were identified comparing the data of the retention times from the previous literature.

2.5 ANTIMICROBIAL ASSAY:

2.5.1 Well diffusion method:

The antimicrobial assay was carried out using well-diffusion technique using two Gram-positive and two Gram-negative bacteria. The strains used were sub-cultured and maintained on TSA (Tryptic soya agar) medium every 2 weeks. The organism was sub-cultured into 5ml TSB in McCartney bottles and incubated at 35ºC for 16 hours. The growth of the culture was monitored at 600nm in a spectrophotometer and the overnight culture was adjusted with TSB to obtain 0.2 OD in a sterile condition. 0.4ml of 0.2 OD organism was added to 40ml antibiotic assay medium (AAM) in 50ml schott bottle at temperature of 40-45ºC. The same procedure was followed for each organism separately. 40ml of AAM containing organism was added to 2 petri plates (20ml each) into which 6mm wells were made using cork borer after the agar was solidified. Amount of 50µl of extract, Vancomycin and Colistin as positive control, and methanol as negative control were inoculated into the well. The plates were incubated for at least 16 hours at 37°C. Following the incubation, the diameter of zone of inhibition was measured in mm using a scale. The antibacterial activity was measured in duplicate for each concentration of the extract, and the mean zone of inhibition was calculated.

2.5.2 Minimum inhibitory concentration (MIC):

2.5.2a Miles and Misra method for enumeration of cells

To perform the MIC, it requires a standardised cell number of $5X10^5$ CFU/ml to analyse the activity of extracts and antibiotics. Ten-fold serial dilution of each concentration of each organism was performed. From each dilution 10µl was dispensed onto TSA plates and incubated overnight at 37°C. The number of colonies was counted and calculated log CFU/ml.

2.5.2b MIC for antibiotics

The minimum inhibitory concentration (MIC) for antibiotics and plant extract were initially determined separately and proceeded to analyse the MIC for the combination of plant extracts with antibiotics. For the antibiotics, stock of 5mg/ml of Gentamicin and Streptomycin were prepared. To the microtiter (96 well) plate, 270µl of TSB was added into 1st well and 150 µl into other 9 wells in a row. 30 µl from 5mg/ml antibiotic stock was added into the first well and mixed. From the first well, 150 µl was taken and mixed into the second well and serial dilution was repeated until the 10^{th} well. 30 µl of standard concentration of organism was added into each well. Again 150 µl of TSB was added into each well. The control well was taken with TSB and organism only. The same procedure was repeated in each row for 4 different organisms with one antibiotic per plate. The plate was incubated overnight at 37°C.

2.5.2c MIC of plant extracts

Methanolic plant extracts of 50 µl of *Panax quinquefolius* and *Nigella sativa* with 100mg/ml concentration was infused into 10 disc each. The infused disc was soaked in 0.5ml distilled water for 2 hours. 300 µl from the extract infused water was transferred into 1.5ml of TSB in a tube making it 1^{st} dilution. In a microtiter plate add 150 µl of TSB into 10 wells in a row. From the 1t dilution 150 µl was added to the third well and serial dilution was continued until 10^{th} well. To each well, 30 µl of $5X10^5$ CFU/ml of organism was added and 150 µl of TSB was again added over making 330 µl. The control well was taken with TSB and organism only. The same procedure was repeated in each with 4 organisms. The plate was incubated overnight at 37°C.

2.5.2d MIC of combination

Seven different stocks of gentamycin and streptomycin was prepared at concentrations of 4µg to 0.125µg to 2ml TSB each. Similarly, stock of plant extract of concentrations 40µg to 2.5µg

in 2ml TSB was prepared. 150µl of each concentration of antibiotic was dispensed into seven wells of each column (fig.2). And 150µl of each concentration of plant extract was dispensed into seven wells in each row. 30 µl of standard concentration of organism was added to each well. TSB with organism was taken as the control for analysing the activity and 150 µl TSB alone as control for antibiotics. The plates were incubated overnight at 37°C.

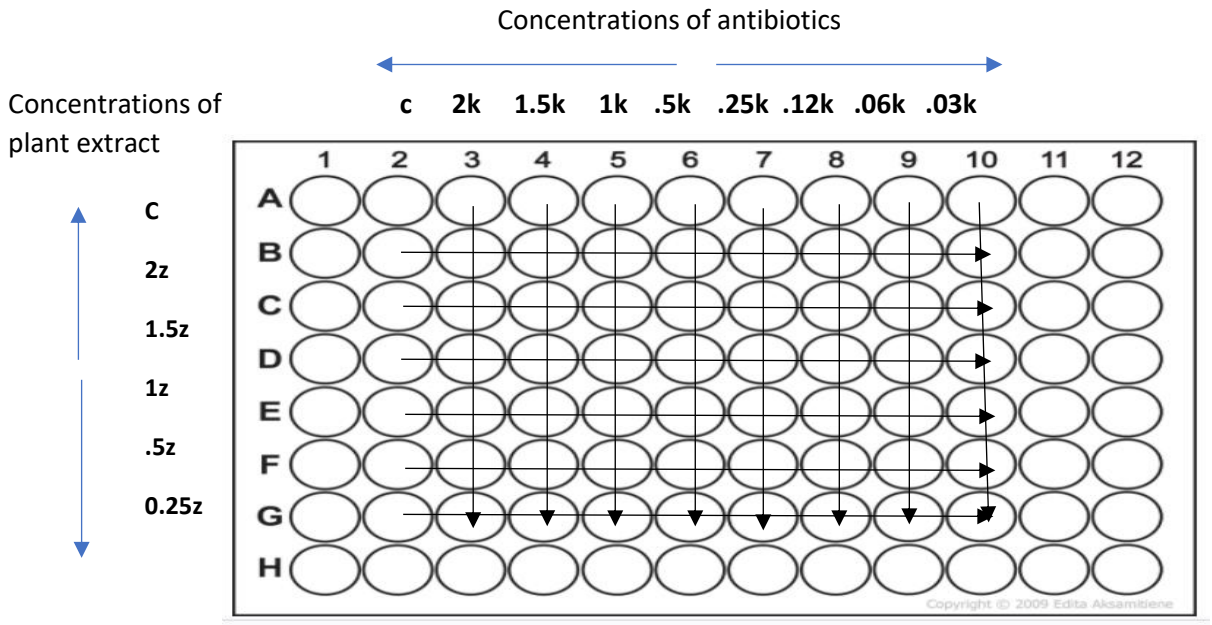


Fig.2. Demonstration of dispensing varying concentrations of Z and K to determine the MIC of the combination therapy, where Z= MIC of plant extract and K= MIC of antibiotics.

2.5.3Minimum bactericidal concentration (MBC)

For the determination of MBC, 10µl of sample from wells that exhibited no growth were dispensed into TSA plates in a grid and incubated overnight at 37°C. The lowest concentration with no visible growth of organism was taken as MBC.

2.6 Statistical data analysis:

Statistical analysis was performed using Microsoft Excel. The data are expressed as mean ± SEM (standard error of the mean).

2.7 Extract yield determination:

The yield from each extraction process was calculated using the formula:

$$\text{Extraction yield (\%)} = \frac{\text{Weight of extract after evaporating solvent and freeze-drying}}{\text{Dry weight of the sample}} \times 100$$

CHAPTER-3

3.RESULTS

3.1 YIELD PERCENTAGE OF EXTRACTIONS

The optimum particle size of the plant powder used for all extractions were 250-500µm. Different solvent systems including water, 100% methanol, 70% methanol and 70% ethanol were used for both *Panax quinquefolius* and *Nigella sativa* in the extractions. The techniques were done to analyse and study the bioactive compounds from the plants exhibiting antimicrobial activity. The yield percentage for the extractions are illustrated in (fig.3) for microwave-assisted extraction, supercritical fluid extraction, ultrasonic-assisted extraction, and shaking-assisted extraction.

SECTION: A

Panax quinquefolius:

3.1.1 Microwave-assisted extraction:

Microwave-assisted extraction (MAE) has the advantages of more efficient heating, faster energy transfer, shorter time, higher extraction rate, and superior yield at a reduced cost (Cheng et al.2007). Different parameters such as microwave power, temperature and time influence the efficiency of extraction.

The highest yield percentage of the extraction for ginseng was 22.5%. There was change in the yield depending on the variation in temperature and microwave power while the time of 15 min and compound to the solvent ratio 1:20 was kept same throughout. The results can be seen in Table.4.

Table.4 Yield percentage of Microwave extract of *Panax quinquefolius* at different variables.

SAMPLE NO.	COMPOUND	SOLVENT	TEMPERATURE	Power	YIELD IN (%)
PQ 1	4g	80mL	60°C	150W	22.50%
PQ 2	4g	80mL	100°C	120W	19.25%

3.1.2 *Supercritical fluid extraction (SFE)*

SFE is an efficient green extraction technique with advantages of using environment friendly solvent such as carbon dioxide, reducing any solvent residues. The extraction is influenced by the variable which must be appropriately implemented to get the target compound.

The *Panax quinquefolius* was unable to be extracted using the supercritical fluid extraction with Carbon dioxide (SFE-CO_2) due its low polarity of the compounds, while CO_2 solvent is used for non-polar compounds and require additional co-solvents to complete the process.

3.1.3 Ultrasonic-assisted extraction:

The ginseng samples were extracted following the parameters as discussed in section 2.1.3 in the previous chapter. The yield was calculated at varied times to analyse the influence of the variable change. The yield percentage for American ginseng is presented in Table.5. The extraction yield has shown to increase in a period of 15-60 minutes with longer sonication.

Table.5 Yield variation at different duration of UAE extraction of *Panax quinquefolius*.

SAMPLE NO.	COMPOUND	SOLVENT	TEMPERATURE	Sonication	Time	YIELD IN (%)
PQ 1	200mg	15mL	25°C	600W	25min	10.50%
PQ 2	200mg	15mL	25°C	600W	60min	20.00%

3.1.4 Shaking-assisted extraction (SAE):

The conventional flask shaking method has been effectively used to extract the ginsenosides from *Panax quinquefolius* roots in literature. Different solvent types including 100% methanol, 70% methanol and 70% ethanol were used for extraction and the yield was calculated (Table.6). The highest yield percentage for American ginseng extracted in 100% methanol was 23.3% and was comparatively high than the other extractions.

Table.6 Differential percentage yields of ginseng extracts in different solvents.

SOLVENT	AMOUNT	SOLVENT	SPEED	TEMP	TIME	YIELD IN (%)
100% MeOH	10g	100mL	150rpm	25°C	24 hrs	23.30%
70% MeOH	10g	100mL	150rpm	25°C	24 hrs	19.60%
70% EtOH	5g	50mL	150rpm	25°C	24 hrs	13.60%

Extraction is the crucial step for analysing any activity in the medicinal plant by choosing appropriate experimental conditions influencing the extraction of the target compound. Hence, with the results achieved methanol extraction has comparatively high yield.

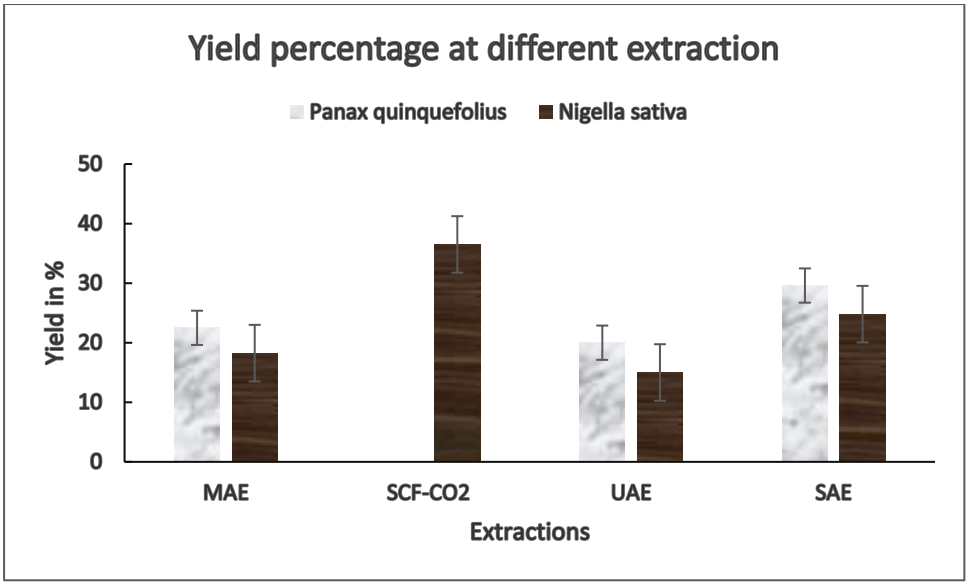


Fig.4 Yield variation among different extractions including microwave-assisted extraction (MAE), supercritical fluid extraction (SFE-CO2), ultrasonic-assisted extraction (UAE) and shaking-assisted extraction (SAE) in *PQ* and *NS.*

PURIFICATION

3.1.4 Thin-layer chromatography

Plant extracts use different solvent system to initially screen and quantify bioactive phytochemical compounds in them. The triterpene saponins in *Panax quinquefolius* was separated using the lower phase of the solvent system of chloroform: methanol: water (65:25:10). The aqueous extract screened 2 spots with rf values of 0.42 and 0.35, and the methanol extract gave 5 spots with Rf values of 0.17, 0.395 and 0.37. The compound spotted in TLC plate can be seen in Fig.5a and Fig 5b.

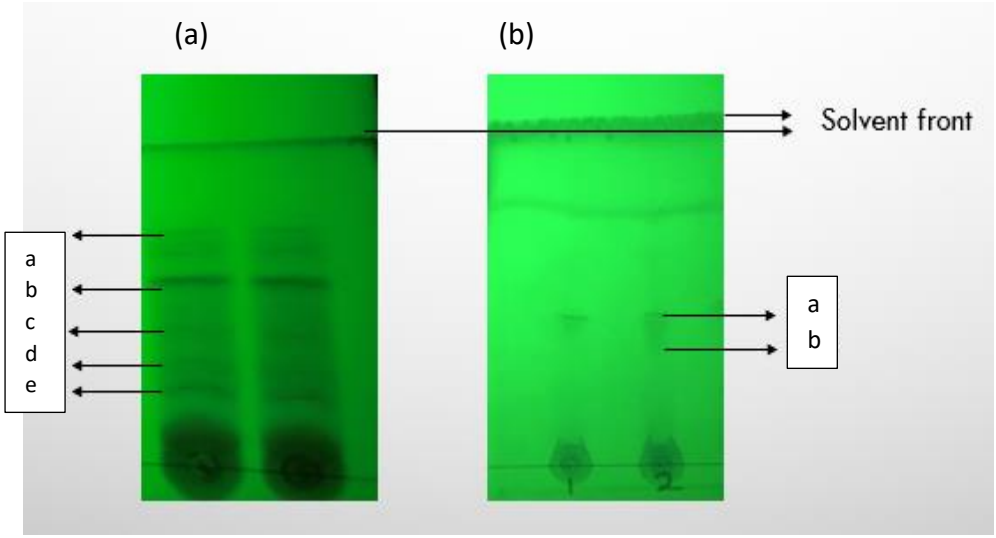


Fig 5 (a)Thin-layer chromatography analysis of methanolic extract of Panax quinquefolius with2 spots of Rf values a= 0.35 and b=0.42 (b) Analysis of aqueous extract of panax quinquefolius to screen compounds using lower phase of $CHCl_3$-MeOH-H_2O (65:25:10), with 5 spots a=0.19, b=0.48, c=0.64, d=0.69 and e=0.94.

High performance liquid chromatography (HPLC):

Under the chromatographic conditions carried out in C-18 column using the mobile phase consisting of deionized water: trifluoro-acetic acid (A; 100:0.01, v/v) and acetonitrile-trifluoro-acetic acid (B; 100:0.01, v/v), the ginsenosides were detected at 203nm wavelength. The HPLC chromatogram produced for *Panax quinquefolius* extracted using MAE, UAE, and SAE, revealed peaks at various retention times with a total running time of 80 minutes (Fig.6). Similar peaks at retention times 71 and 76 minutes were observed in all the 3 extracts. The MAE and UAE samples had more similar peaks compared to the methanolic extract.

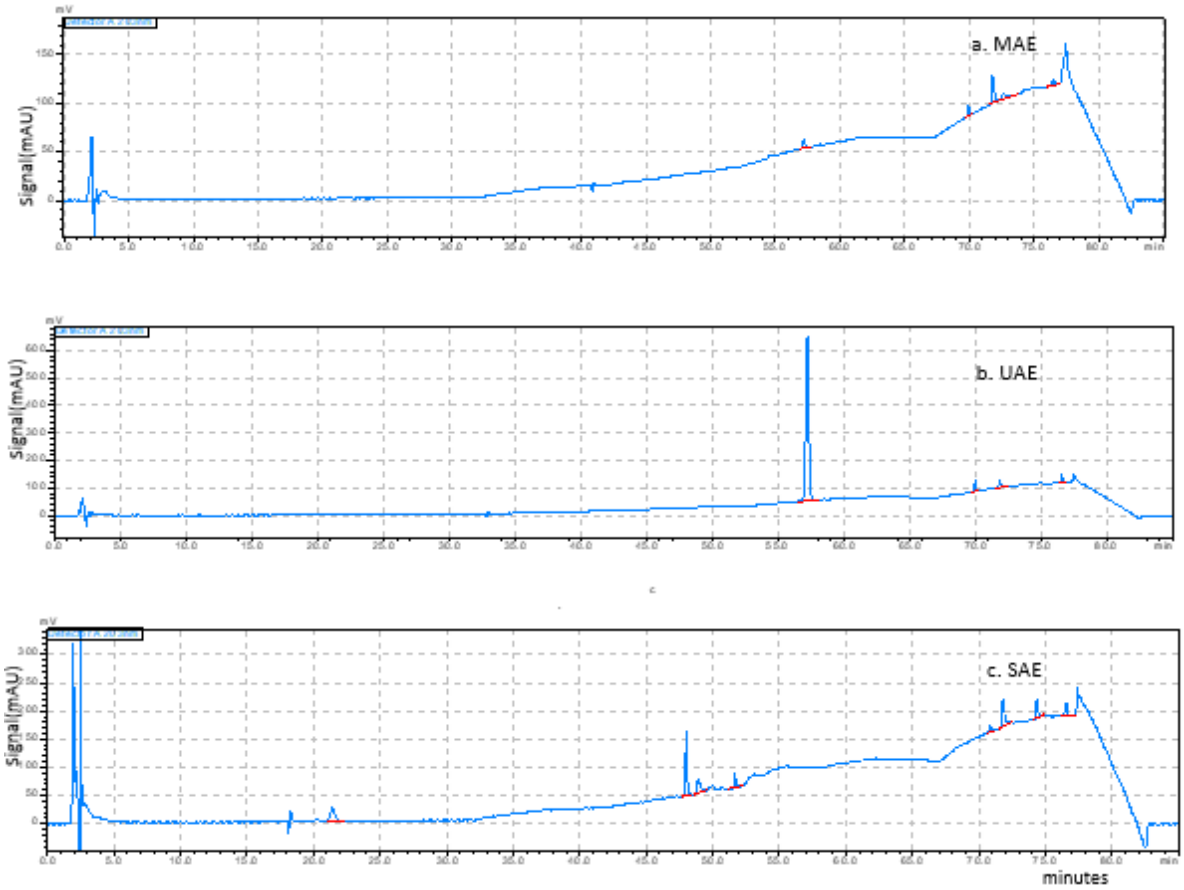


Fig.6 HPLC Chromatograms of *Panax quinquefolius* of (a) MAE, (b) UAE and (c) SAE at 203nm

3.2 **ANTIMICROBIAL SUSCEPTIBILITY ASSAY**

3.2.1a Well diffusion assay:

The assay of the extracts of *Panax quinquefolius* from the MAE, UAE and SAE was tested against Gram positive organisms-*Staphylococcus aureus*, *Bacillus pumilis* and Gram-negative organisms- *Escherichia coli* and *Pseudomonas aeruginosa* to analyse the presence of antimicrobial activity. The standard antibiotics including Vancomycin and Colistin, both at 40ug/ml, were taken as positive control for Gram Positive and Gram-negative bacteria, respectively.

The *Panax quinquefolius* extracted by microwave assisted extraction (MAE) was re-constituted in 100% methanol at 100mg/ml concentration and analysed for its antimicrobial activity. To evaluate the standardised micro-organism value, 50 µl of sample extract was tested against each organism at 1%, 2% and 4% of inoculum using the diffusion assay. The MAE of American ginseng extract at 100 mg concentration showed no inhibition zones for any organism at all (Table.7)

Table.7 Antimicrobial assay of MAE of *Panax quinquefolius* against gram positive and gram-negative organisms at 1%, 2% and 4% inoculum.

SAMPLE	*P. aeruginosa*			*E. coli*			*S. aureus*			*B. pumilis*		
	1%	2%	4%	1%	2%	4%	1%	2%	4%	1%	2%	4%
PQ	0	0	0	0	0	0	0	0	0	0	0	0
NS	9	10	0	0	0	0	0	0	0	9	0	0
SFE-NS	7	0	0	14	10	10	13	12	11	21	20	24
VANCOMYCIN	0	0	0	0	0	0	14	16	15	25	20	19
COLISTIN	15	18	18	14	18	15	0	0	0	0	0	0
CARVACROL	26	24	26	26	33	26	21	25	20	30	31	33

Following the MAE antimicrobial assay, the standardised inoculum was the 2% of organism used further. The results of assay for other extracts of American ginseng including UAE, and SAE using different solvents of 100% methanol, and 70% methanol at 100mg/mL concentration done against all organisms is presented in Table.5. Controls were used to ensure that the inhibitory effect was due to the extract rather than the alcohol solvent. Inhibition zones of 10-15mm of diameter was exhibited by both Gram positive and Gram-negative organism for all the extracts except the negative controls. The sonicated extract was susceptible to all organisms other than *E. coli*. Ginseng extracted with absolute methanol exhibited the highest inhibition zone of 15mm against *P. aeruginosa*, 14mm for *E. coli*,

14mm for *S. aureus* and 11mm for *B. pumilis*. Hence, 100% methanol is the best solvent comparatively.

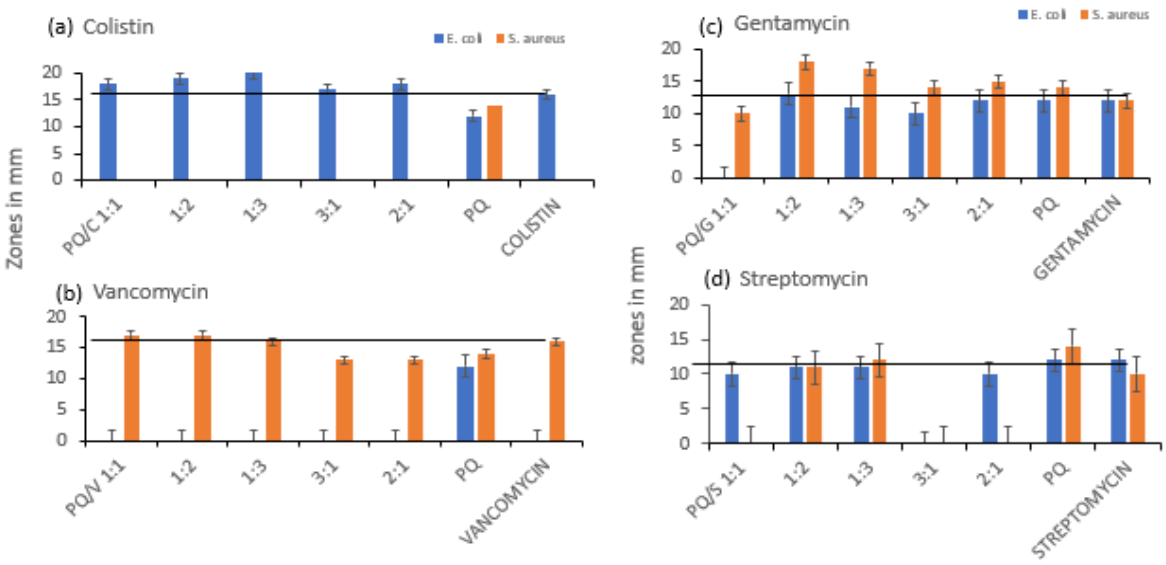
Table.8 Antimicrobial assay (zone of inhibition(mm)) of samples of *Panax quinquefolius* in different solvent system with positive control against Gram positive and Gram-negative organism.

SAMPLE TYPE	*P. aeruginosa*	*E. coli*	*S. aureus*	*B. pumilis*
PQ- 70% EtOH	0	14	13	10
PQ-UAE	11	0	13	11
PQ- 100% MeOH	15	14	14	10
Colistin (20µg/ml)	23	22	0	0
Vancomycin (20µg/ml)	0	0	18	30
MeOH	0	0	0	0
EtOH	0	0	0	0

With the positive result of American ginseng exhibiting antimicrobial activity at 100mg/ml concentration against both Gram-negative and Gram-positive organisms, the interaction of the extract with antibiotics was explored. For this, initially assay of serial dilution of American Ginseng with methanol was studied to understand the interaction as the combination reduces the concentration of ginseng (Table.1 Appendix). Two-fold serial dilution from 5mg/ml concentration of each antibiotics including Colistin, Vancomycin, Gentamycin and Streptomycin was assayed against each organism. The concentration of antibiotic- Colistin (64µg/ml, 32µg/ml), Vancomycin (16µg/ml), Gentamycin (16µg/ml, 8µg/ml) and Streptomycin (64µg/ml, 32µg/ml) exhibited similar inhibition zones as produced by 100mg/ml ginseng extract for each organism was explored to analyse ginseng with antibiotic interaction (Table.2 Appendix). The results of combination of American ginseng with antibiotics against each of Gram-positive and Gram-negative organism is presented in Fig.7. According to the results, American ginseng with Colistin exhibited high zones at 1:3 dilution, due to an addition effect in Gram negative organism while no zones are seen in Gram-positive organism.

Vancomycin combination did not have much interactive effect in the combination. And with antibiotics-Gentamycin and Streptomycin, Gram-positive bacteria exhibited slight additive interaction. Hence, the synergy of American ginseng with only colistin was explored for *E. coli*.

Fig.7 Graphical presentation of antimicrobial assay (zone of inhibition(mm)) of *Panax quinquefolius* (PQ) in combination with antibiotics dilution series against *E. coli* and *S. aureus.* (a)PQ (100mg/ml) in combination with (a) Colistin (64µg/ml) (b) Vancomycin (16µg/ml), (c) Gentamycin (16µg/ml) and (d) Streptomycin (32µg/ml)



Minimum inhibitory concentration:

The MIC value of American ginseng was exhibited for *S. aureus* as 2.5mg/ml while the *E. coli* exhibited inconsistent results from serially diluted extract from stock of 100mg/ml of plant extract. Hence, without which the MIC of the combination was unable to proceed.

DISCUSSION:

Panax quinquefolius also known as American ginseng is one of the important herbal remedies with many pharmacological properties. It has various bioactive components including

ginsenosides, polysaccharides, peptides, polyacetylene alcohols, and fatty acids. The Ginsenosides in American ginseng possess antidiabetic, antiadhesive, antitumor, anti-inflammatory, antioxidation, inhibition of cell apoptosis and antimicrobial properties (Battinelli et al., 1998). The ginsenosides are also called triterpenoid saponins with more than 280 different types identified based on their structural variability and quantitatively depending on the species, growing environment, soil and its fertility, parts, and age of the plant as well as extraction techniques used. The quantity, arrangement, and orientation of the sugar units, as well as the number and types of sugar chains linked to the aglycone moiety, all contribute to the difficulty of saponin extraction (Majinda & RR 2012). Saponins are polar compounds that are chemically and thermally labile, non-volatile, and found in low amounts in plants (Sasidharan et al. 2011).

The saponins have been topic of interest recently due to its great potential in industrial applications. It is very important to select the appropriate extraction technique to get the target compounds. Hence, extraction is challenging as it is the first step towards isolating and characterising the desired component from the plant (Sasidharan et al. 2011). Variation in characteristics of the compounds is analysed to choose the appropriate extraction. So far, the extraction of saponins has been divided into two categories: conventional and green approaches.

Solvent selection is essential in designing efficient extraction methods due to its economic and environmental impact (Zhang et al. 2018). The green technology requires replacing the hazardous chemical solvents with safer and renewable resources. Water is basically non-toxic, non-inflammable and economic greenest solvent. It also has significant limitations in terms of being converted into a universal, sustainable alternative for solvent extraction processes (Flórez et al. 2015). According to the result in Table.1, the MAE exhibits higher yield in comparison with other green technology like Ultrasonic-assisted extraction (UAE). The yield may be due

to water being reported to be an excellent microwave absorbing solvent with enough polarity to be heated by microwave power (Tatke, P & Jaiswal, Y 2011). The solvent can act on its affinity for the target chemical as well as its capacity to absorb microwave energy into the metabolites. In MAE, the microwaves can permeate biomaterials and generate heat by reacting with polar molecules such as water. Microwave penetration depth into plant matrix depends on the moisture content, temperature, and electrical field frequency. Water in plant materials absorbs microwave energy, causing internal superheating and cell structural rupture, which enables the diffusion of bioactive compounds from the plant matrix (Takeuchi et al., 2009).

Ultrasonic-assisted extraction (UAE) is also a simple and effective green technology used for recovering and purifying bio-actives. Suitable solvent system for saponin was the combination of absolute methanol with water (Ngo et al. 2017). The ginseng powder with 70% methanol solvent was sonicated and the yield was measured at 2 different periods of time. The result in Table.2 shows that the yields increased significantly with the sonication extending from 25 to 60 min. With further sonication for 90 min, the yield increased only slightly which suggest 1-2 hours to be the optimal period for saponin extraction. Methanol is believed to be an excellent solvent, the ultrasound waves create cavitation through which the additional amount of water efficiently diffuse into the matrix and the heat released accelerate mass transfer of compounds into the solvent, boosting the yield (Vardanega et al. 2014).

Shaking-assisted extraction is a commonly used conventional technique for extracting saponins using solvents like methanol. Previous research has used the SAE for extracting different ginsenosides from American ginseng and greater yield efficiency (Ligor et al. 2005). The result exhibited in Table.3 shows the variation in the yield efficiency with different solvent system. Hence, absolute methanol has the highest saponin recovery. The disadvantage of SAE is its longer extraction time of 24 hours and larger solvent amounts as compared. The methanol

can affect the chemical labile compounds which can conflict in isolating the target compound (Zhang et al. 2018).

Temperature variability on extraction: According to the results exhibited for MAE in Table.1, the yield percentage of the compound shows variation with the alteration in temperature and microwave energy. The solid to liquid ratio was taken as 1:20 throughout the experiment and the variability of solid liquid ratio was not analysed. Temperature is also an influential parameter as it can alter morphological changes and induce faster mass transfer of plant secondary metabolites. Higher temperatures are expected to improve extraction efficiency by increasing elution of solutes from active areas in the matrix (Li et al. 2010). At high temperature the yield has been less as compared to lower temperature yield, which may be reasoned due to the thermolabile property of the compound. Previous research on saponin microwave extraction have shown increase in yield at lower temperature and decline in yield with increase in temperature (Yao et al.,2016). Hence, the mass transfer at high temperature in aqueous solvent have shown to give low yield of ginsenosides and an appropriate temperature of 60°C maybe optimal for MAE of American ginseng.

UAE has the advantage of using lower temperature for efficient recovery of thermolabile saponins as compared to other green techniques as well as conventional methods (Vinatoru et al., 2017).

A summation of the yield efficiency of plant extract in *Panax quinquefolius* is displayed in Fig 1, which shows using 3 techniques of green technology and a conventional method. Hence, the highest is exhibited by the shaking assisted method. Considering the other facts like extraction duration and renewable sources, MAE has been efficient in recovering the bio-actives with slight difference in the yield at much lower time. Optimisation in the solvent system in MAE

might make a difference, as previously discussed combination solvent system of absolute methanol/ethanol with water can efficiently recover compounds.

TLC is a simple, easy, and fast screening technique to analyse any bioactive compounds in the mixture based on the polarity of compounds and the solvent system used. It has been successful in separating and determining many saponins in plants. In the *Panax quinquefolius* extracts, ginsenosides from each group of protopanaxadiols and protopanaxatriols in which compounds are differentiated on basis of the sugar moieties, were simultaneously determined, and well separated. According to previous literature, the Rf value of compounds identified are assumed to be ginsenosides Rb1 and Re in aqueous extract and Rb1 in methanolic extract. Hence, the predominant ginsenosides in roots are Re and Rb1 and it accounts for 75% of the total ginsenosides in the root (Li et al.1996). For further analysis and confirmation of compounds the extracts were run through HPLC.

The separation of compounds depends on various factors including the absorbents, solvents, the extraction technique as well as any chemical treatment to visualise the colourless compounds (Laila et al. 1995). The solvent system used for most saponins is the combination of chloroform: methanol: water so that the lower phase can separate these strong polar compounds. The triterpene saponins are colourless and optically active, but they lack reactivity, making them difficult to characterise due to their lack of visibility in natural light and even when exposed to UV radiation (Oleszek et al.2008). The spots visible in 254nm were infused into the silica gel plates, which had been activated at 100°C for 1 hour. The advantage of these plates are they do not require spraying with any toxic chemicals.

The initial and fast antimicrobial activity screening is made easy by performing the bioautography by placing the TLC plates with separated compounds on agar plates infused with organisms and incubated for any inhibition zones (Oleszek et al.2008).

According to Xue et al. 2017, the antibacterial activity of ginseng depends on the extraction and processing techniques. As the solvents can influence the type of ginsenoside being extracted and polar solvent like water can only extract polar ginsenosides. Another study states that the antimicrobial activity is closely related to the polarity of the ginsenosides in the extract (Zheng et al.2017).. Further, less polar ginsenosides destroy integrity of the bacterial membrane system and destroy the cell. The ginseng usually possesses polar ginsenosides which on physical, chemical or biotransformation treatments modify the sugar moieties in the structure and convert to less polar ginsenoside (Zheng et al.2017). Therefore, the ginsenosides present in the MAE might have been polar compounds and require temperature more than 70ºC for the process. Hence, research papers like Onivogui et al. 2016 and Ashafa et al. 2008 reports the aqueous extract exhibits low antimicrobial activity compared to methanol and ethanol. The antibacterial activity exhibited by Ginseng shows the presence of active ginsenosides and confirmed with the presence of peaks analysed in the HPLC. The interaction with antibiotics in the well diffusion assay exhibited an additive effect in combination with Colistin against *E. Coli* and in combination with Gentamycin against *S. aureus* as in (Fig.6). Hence, the combination of American ginseng with Colistin exhibited higher synergy for *E. coli* comparatively and was aimed to further explore for the novelty of the combination. Colistin have been previously used for combination therapy with other antibiotics for improving the antimicrobial activity. The Colistin combination studied proved to have definite synergy with selected antimicrobial agents (Petrosillo et al.2008). Using Colistin in combination with American ginseng was a novel idea, but in current study the inconsistent results by the MIC of plant extract along with limitation in time to perform repeat experiments. Secondly, the extract used was from the American ginseng powder readily available in market which may point towards ineffectiveness as the quality and active ingredients vary and the extraction technique used. With effective activity displayed by American ginseng roots towards *E. coli*, the future

direction would be to study combination therapy with Colistin. Colistin is commonly used to treat MDR Gram-negative infections (Luo et al.2020), the combination can help slow the spread of Colistin resistance by lowering the amount of the antibiotic is being utilised.

SECTION: B

NIGELLA SATIVA

EXTRACTION

3.1.1b. Microwave-assisted extraction:

The highest yield percentage obtained from the microwave extraction was 18.25%. The parameters like temperature and microwave power were altered which gave a variation in the yield as in Table.9.

Table.9 Yield percentage of MAE samples of *Nigella sativa* at different variables.

SAMPLE NO.	COMPOUND	SOLVENT	TEMPERATURE	POWER	YIELD (%)
NS TRIAL1	4g	80mL	70°C	350W	18.25%
NS TRIAL 2	4g	80mL	45°C	200W	15.50%

The extraction was focused on separating the bioactive compounds Thymoquinone and Carvacrol which are monoterpene phenol compounds and non-polar in nature.

3.1.2b Supercritical fluid extraction:

The parameters set at 350 bar pressure and 70ºC temperature was for the extraction of thymoquinone in *Nigella sativa*, which exhibited strong antimicrobial activity against several organisms. The oil produced from the seeds exhibited a yield of 36.5%, which was close to the results reported in the literature. Hence, the temperature was reduced to 40°C and pressure increased to 400 bars to analyse the yield comparatively.

3.1.3b Ultrasonic assisted extraction

The phenolic compound in Nigella seed was extracted using ultrasonic power of 600W for maximum duration of 30 minutes. The yield was compared at 15 minutes and 30 minutes showing a variation in the yield as shown in Table.10. An increase in yield was observed with the longer sonication period.

Table.10 Yield variation at different duration of UAE extraction of *Nigella sativa*.

SAMPLE NO.	COMPOUND	SOLVENT	TEMPERATURE	Sonication	Time	YIELD IN (%)
NS1	200mg	15mL	25°C	600W	25min	9.20%
NS2	200mg	15mL	25°C	600W	60min	15.00%

3.1.4b Shaking assisted extraction:

The *Nigella sativa* was extracted using solvents including ethanol and methanol at different concentrations to evaluate the highest yield for thymoquinone extraction. The yield percentage for 100% methanol, 100% ethanol and 70% ethanol are in (Table.11) in which the highest is exhibited by the 100% methanol extraction at 24.8%.

Table.11 Percentage yields of Nigella extracts in different solvents.

SOLVENT	AMOUNT	SOLVENT	SPEED	TEMP	TIME	YIELD IN (%)
100% MeOH	10g	100mL	150rpm	25°C	24 hrs	24.80%
70% MeOH	10g	100mL	150rpm	25°C	24 hrs	17.70%
100% EtOH	10g	100mL	150rpm	25°C	24 hrs	22.40%

PURIFICATION

3.2bThin layer chromatography:

The bioactive compounds comprising of monoterpene phenols from *Nigella sativa* were analysed using chloroform as the single solvent system. The mixture used were methanolic extracts of Nigella obtained from shaking-assisted extraction and oil from supercritical-fluid extraction. The spots were confirmed by comparing with data in literatures following the similar protocol. The extracts were run for a period of 30 minutes, 2 compounds spotted at retention factor (Rf) values 0.45 and 0.22 in methanol extract and 3 compounds were spotted in oil extract with Rf values 0.07, 0.11 and 0.52. Carvacrol standard was run to compare the results which gave a spot with Rf value of 0.59 (Fig.8).

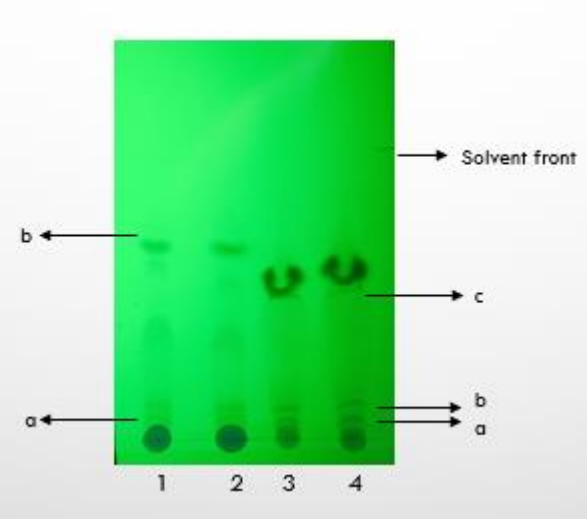


Fig.8 Thin layer chromatographic analysis of aqueous Nigella extract in 1 and 2, giving 2 spots with rf values a=0.10 and b=0.71. The oil extract from SCF placed on 3 and 4, screened compounds with rf values a=0.07, b=0.11 and c=0.52.

QUANTIFICATION

3.3bHigh partition liquid chromatography (HPLC):

With the chromatographic conditions carried out in C-18 column using the mobile phase consisting of deionized water: trifluoro-acetic acid (A; 100:0.01, v/v) and acetonitrile-trifluoro-

acetic acid (B; 100:0.01, v/v), the compounds were detected at 203nm wavelength. The HPLC chromatogram obtained for analysis of Nigella extracted using MAE, UAE, and SAE, exhibited peaks at different retention time with a total running time of 80 minutes (Fig.9). The peak at 71 minute was commonly seen in all the three extracts. The RT was compared with other research papers to identify the peaks. The extracts obtained using green technology of MAE and UAE had more 4 similar compounds at retention time of 57, 71, 74 and 76 minutes, respectively.

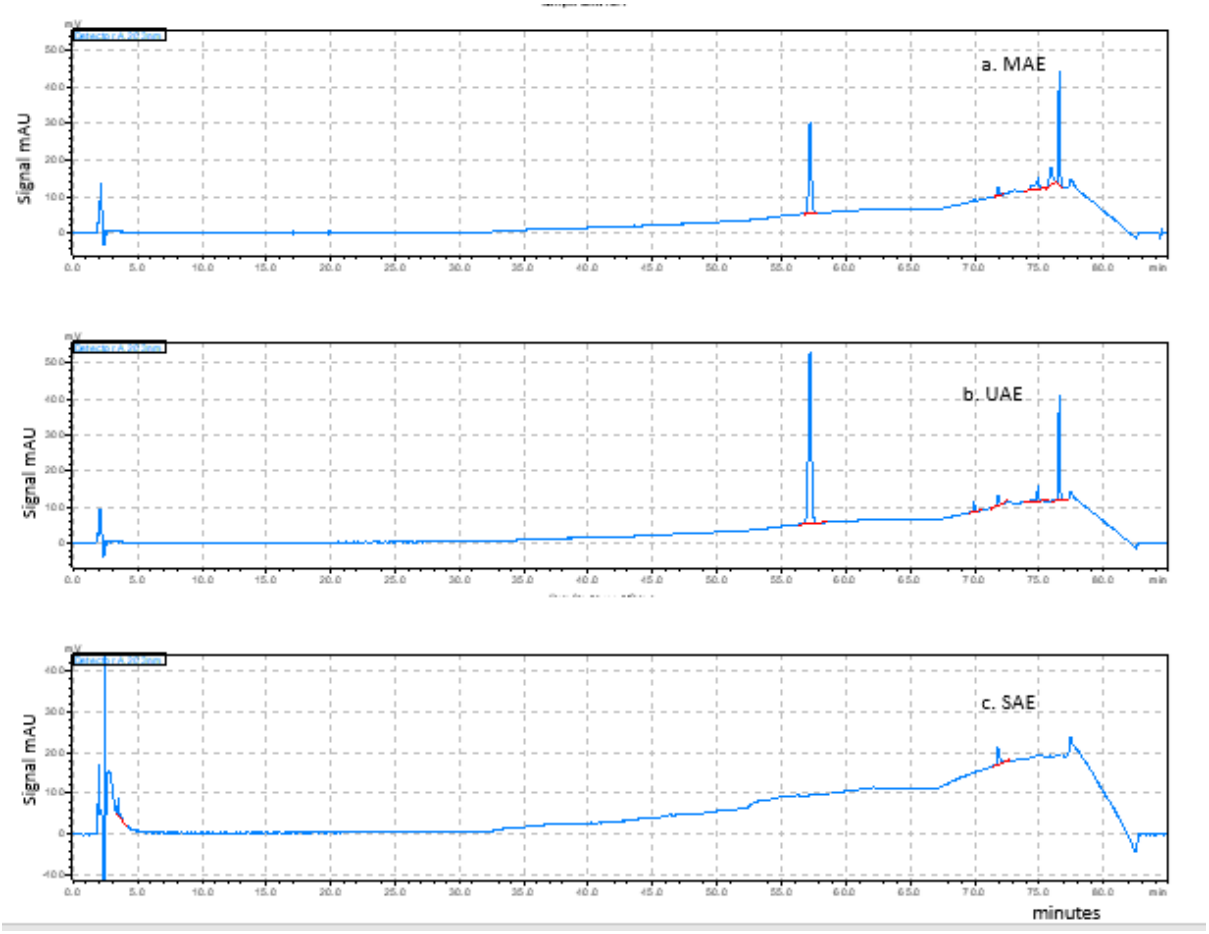


Fig.9 HPLC Chromatograms of Nigella extract of (a) MAE, (b) UAE and (c) SAE at 203nm.

Antimicrobial susceptibility assay:

3.4b Well diffusion assay:

Initially the antimicrobial activity of the microwave aqueous extracts of *Nigella sativa* was analysed and no inhibition zones were detected in *E. coli*, *Staphylococcus aureus*, and *Bacillus pumilis* while *Pseudomonas aeruginosa* exhibited faint zones of 9mm. And the oil from SFE extraction of the *Nigella sativa* gave activity zones as in (Fig.10).

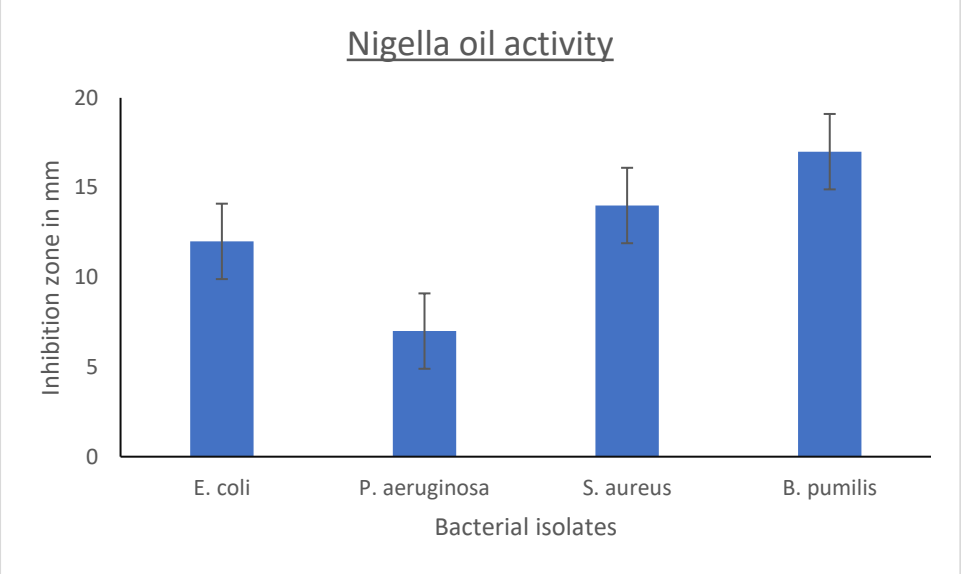


Fig.10 Antibacterial activity of Nigella oil from SFE in well diffusion technique

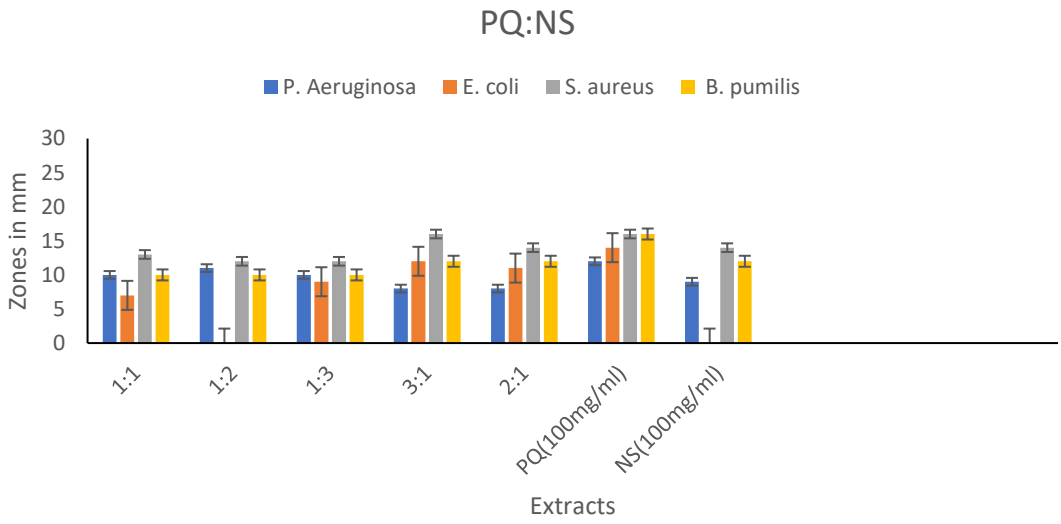
The effective Nigella extract from different extraction was analysed against Gram positive and Gram-negative organisms, among which absolute methanol extracts exhibited activity consistently for the Gram-positive organisms and *P. aeruginosa*. (Table.12)

Table.12 Antimicrobial assay (zone of inhibition(mm)) of samples of *Nigella sativa* in different extraction with positive control against Gram positive and Gram-negative organism.

SAMPLE TYPE	*P. aeruginosa*	*E. coli*	*S. aureus*	*B. pumilis*
NS -SAE	11	0	15	16
NS oil-SCF	0	0	14	11
NS -UAE	14	0	14	11
NS oil- standard	14	0	14	11
Colistin (20µg/ml)	23	22	0	0
Vancomycin (20µg/ml)	0	0	18	30
MeOH	0	0	0	0
EtOH	0	0	0	0

With effective inhibition zones using exhibited by the methanol extract of Nigella from SAE, further experiments were conducted using the same. The extracts were effective mostly against the gram-positive organisms and *P. aeruginosa*, and completely ineffective on *E. coli*. The two-fold serial dilutions of *Nigella sativa* with methanol were assayed to calculate the methanol interaction and its effect on the concentration of the plant extract to understand the concentration variation during combination with antibiotics (Table. 3 Appendix). The result of *Nigella sativa* combined with American Ginseng exhibited no additive activity together in Fig.11.

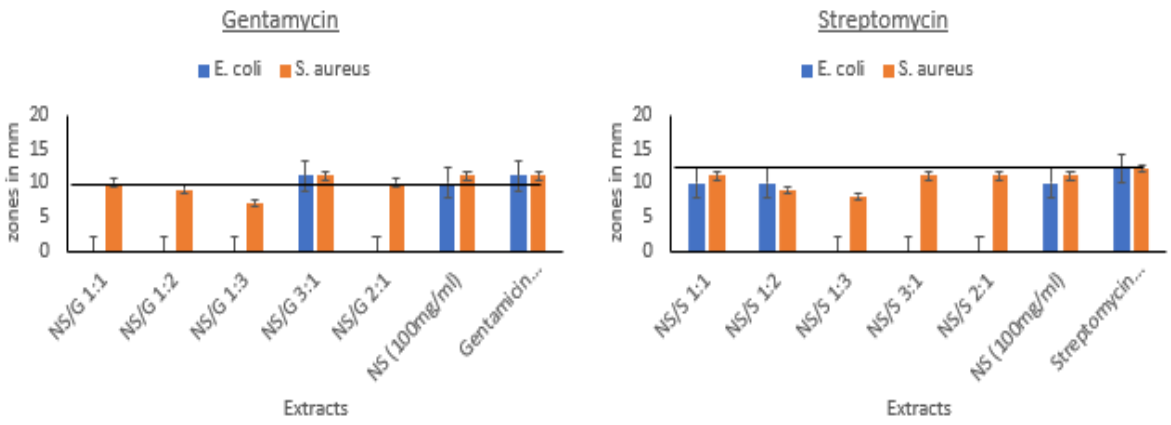
Fig.11 Antimicrobial assay (zone of inhibition(mm)) using combination of methanol extract dilute series of *Panax quinquefolius* and *Nigella sativa*, PQ: NS (100mg/ml) on Gram negative and Gram-positive organism.



The concentration of the antibiotics-colistin, vancomycin, gentamicin and streptomycin exhibiting similar zones of 100mg/ml *Nigella sativa* was analysed (Table.2 Appendix). In the results of combination of *Nigella sativa* with antibiotics, an antagonistic activity was shown by the combination with vancomycin and streptomycin and colistin had no effect (Table.4.

Appendix) While the gentamicin exhibited larger zones in the combination which shows the synergy (Fig.12) at 3:1 dilution.

Fig.12 Antimicrobial efficacy (zone of inhibition(mm)) of *Nigella sativa* (NS) in combination with antibiotics-Gentamicin (16µg/ml and 8µg/ml) and Streptomycin (64µg/ml, 32µg/ml) against one Gram-positive and Gram-negative organism highlighting the effect.



3.5b Minimum inhibitory concentration for synergy confirmation:

The MIC value of nigella extract against *Staphylococcus aureus* and *Bacillus pumilis* were 10 mg/ml respectively, while for the *Escherichia coli* and *Pseudomonas aeruginosa* were >10 mg/ml. From the MIC determined, the MBC value for both *S. aureus* and *B. pumilis* were 10 mg/ml.

MIC for the standard compound Carvacrol was also determined to be 1.90 mg/ml.

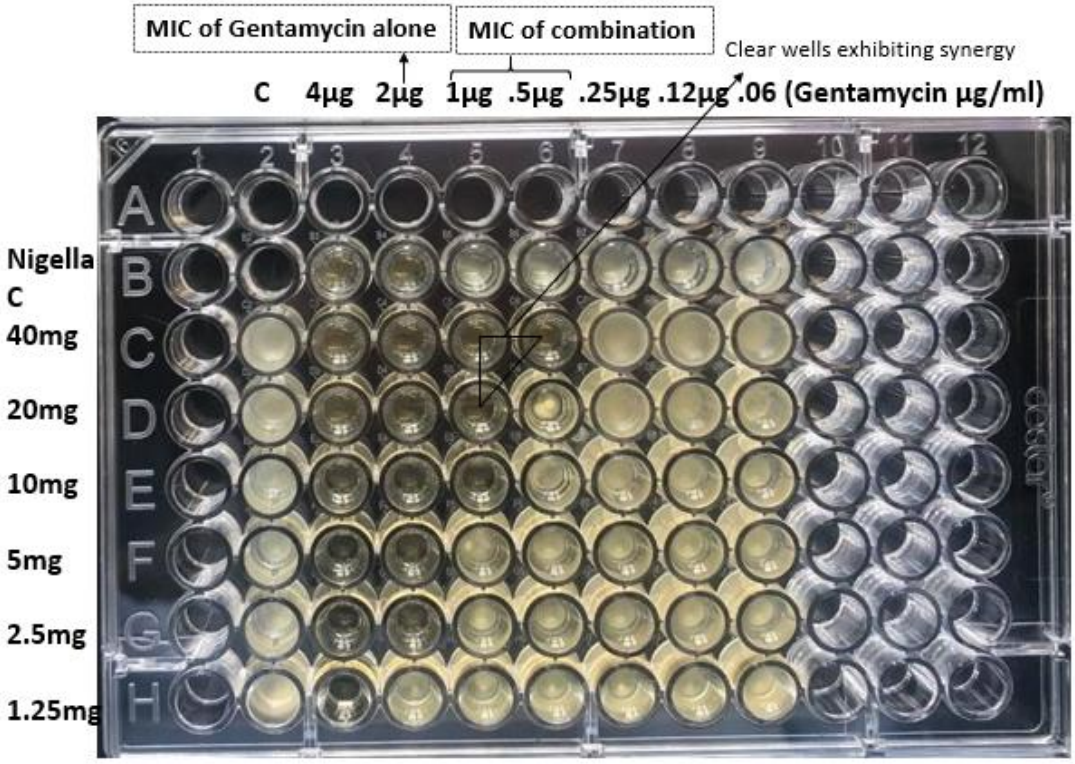
The MIC and MBC values for Gentamycin for both Gram-positive organisms and Gram-negative organisms are as in the Table.13.

Table.13 MIC value determination of Gentamycin(5mg/ml) against Gram-positive and Gram-negative organisms.

	MIC (µg/ml)	MBC (µg/ml)
Gentamycin(5mg/ml)		
Escherichia coli	7.5	15
Staphylococcus aureus	1.8	3.75
Pseudomonas aeruginosa	15	30
Bacillus pumilis	3.7	7.5

Following the evaluation of MIC of the plant extracts and antibiotics, the combination MIC value was analysed for the synergy. The MIC value for the combination *Nigella sativa* (10 mg/ml) with Gentamicin (2µg/ml) in *Staphylococcus aureus* exhibited synergy at the combination of concentration of *Nigella sativa* at 40mg/ml and Gentamycin at 1 µg/ml and 0.5 µg/ml. This indicates that in the combination, 40mg of Nigella extract reduced 25-50% of the MIC of Gentamycin of 2 µg/ml which alone can effectively inhibit *S. aureus*. Another synergy was exhibited at the concentrations of 20mg/ml of Nigella which reduced 50% of gentamycin concentration to1 µg/ml for combined inhibition as in Fig.13.

Fig.13 The MIC of synergy exhibited from the combination of Nigella extract at 40 and 20 mg/ml with Gentamycin at 2 and 1 µg/ml, respectively with MIC of Gentamycin alone of 2 µg/ml.



***Discussion*:**

Nigella sativa, also known as the black cumin seed, is a traditional medicinal plant used since ancient times. It is composed of fixed oil -32-40%, proteins, alkaloid, saponin and essential oil-0.4-0.45% (volatile oil). The volatile oil contains the main bioactive compounds like Thymoquinone (TQ), Di thymoquinone (THQ), Carvacrol and Thymol exhibiting great pharmacological properties (Forouzanfar et al.2014). These compounds are monoterpene phenols which are lipophilic hydrocarbons and vary in their structural property and polarity accordingly which the extraction technique for target compounds is fixed. Mostly the terpenes are non-polar or less polar due to the presence of the hydrocarbons in the structure (Liu et al.2011).

MAE results demonstrates that the final yield of the target compound depends on factors such as irradiation power and the temperature. It uses temperature to power combination to optimise

the extraction of bio active. At higher temperatures, there is an increased solvent diffusivity into the matrix, as well as adsorption and segregation of solutes into the solvent, improving the yields (Florez et al. 2014). However, the temperature optimal for Nigella bio-active compounds is not more than 70ºC, above which it can cause depolymerisation and de esterification leading to lower recovery (Salea et al. 2013). And with increase in irradiation power, it improves the molecular interaction between the electromagnetic field and the target compound, making it easier for solvent to penetrate the plant matrix and for solutes to dissolve and be recovered (Alara et al. 2019)

The supercritical fluid extraction with carbon dioxide (SFE-CO2) of *Nigella sativa* seeds produced oil with an excellent yield efficiency of 36.5%. The main short coming is the time-consuming extraction process. Certain factors like particle size, temperature, and pressure are critical parameters influencing the extraction. As for the antimicrobial active compounds, the best extraction conditions are high pressure and low temperature (Santoyo et al. 2006). This study was carried out at 300bar and 75°C giving the yield. Another study was done with increase in pressure to 400 bar and reducing the temperature to 45ºC. The result was unsatisfactory as the oil production was halted giving incomplete extraction and the yield could not be calculated. The explanation for this could be due to the decreased temperature, which prevented the solute from the plant from diffusing into the solvent system.

Ultrasonic-assisted extraction of *Nigella sativa* showed increase in yield with the longer period of sonication. According to previous studies, the period of more than 60 minutes has only slight variation in the yield, which confirms 60 minutes as the optimal time for sonication (Wu et al.2001). Other parameters such as frequency and the solvent system are highly influential, a high yield is exhibited with pure methanol/ethanol solvent (Gimbun et al. 2014). Hence, use of different solvent system was a limitation in my study, and was unable to analyse the variation.

Shaking-assisted extraction (SAE) is a commonly used conventional technique for extraction. The extraction yield can be affected by the solvent's polarity, pH, temperature, extraction time, and sample composition. Solvent composition and sample are the most critical parameters when using the same extraction time and temperature. The yield extraction by different solvents were in the order: 70% methanol>100% ethanol>100% methanol. Hence, extraction using 100% methanol is higher than absolute ethanol proving that yield increases with increase in polarity (Nawaz et al. 2020). According to the study, supercritical extraction using carbon dioxide is a viable approach for the isolation of active compounds from *Nigella sativa*.

Thin layer chromatography (TLC) has been used as a preliminary detection method as it has proved to be a simple and economical approach for chemical and biological screening of plant extracts (Sewara et al. 2019). The method was employed to analyse the presence of antimicrobial activity exhibiting compounds Thymoquinone (TQ), di thymoquinone (THQ) and carvacrol. With the obtained extraction samples, the chromatogram was analysed under UV lamp at 254 nm, and the detect spots were compared to the literature data of the standards. To analyse the target compound, the extraction method and the solvent used are very significant. Hence, referring to previous research, the screened compounds are assumed to be like Rf values of thymoquinone and Di thymoquinone which make up the main bioactive compounds of *Nigella sativa*. The oil from SFE showed a strong visible spot of compounds. Rather than spots, extremely polar compounds frequently develop streaks (Anon, 2019). Hence, the polar nature of chloroform system was used to detect and elute nonpolar compounds of monoterpenes in the *Nigella sativa*.

The HPLC results exhibit peaks at various time interval, showing the presence of bio-actives. A common peak at 71 minutes is present in all the extracts. In reference to other papers, most of the analysis is done at 294nm. Hence, the compounds were not identified with no standards

run at the similar protocol but presence of bioactive was validated with the antimicrobial activity exhibited in the well diffusion assay.

The Nigella seeds and its oil have been reported to show antimicrobial efficacy towards wide range of Gram-positive and Gram-negative organisms (Salman et al.2007). In this study, the methanolic extract of *Nigella sativa* seeds exhibited high antibacterial activity specifically towards the Gram-positive organisms-*S. aureus* and *B. pumilis*. In previous reports, the bio-active compound thymohydroquinone was particularly active in inhibiting gram-positive organisms (Kokoska et al2008). The Gram-negative organisms have an outer coating of lipo-polysaccharides restricting the penetration of amphipathic compounds across the barrier (Ropponen et al. 2021). Hence, this can be reasoned for the inactivity of *Nigella* on Gram-negative organism particularly *E. coli*. The second aim of the study was to observe the change in antibacterial activity of Nigella extract in combination with antibiotics. Hence, Nigella with the combination of Gentamicin exhibited synergistic effect. This was confirmed with evaluating the MIC values, where the synergy was exhibited at combination of 40mg/ml to 1 µg/ml, 40 mg/ml to 0.5 µg/ml and 20mg/ml to 1 µg/ml of Nigella and Gentamicin, respectively against *S. aureus*. For comparison, the MIC of the standard compound carvacrol in combination with gentamycin was tested against *S. aureus*. The combination of *Nigella sativa* and gentamicin has previously been studied in vivo to corroborate Nigella′s preventive function against gentamicin-induced nephrotoxicity (Yaman et al. 2010). Gentamicin is a commonly used antibiotic effective against Gram negative bacterial infection in human and animals (Reiter et al., 2002). High dose has reported to be causing complication in human (Hayward et al. 2017). As a result, the Gentamicin concentration can be lowered in combination therapy, and overall toxicity can be minimised because to Nigella's protective characteristics.

RESEARCH LIMITATIONS:

Bioactive substances have been extracted using diverse techniques for various uses, as a result of major research efforts. Depending on applications of the compounds, different methodologies are developed. With each type of extractions certain limitations come such as low efficacy, low yield, high energy and so. The protocols from different papers for the same compound may not report the same results depending on the nature of compound which may contain compounds with effect of the growth conditions and other factors of influencing the change. Hence, optimised conditions of pre-treatment strategies, solvent selection, available extractor conditions were to be applied with each result outcome. It was challenging to use the available solvents and optimise accordingly. The microwave extraction was limited to use only water as the solvent, hence other solvent extraction was not possible which limited my study and the results.

Antimicrobial assay is an essential and simple technique to determine the activity of any compounds. Dealing with micro-organisms give varying results and time consuming in their growth and re-growth. The limitation was inconsistent maintaining of the cultures, which resume growth with time and storage conditions.

Research in times of covid-19 has been difficult in causing delivery delay with the products purchased online. For the initial trials, American Ginseng roots were purchased, which showed good activity; but, due to the delayed delivery time, different ginseng powder was purchased locally, which had lower activity. As a result of the change in product, my final results were inconsistent, making it impossible to determine synergy.

CONCLUSION

The current study on the antimicrobial activity exhibited by the green technology extracts and the conventional extracts of *Panax quinquefolius* and *Nigella sativa* has confirmed its activity against Gram-positive and Gram-negative organisms. The methanolic extracts of the plant extracts exhibited activity against both type of bacteria. With the MIC of PQ and NS exhibited at 10 mg/ml, it proves to be effective against pathogenic bacteria mostly the Gram-positive organisms. The antimicrobial activity depends on the extraction technique used. However, this has been proven in previous research and with the change in the extraction conditions and solvents in the study, there was variation in activity towards each bacterial strain (Seleshe et al. 2019). However, developing optimised extraction conditions for application in green technology for upscaling would be economic and environmentally sustainable.

In addition, the combinations of the plant extracts and antibiotics exhibited synergistic effect against Gram-positive organisms. The combination of Nigella with Gentamycin exhibited synergistic effect against *S. aureus*. Similarly, the synergy of American ginseng and colistin is promising with antimicrobial assay displaying larger zones. Moreover, the plants have shown to reduce the toxicity and concentration of the antibiotics in the combined application (Yaman et al. 2010). The next approach for getting an effective synergistic action should be identifying and isolating any single or multiple components in the extract responsible for the synergy and their composition in the plant. The cytotoxicity of the combination on mammalian cell lines such as normal liver cells are to be tested for developing skin treatment topicals. According to the reports from Iwaki et al. 2011, 895 µg/ml and 0.1% of commercially available Gentamicin sufficiently inhibits bacteria including gentamycin resistant strains causing skin infections. While in this study in the combination with *Nigella sativa*, enough Gentamicin was as low as 1-2 µg/ml and *Nigella* has also proven to be effective in treating skin infections (Rafati et al. 2014). Hence, further studies should be followed for drug application using the combination.

FUTURE DIRECTIONS:

The natural bio actives are being studied for their varying pharmacological properties which have the industrial potential of drug application. Because of the unrivalled abundance of chemical variety, natural products, such as plant extracts, either as pure chemicals or as standardised extracts, give limitless prospects for new drug discoveries (Cos et al., 2006). Consequently, chemically manufactured medications have developed side effects and bacteria resistance. Thousands of phytochemicals from plants were discovered to be safe and effective alternatives with fewer side effects. Nearly 20,000 therapeutic plants exist in 91 countries, according to the World Health Organization (WHO). American Ginseng and *Nigella sativa* has been part of traditional medicines from a long time. About 280 types of ginsenosides in Ginseng have been identified and expected to have more present. Due to the thermolabile nature of compound, temperature is an influential factor for extraction. In comparing, the conventional and the green technology extraction, there is only a slight variation in the yield, but the activity was higher for the methanol-based extract. Application of a suitable solvent in the techniques like microwave assisted extraction and ultrasonic assisted extraction with a biorefinery of the products maybe more valuable as these plants contain more bioactive compounds which can be utilised for other application. As in the case of American ginseng contain saponin and polysaccharides both of which have been reported for the possible adjuvant activity (Ghosh et al.2020). Similarly, the compounds in *Nigella sativa* exhibit excellent pharmacological properties, but the in-vivo studies have not been implemented due to the instability of thymoquinone during extraction. Other bio-active compounds like carvacrol have not been explored enough while the same compound from oregano and thyme have been analysed well.

With effective activity of the medicinal compounds combination with drugs will be giving promising results. To reap such benefits, innovative research for better quality control of herbs,

identification of active components, and knowledge of the molecular process would be required.

APPENDIX

Table.1 Antimicrobial assay (zone of inhibition(mm)) of *Panax quinquefolius* (AG) and *Nigella sativa* (NS) with methanol at different dilutions against Gram positive and Gram-negative organisms

	DIAMETER OF INHIBITION ZONE(MM)		
Sample	***E. coli***	***S. aureus***	***P. aeruginosa***
NS/M 1:1	12	9	9
NS/M 1:2	11	8	11
NS/M 1:3	13	8	9
NS/M 3:1	16	11	9
NS/M 2:1	12	10	11
AG/M 1:1	13	12	10
AG/M 1:2	9	7	11
AG/M 1:3	11	7	10
AG/M 3:1	9	15	10
AG/M 2:1	10	14	11
AG	11	12	12
NS	11	14	10
COLISTIN	14	0	16
VANCOMYCIN	0	18	0
METHANOL	0	0	0

Table.2 Antimicrobial assay (zone of inhibition(mm)) of antibiotic-Colistin, Vancomycin, Gentamycin and Streptomycin(1mg/ml) dilution series.

	DIAMETER OF INHIBITION ZONE(MM)			
Sample(1mg/ml)	*E. coli*	*S. aureus*	*P. aeruginosa*	*B. pumilis*
COLISTIN 1	22	0	19	0
2	20	0	18	0
3	18	0	15	0
4	16	0	14	0
5	13	0	12	0
6	11	0	10	0
VANCOMYCIN 1	15	23	0	28
2	13	22	0	26
3	11	20	0	26
4	9	18	0	25
5	8	16	0	23
6	0	14	0	18
7	0	12	0	15
GENTAMYCIN 1	24	24	21	24
2	23	24	20	22
3	21	23	19	21
4	19	19	16	19
5	16	17	16	17
6	14	15	14	16
7	12	13	12	15
8	11	12	11	13
STREPTOMYCIN 1	20	18	18	24
2	18	17	17	24
3	16	14	14	21
4	16	14	13	18
5	14	12	11	17
6	12	11	9	15

Table.3 Antimicrobial efficacy (zone of inhibition(mm)) of *Panax quinquefolius* in combination with antibiotics-Gentamicin (16µg/ml and 8µg/ml) against gram negative and gram-positive organism.

Zones in diameter(mm)

AG:G	*E. coli*	*P. aeruginosa*	*B. pumilis*	*S. aureus*
	Plate1	Plate1	Plate1	Plate1
1:1	12	10	13	9
1:2	12	11	13	10
1:3	13	10	13	10
3:1	11	9	11	8
2:1	9	11	0	8
AG(100mg/ml)	12	8	9	0
Gentamicin	12	11	12	11

Table.4 Antimicrobial efficacy (zone of inhibition(mm)) of *Panax quinquefolius* in combination with antibiotic-Streptomycin (64µg/ml, 32µg/ml and 8µg/ml) against gram negative and gram-positive organism.

DIAMETER OF INHIBITION ZONE(MM

AG:S	*E. coli*	*P. aeruginosa*	*B. pumilis*	*S. aureus*
1:1	11	10	13	9
1:2	11	11	13	10
1:3	12	12	14	10
3:1	10	9	12	11
2:1	11	10	13	8
AG	0	0	8	10
STREPTOMYCIN	12	12	12	10
COLISTIN	14	16	13	0
VANCOMYCIN	0	0	14	11

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