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Table of Content

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REVIEW ARTICLE

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Synergistic Interaction Between Combination of Existing Therapy with Polyphenols in Several Human Diseases: A Review

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ABSTRACT

Introduction: The complicated pathology of current diseases requires an intricate treatment. Today, current application of individual single-target drugs or therapeutic approaches is inadequate to target these diseases not to mentioned perceived shortcomings and presented with numerous adverse effects. The extensive and successful documented findings in natural product researches urges the need to make use of these knowledge in the development of new generation of medicine. Polyphenols are compounds naturally derived from plants and have been describe in many research to have tremendous medical benefit. Therefore, a synergistic combination of readily available drugs or other therapeutic approaches is a favourable approach to enhance efficacy, overcome toxicity and optimize safety. The objective of this review is to describe the synergistic effects between the combination of a variety of polyphenols with synthetic drugs or other therapeutic approaches which can help to improve therapeutic efficacy subsequently minimize the adverse effects of a substance targeted in various diseases focusing mainly on cancer, diabetic, microbial infections and tissue regeneration along with their underlying mechanism.

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Introduction

Natural products from plants have been widely applied by humans for the past centuries for treatment of various diseases (Firenzuoli & Gori, 2007). The use of natural products for medicinal purpose were being practiced by about 75% to 80% of the world population mainly because of its cultural acceptability as well as superiorly compatibility with human body (Set et al., 2010). Contrary to synthetic drugs which typically are chemically isolated compound, natural products consist of multicomponent of phytoconstituents (Ginsburg & Deharo, 2011). There are numerous elements that influence the efficacy mechanism of natural products which includes the geographical origin, plant parts (leaves, stem, root, and fruit), storage, processing, extraction as well as types of solvents used (Doughari, 2012; S.-Y. Pan et al., 2013). Over the recent years, researchers are currently focusing on natural products for drug discovery targeted for various diseases.

In spite of the facts that the process of drug discovery is now moving forward resulting in advancement of various technology platforms, drug development remains a considerable long process with a low rate of success and large investment. It takes up to years for a newly discovered chemically synthetic compound to become a successful marketable therapeutic agent (Barden & Weaver, 2010). Not to mention, readily available synthetic drug usually perceived shortcomings and presented with numerous adverse effects (Pöch, 2012). Today, thanks to scientists over the world, approximately 80% of antimicrobial, cardiovascular, immunosuppressive, and anticancer drugs are from plant origin or developed from a natural compound (Krief et al., 2004). Polyphenols are one of the natural products that has been extensively researched upon and has been described successfully either *in vitro* (Curti et al., 2017; H. Sun et al., 2018); *in vivo* (Kujawska & Jodynis-Liebert, 2018; Wang et al., 2017) or in clinical trial (Borges et al., 2016; Wauquier et al., 2019) to effectively target various disease documented with low toxicity and minimum adverse effects to human (Cory et al., 2018; Edwards et al., 2012; Nash et al., 2018).

Throughout recent years, more attention have been given by researcher to study the potential interaction between synthetic drugs with natural product targeting diverse type of diseases (Lehár et al., 2009). Thus, with the presence of recent technological advancement and abundant discovery on the effects of natural product and its health benefits; the development of a new generation medicine can be expedited by combining natural products with synthetic drugs or other therapeutic approaches (Pan et al., 2013). The potential benefits that can be gained from interaction of natural product with synthetic drug or other recent therapeutic approaches includes increased efficiency, reduction of undesirable effects increase in bioavailability of the free agents as well as gaining sufficient therapeutic effect with relatively small doses in comparison with a synthetic medication (Chanda & Rakholiya, 2011). The focal point of this review is on the discovery of synergistic effect of combination of naturally derived polyphenols with synthetic drugs or other therapeutic approaches on various diseases and medical discoveries.

The medicinal role of Polyphenols

Polyphenols represent a group of common phytochemicals that are structurally characterized by the presence of one or more phenol units which includes hydroxybenzoic acids, hydroxycinnamic acids, anthocyanins, proanthocyanidins, flavonols, flavones, flavanols, flavanones, isoflavones, stilbenes, and lignans (Gupta et al., 2008) . Flavonoids for example can be further subdivided into flavones, flavonols, flavanones, isoflavones, anthocyanidins, chalcones, and catechins predominantly found in fruits, vegetables, legumes, red wine, and green tea and have a potential effect on radical scavenging activity and inflammatory reactions (Xiao et al., 2011). Additionally, stilbenes or resveratrol are polyphenols found in product of grapes, red wine, and peanuts. Meanwhile, phenolic acids found in coffee, tea, cinnamon, blueberries, kiwis, plums, apples, and cherries; all has been reported to elicit tremendous health benefits (Hasan et al., 2013; Sales & Resurreccion, 2014).

It has been documented that different groups of polyphenols have shown to have different biochemical mechanism and acts differently in response to various modes of diseases. Numerous studies confirm that treatment by using variety of polyphenolic compounds either single compounds or in groups as well as dietary intake of natural sources rich in polyphenols, reduced incidence of chronic diseases. Polyphenols mainly acts by interacting with reactive oxygen species (ROS). ROS are typically formed within human body in a controlled amount and are vital compounds that are related in the regulation of processes in maintaining cell homeostasis and functions such as signal transduction, gene expression, and activation of various signaling receptors (Kumar & Pandey, 2015). The involvement of polyphenols on ROS has suggested to be associated with the etiological effects on prevention of different disease pathologies (Lima et al, 2014).

Tremendous researches have shown the therapeutic potential of polyphenols (Figure 1)(Ganesan & Xu, 2017) such as anti-diabetic (Cao et al., 2017), anticancer (Devi et al., 2017), anti-inflammatory (Yahfouf et al., 2018), cardioprotective (Arbeláez et al., 2018), osteoprotective (Brzóska et al., 2016), neuroprotective, antiasthmatic, antihypertensive, antiageing, antiseptic, hepatoprotective, antifungal,

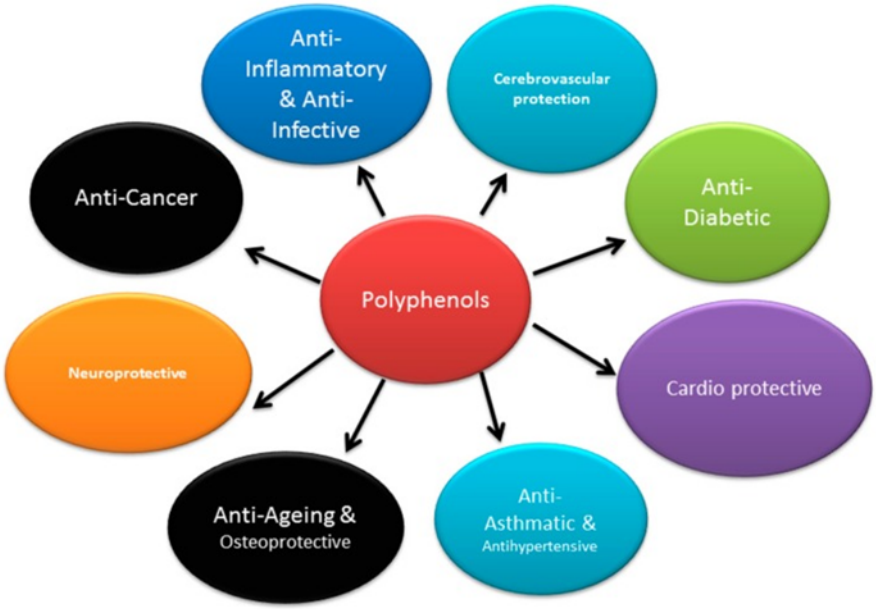

Figure 1: Role of polyphenols in humans (Ganesan & Xu, 2017)

antibacterial and antiviral properties (Ganesan & Xu, 2017; Gorzynik-Debicka et al., 2018; Gupta et al., 2008).

Synergistic mechanism

Development of combined pharmaceuticals or therapeutics substances primarily aimed to achieve synergism in order to remain clinically significant. Researcher has denoted how latest molecular biological methods and new genomic technologies allow us to unwinds the various synergistic mechanisms underlying these effects (David et al, 2015). However, recognition of successful drug combination is complicated and often remains a setback due to lack of standardization in the aspect of terminology, experimental protocols as well as drug modelling.

Synergistic effects are produced when compounds interact with one another of the same constituents or in combination with other compounds such as synthetic drugs, biomaterials and other therapeutics approaches. The mechanism of synergy will effect different targets in order to improve the solubility and by that means enhance the bioavailability of one or several substances of a compounds (Pöch, 2012). The general standardized method used to measure synergisms and antagonisms is the isobologram method in which concludes the iso-dose effect of two or more substance acting together (Berenbaum, 1989). Moreover, the construction of a dose response curve also allows investigation of combined effects of investigated compounds (Chou, 2006). Additionally, to achieved a successful combined therapy synergism it is important to also consider numerous factors affecting drug-drug interactions or compounds interactions (natural products) such as compound target site, affected pathway of compound, process or pathogenesis the compounds acts on and most importantly patients' ability to absorb, metabolize and excretes a substance or compounds (Pemovska et al., 2018; X. Sun et al., 2013).

Methodology

The relevant articles were searched through PubMed, Google Scholar and ScienceDirect database. The following keywords and search terms were used: "synergistic combination of polyphenols therapy" in "anti-cancer", "anti-microbial", "anti-diabetic," "cardio-protective" and "regenerative effect". The articles were screened and the articles that are related to the search keywords dated from the year from 2000 to 2020 were included in the review.

Results

Synergistic interaction between combination of existing therapy with polyphenols on various diseases

Over the recent years, consistent efforts have been made to translate the benefits offered by natural product into clinically relevant substances or therapy in many diseases setting. In this review, we listed the successful work of the combination treatment; by employing existing standardized therapy such as synthetic drugs and biomaterials commonly used in clinical setting with polyphenols (Table 1) and its mechanism. In most cases, transforming experimental research for clinical based trial might be hard, however, in combination therapy; through the use of existing standardize regimes and protocol; polyphenols can simply be introduced safely in clinical trials for example through diet or in the form of

Table 1: Therapeutic combination of existing therapy with polyphenol on various diseases target.

Drugs or therapeutics approach	Polyphenols	Targets	Experimental models	Experimental findings	Ref
Anticancer					
Tamoxifen	Green tea extract	Breast cancer	*In vitro* MCF-7, ZR75, T47D breast cancer cell line and *in vivo* breast cancer induced mice model	i. *In vitro* green tea extract increased the inhibitory effect of tamoxifen on the proliferation of estrogen receptor (ER) -positive MCF-7, ZR75, T47D human breast cancer cells. ii. *In vivo* mice treated with both green tea and tamoxifen showed highest apoptosis in tumor tissue compared with either agent administered alone.	(Sartippour et al., 2006)
Trastuzumab	Oleuropein aglycone from extra virgin olive oil extract	Breast cancer	*In vitro* SKBR3 breast cancer cell line	Increased efficacy of trastuzumab in the presence of oleuropein aglycone via significant reduction of HER2 gene; that are associated with unfavorable breast cancer prognosis includes high fatality and relapse rate.	(Menendez et al., 2007)
Docetaxel	Curcumin	Breast cancer	Phase I clinical trial on advanced metastatic breast cancer patients	Fourteen patients were accrued in the trial. Findings showed combination dose of curcumin for seven consecutive days every 3 weeks with a standard dose of docetaxel improved biological response and clinical presentation in most patients indicated encouraging efficacy results.	(Bayet-Robert et al., 2010)
Doxorubicin (DOX)	Quercetin	Breast cancer	*In vivo* mice model transplanted with 4T1 breast cancer cells.	Combination of dietary quercetin with intratumoral DOX injection synergistically induced potent rejection of 4T1 breast cancer, induced T-cell tumor specific response that results in long-term, tumor-free survival in mice.	(Du et al., 2010)
Doxorubicin (DOX)	Silymarin (SLM) from seeds of Silybum Marianum	Breast cancer	*In vitro* 4TI breast cancer cell line	Combination of SLM-DOX exerts synergistic growth inhibitory effects on 4TI breast cancer cell line.	(Gheybi et al., 2019)

Table 1 (cont.): Therapeutic combination of existing therapy with polyphenol on various diseases target.

Drugs or therapeutics approach	Polyphenols	Targets	Experimental models	Experimental findings	Ref
Doxorubicin (DOX)	Epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) from green tea extract	Liver cancer	*In vitro* human hepatocellular carcinoma (HCC) cell line; BEL-7404 and BEL-7404/DOX and *in vivo* mice model transplanted with BEL-7404/DOX HCC cells.	ECG and EGCG increased chemosensitivity to DOX and increase DOX cytotoxicity in BEL-7404/DOX cells by inhibiting P-gp pump function that contribute to the reversal of multidrug resistance (MDR) *in vitro* and *in vivo*.	(Liang et al., 2010)
SAHA-suberoylanilidine hydroxamic acid Zolinza (vorinostat)	epigallocatechin-3-gallate (EGCG) from green tea extracts	Melanoma	*In vitro* A-375, Hs-294T and G-361, human melanoma cell line.	Combination treatment EGCG with varinostat resulted in significantly higher inhibition of cell proliferation, increased apoptosis via modulation of the cyclin-cdk-cki network, Bcl2 family proteins and NF-κB activity.	(Niha et al., 2010)
Cisplatin	Theaflavin (TF) and epigallocatechin-3-gallate (EGCG) encapsulated in biodegradable nanoparticulate (TF/EGCG-NPs)	Lung cancer, acute monocytic leukemia (AML) and cervical cancer	*In vitro* A549 human lung adenocarcinoma cell line, THP-1 human acute monocytic leukemia cell, HeLa human epithelial cervical cancer and *in vivo* ascites carcinoma induced mice.	Combination of TF/EGCG-NPs with cisplatin inhibited NF-κB activation and suppressed cyclin D1 activation, matrix metalloproteinase-9, and vascular endothelial growth factor (VEGF), involved in cell proliferation, metastasis, and angiogenesis *in vitro*. *In vivo* findings showed that combination treatment increased the life span of mice model with apparent regression of tumor volume compared to either agent alone.	(Singh et al., 2015)
Leptomycin B (LMB)	Epigallocatechin-3-gallate (EGCG)	Lung cancer	*In vitro* A549 human lung adenocarcinoma cell line	Combination treatment of EGCG enhanced LMB cytotoxicity through enhanced ROS production and modulation of drug metabolism via p21/survivin pathways.	(Cromie & Gao, 2015)
Doxorubicin (DOX) and etoposide	Quercetin, apigenin, emodin, rhein and cis-stilbene (commercial compounds)	Lymphoid and myeloid leukemia	*In vitro* TIB-152 peripheral blood T cell leukemia, CCRF-CEM acute lymphoblastic leukemia, THP-1 acute monocytic leukemia and KG-1a acute myelogenous leukemia	DOX alone combined with quercetin, apigenin, emodin, and cis-stilbene synergistically leads to the downregulation of glutathione and increased apoptosis via caspase 8 and 9 activation in myeloid leukemia.	(Mahbub et al., 2015)

Table 1 (cont.).: Therapeutic combination of existing therapy with polyphenol on various diseases target.

Drugs or therapeutics approach	Polyphenols	Targets	Experimental models	Experimental findings	Ref
Bleomycin (BLM)	Tea polyphenols (TPP)	Antioxidant based therapy for cervical cancer	*In vitro* SiHa cervical cancer cell line	The TPP-BLM treatment synergistically induced apoptosis through caspase-3, caspase-8 and caspase-9 activation, Bcl-2 upregulation and p53 overexpression as well as increased the percentage of apoptotic nuclei in nuclear staining.	(Alshatw et al., 2016)
Tamoxifen (TAM)	Genistein (soy-based extracts)	Hepatocellular carcinoma	*In vitro* HePE2 human hepatocellular carcinoma cell line	Genistein and TAM significantly inhibit proliferation and induce apoptosis in HepG 2 cell line.	(Sanaei et al., 2017)
Dacarbazine and evorolimus	Oleuropein	BRAF mutated melanoma	*In vitro* A375 human melanoma cell line induced with BRAF mutation	Oleuropein successfully increased the cytotoxic effect of Dacarbazine and significantly enhanced Everolimus effects on BRAF melanoma cells, via inhibition of the pAKT/pS6 pathway.	(Ruzzolini et al., 2018)
Cisplatin	Theaflvineflavin-3,3'-digallate (TF3)	Ovarian cancer	*In vitro* A2789, CP70 and OVCAR3 ovarian cancer cell line	TF3 and cisplatin synergistically induced apoptosis and G1/S cell cycle arrest in ovarian cancer cells as well as downregulated Akt phosphorylation in ovarian cancer cells.	(H. Pan et al., 2018)
2-methoxyestradiol (2-ME)	Oleuropein	Osteosarcoma	*In vitro* 143B human osteosarcoma (OS) cell line	Oleuropein significantly enhanced anti-cancer effects of 2-ME on highly metastatic 143B OS cells.	(Przychodzen et al., 2019)
Antimicrobial					
Amphotericin B	Epigallocatechin-3-gallate (EGCG)	Antimycotic-susceptible and -resistant Candida albicans	*In vitro* microbial culture	Combined treatment between EGCG and amphotericin B enhances the antifungal effect of amphotericin B by inhibiting growth of antimycotic-susceptible and -resistant C. albicans by 98.5%–99.7%. as well as allows the use of lower doses of antimycotics.	(Hirasawa & Takada, 2004)
Norfloxacin (NOR), ampicillin (AMP), oxacillin (OXA), ciprofloxacin (CIP)	Curcumin	Methicillin-resistant Staphylococcus aureus (MRSA)	*In vitro* microbial culture	Curcumin in combination with all the four antibiotics effectively inhibit S.aureus growth via reduction in minimal inhibitory concentration (MIC) against MRSA.	(Mun et al., 2013)

Table 1 (cont.): Therapeutic combination of existing therapy with polyphenol on various diseases target.

Drugs or therapeutics approach	Polyphenols	Targets	Experimental models	Experimental findings	Ref
Ciprofloxacin	Phenolic-rich maple syrup extract (PRMSE); active components catechol	Gram-negative clinical strains of Escherichia coli, Proteus mirabilis, and Pseudomonas aeruginosa	*In vitro* microbial culture	PRMSE with ciprofloxacin exhibit synergistic interaction by targeting bacterial biofilm which helps reduced biofilm formation and increased the susceptibility of bacterial biofilms to antibiotics.	(Maisuria et al., 2015)
Fluconazole and amphotericin B	Catechins from Assam and Himachal Pradesh green tea	Candida albicans and candida glabrata	*In vitro* microbial culture and *in vitro* vero cell line	Purified catechins showed synergistic activity with fluconazole and amphotericin B against Candida species with twice MIC compared to any agent alone. Cytotoxicity analysis of the combined treatment depicted high percentage viability from 91.4% to 100% of Vero cell line; suggesting non-cytotoxic activity of proposed composition on healthy cells.	(Anand & Rai, 2017)
Rimfampin (R) and isonic acid (INH)	Polyphenols from Punica stranatum or pomegranate extracts	Multidrug resistance turbeculosis (MDR-TB)	*In vitro* microbial culture	Synergistic effects were observed between R and INH with Punica stranatum extracts against MDR-TB strains. However, combination therapy of R was more effective than INH. Combination of R with Punica stranatum extracts at 15% inhibited 100% (MIC 200%) against MDR-TB strains.	(AlMatar et al., 2019)
Anti-diabetic					
Insulin	Curcumin	Diabetes	*In vitro* C2C12 mouse myoblast cell line	Treatment of insulin and curcumin synergistically and strongly induced glucose uptake and the phosphorylation of AMP-activated protein kinase *in vitro* with increased insulin sensitivity in muscle cells.	(Kang & Kim, 2010)
Oral hypoglycemic drugs (OHD), namely, thiazolidinedione (THZ) and metformin,	Ferulic acid, p-coumaric acid, eugenol, chlorogenic acid, and caffeic acid from dietary polyphenol	Diabetes	*In vitro* 3T3-LI adipocytes cell line	Cinnamic acid, ferulic acid, p-coumaric acid, eugenol, chlorogenic acid, and caffeic acid in combination thiazolidinedione (THZ) and metformin, increases glucose metabolism via increased uptake of 2-deoxyglucose (2DG) by 3T3-L1 adipocytes cells.	(Prabhakar & Doble, 2011)

Table 1 (cont.): Therapeutic combination of existing therapy with polyphenol on various diseases target.

Drugs or therapeutics approach	Polyphenols	Targets	Experimental models	Experimental findings	Ref
Pioglitazone	Ellagic acid	Type II diabetes	*In vivo* diabetes induced rat	Diabetic rats received combination of 10 mg of ellagic acid/kg with 10 mg of pioglitazone/kg showed improvements in all biochemical parameters in comparison to single treatment along with increased the expression levels of GLUT4, PPAR-γ and adiponectin in skeletal muscle.	(Nankar & Doble, 2017)
Cardio-protective effects					
Simvastatin	Chokeberry flavonoid extract (anthocyans, polymeric procyanidines and phenolic acids)	Myocardial infraction (MI)	Clinical trial in patients that survived MI and have received statin therapy at least 6 months	Forty-four patients with mean age 66 years undergone double-blind, placebo-controlled trial. Together with simvastatin, flavonoids from chokeberry extract reduce the severity of inflammation by significantly decreased serum 8-isoprostans and Ox-LDL levels, as well as hsCRP and MCP-1 levels with the reduction in systolic and diastolic blood pressure.	(Naruszewi et al., 2007)
Simvastatin or atorvastatin	Flavonoid-enriched chocolate contained short-term flavan-3-ol and isoflavone	Cardiovascular disease risk	Randomized, double-blind, placebo-controlled clinical trial in postmenopausal women with type II diabetes receiving mg simvastatin or atorvastatin	118 participants aged less or equal to 75 years old undergone the clinical trial. The chocolate enriched flavonoid intervention with existing therapy (simvastatin or atorvastatin) improved pulse pressure variability equated to a 10% cardiovascular disease risk reduction with larger reductions in diastolic blood pressure and mean arterial pressure indicating clinically relevant improvements in arterial stiffness.	(Curtis et al., 2013)
Atorvastatin calcium	Curcumin	Atherosclerosis	*In vitro* human aortic endothelial cells and *in vivo* ApoE knockout (ApoE-/-) mice	Synergistic suppression of adhesion molecules (E-selectin and ICAM-1) and plasma lipid along with secretion of inflammatory factors (IL-6 and MCP-1) on combined atorvastatin calcium and curcumin delivery in vitro. Reduced Ato-inducible cytotoxicity *in vivo* observed in combined treatment. Both *in vitro* and *in vivo* results demonstrated drastically reduces atherosclerotic lesions.	(Li et al., 2019)

Table 1 (cont.): Therapeutic combination of existing therapy with polyphenol on various diseases target.

Drugs or therapeutics approach	Polyphenols	Targets	Experimental models	Experimental findings	Ref
Atorvastatins	Resveratrol	Percutaneous coronary intervention (PCI) for coronary artery disease	*In vivo* rabbits with induced abdominal aorta injury followed by drug - eluting stents (DESs) implantation and *in vitro* bone marrow stem cells (BMSCs)	The area of proliferation and migration of vascular smooth muscle cells in the tunica intima and mean thickness were greater in the combined atorvastatin and resveratrol treatment *in vivo*. Biochemical assays on *in vitro* BMSCs resulted in significantly upregulated Akt, p -Akt, eNOS, p -eNOS , and CXCR4 expression. Both findings exhibited improved re-reendothelialization.	(Chen et al., 2020)
Regenerative effects					
Bone marrow stromal cells (BMSCs)	Green tea polyphenols (GTPs)	Blood-spinal cord barrier (BSCB) after spinal cord injury	*In vivo* rat model with spinal cord injury	Combination of BMSCs and GTPs shown to decrease BSCB permeability that helps to improve spinal cord compression, improve motor function, up-regulated expression of tight junction associated proteins claudin-5, occludin and ZO-1 in rat model with spinal cord injury.	(Yu et al., 2015)
Collagen scaffold	Curcumin based chitosan nanoparticle	Wound healing	*In vivo* rat model	Synergistic combination of Curcumin based chitosan nanoparticle and collagen scaffold indicated faster contracted wound in wound closure analysis and complete epithelialization with thick granulation tissue formation *in vivo*.	(Karri et al., 2016)
Pamidronate	Quercus infectoria extracts	Bone regeneration	*In vitro* hFOB 1.19 human osteoblast cell line	Increased rate of proliferation and significant elevation of Runx2 and Osx expression; biochemical markers for bone tissue regeneration in cells treated with combination of Quercus infectoria extract and pamidronate.	(Raudhah et al., 2018)
Collagen scaffold	P-coumaric acid	Mandible tissue regeneration	*In vivo* rat model with critical size mandible defect.	Impregnation of collagen scaffold loaded with p-coumaric acid and cartilage oligomatrix protein (COMP) enhanced formation of new bone and showed up-regulation of osteogenesis related biochemical markers; Osx, OCN and OPN as well as angiogenesis markers; fibroblast growth factor-2 and VEGF in rat model with critical size mandible defects.	(Bhattarai et al., 2019)

capsulation. The main aims are primarily to increase the efficacy and effectiveness of the readily available therapy; determining the required doses; thus, allowing synergistic interaction.

Mechanism of interaction of combined existing therapy with polyphenols

The capability of polyphenols to regulate the activity of various enzymes and thus to interfere in signaling mechanisms in various cellular processes may be ascribed in part to the physiochemical properties of these compounds that allow them to participate in different metabolic cellular oxidation–reduction reactions. Most documented mechanism of action of polyphenols are via savaging ROS. As mentioned, ROS are produced by human body in a controlled quantity and are capable of unrestricted oxidation of various cellular components that controlled different signal transduction in cells; it can also lead to the oxidative destruction of the cells (Mittler, 2002). Commonly, oxidatively modified forms of proteins accumulate during aging, oxidative stress, and in most pathological conditions of diseases; these had scientist to focused their attention on the modification of biological molecules by various kinds of ROS. Collectively, these ROS can lead to oxidation of amino acid residue side chains, formation of protein-protein cross-linkages, and oxidation of the protein backbone and deoxyribonucleic acid (DNA) modification that may help in development of targeted therapy for management of various disease (Balaban et al., 2005; Buttke & Sandstrom, 1994; Finkel & Holbrook, 2000).

As previously mentioned, development of newly discovered drugs or synthetic compounds could take up to years involving tremendous experimental research and multiple clinal trial in order for it to be marketable and confirmed the safety of its use (Barden & Weaver, 2010). Hence, taking into consideration the immense benefits offered by polyphenolic compounds; as listed in table 1; this review uncovers successful studies on the combination of polyphenols with readily available drugs or therapeutic approaches targeted on various diseases and its ability to increase drugs along with therapeutic efficacy. As a consequence, may fast tract the process of new drug-natural product discovery.

Combination of chemotherapeutic agents with polyphenols

Despite many advances in the treatment for most forms of cancer, the mortality and relapse rate remain high. Since there is no definitive treatment for cancer, many efforts have been made in order to find a breakthrough in cancer. We have compiled a list of successful combination treatment in cancer therapy employing the use of standard chemotherapeutics drugs with polyphenols targeted for different types of cancer. An ideal treatment combination should achieve synergistic effects via measurement of various biochemical markers. Studied on the successful *in vitro* assessment of combined treatments between chemotherapeutics agents and polyphenols on targeted cancer cell lines exhibited improved or increased inhibition of cancer cells via direct anti-proliferative and pro-apoptotic effects (Alshatwi et al., 2016; Cromie & Gao, 2015; Gheybi et al., 2019; Liang et al., 2010; Mahbub et al., 2015; Menendez et al., 2007; Nihal et al., 2010; Przychodzen et al., 2019; Ruzzolini et al., 2018; Sanaei et al., 2017; Sartippour et al., 2006; Singh et al., 2015). Additionally, some of the studies has shown that the presence of polyphenols increased the efficacy of chemotherapeutic agents used against the studied cancer cell line compared to any of the agents alone (Cromie & Gao, 2015; Liang et al., 2010; Mahbub et al., 2015; Menendez et al., 2007; Ruzzolini et al., 2018; Sartippour et al., 2006). The biological activity of combined treatment between chemotherapeutic drugs and polyphenols has been extensively studied in the preclinical assays. Some combined treatments are more effective in overcoming cancer chemotherapeutic resistance by modulating cancer cells with multiple drug resistance (MDR) overexpression phenotype (Liang et al., 2010; Sartippour et al., 2006). A part from that, some of the studies has shown that synergy combination between chemotherapeutic drugs and polyphenols, exert an important role in apoptosis induction, cell cycle arrest and oxidative stress *in vitro* (Alshatwi et al., 2016; Kiemlian Kwee, 2016; Pan et al., 2018). Moreover, *in vivo* study demonstrated suppression of angiogenesis is translated to larger areas of necrosis and lower blood vessel density in the treated xenografts (Sartippour et al., 2006). *In vivo* experimental results also showed promising potential of long-term tumor free survival in the experimental model (Du et al., 2010; Singh et al., 2015). Regardless of the successful preclinical findings documented, clinical trial has yet to be truly investigated for combined treatment between chemotherapeutic drugs and polyphenols. Bayet-Robert et al, however; demonstrated significant improvement in clinical presentation and biological response in a phase I clinical trial conducted among advanced metastatic breast cancer patients receiving doxetacel (chemotherapeutics drugs) and curcumin (polyphenols) (Bayet-Robert et al., 2010). Hence, this has shown the significant possibility of successful clinical trial of the combined treatment.

Combination of antimicrobial drugs with polyphenols

Antimicrobial drugs act by interfering with the life cycle of an organism in various ways by binding to a cellular target that results in the alteration of the normal function of the microorganism, leading to either inhibition of growth or cell death. A part from that, in order for the antimicrobial agent to reach its target site, the drug must also possess sufficient affinity for its receptor (Neely &

Jelliffe, 2017). These pharmacological characteristics are the primary determinants of antimicrobial activity. Some of these drugs is notorious for developing rapid resistance to antibiotics, caused primarily by antibiotic selection and the horizontal transfer of resistance genes. Based on the previous findings, it has been reported that polyphenols also serve a beneficial role as potent antimicrobial agents. Therefore, this study was investigated to assess whether polyphenols in combination with antibiotics has the potential qualities of alternative therapeutic agents to overcome the antibiotic resistance of various microbial strains. Studies from various documented literature has shown synergistic interaction between combination treatment of antimicrobial drugs and polyphenols causing increased in drug's efficacy against various MDR strains includes *candida albicans* (Anand & Rai, 2017; Hirasawa & Takada, 2004), MRSA (Mun et al., 2013) and MDR-TB (AlMatar et al., 2019) in *in vitro* microbial culture. Moreover, research by Maisuria *et al*, indicated the successful combined treatment between PRMSE and ciprofloxacin on targeting bacterial biofilm and reducing its formation in clinical strains of *Escherichia coli, Proteus mirabilis*, and *Pseudomonas aeruginosa* (Maisuria et al., 2015). Based on the successful synergistic combination recorded, there's the need for further *in vivo* testing for development of an efficient and safer combinational drug against various microbial agent.

Combination of antidiabetic agents with polyphenols

Diabetes is a group of heterogeneous disorders that are commonly presented with hyperglycemia and glucose intolerance, due to insulin deficiency, impaired insulin action or sometimes could be both. According to the World Health Organization (WHO), diabetes mellitus (DM) is the most common endocrine disorder that are now affecting at least 171 million people worldwide (Wild et al., 2004). Hence, there is a necessity for new antidiabetic agents with a better therapeutic efficacy and less adverse effects. Previous study has demonstrated significant roles of polyphenols in glucose metabolism through scavenging of free radicals, and its role on oxidative stress-linked cell signaling which are the key towards uncovering the therapeutic intervention of DM (Kamalakkannan & Prince, 2006; Veerapur et al., 2017). Hence, in this literature, we present the successful increase in efficacy of antidiabetic drugs when combined together with polyphenols *in vitro* (Kang & Kim, 2010; Prabhakar & Doble, 2009; Prabhakar et al., 2011) and *in vivo* (Nankar & Doble, 2017) experimental model. The ability of polyphenols; curcumin as a potent antioxidant in glucose metabolism was shown through AMPK/ACC pathway activation that results in enhanced insulin sensitivity *in vitro* (Kang & Kim, 2010). Meanwhile, dietary polyphenols from cinnamic acid, ferulic acid, p-coumaric acid, eugenol, chlorogenic acid, and caffeic acid in combination thiazolidinedione (THZ) and metformin also demonstrated to help increases glucose metabolism (Prabhakar & Doble, 2011). The dietary intake from plant source and their ingredients could be a more effective strategy for the management of DM because of the likelihood of high compliance not to mention; these dietary polyphenols are free from side effects, have better effectiveness, act on multiple target sites, and relatively cost effective. Hence, these studies has shown the potential reduced therapeutic concentration of anti-diabetic drugs when combined with polyphenols, that in returns will cause the side effects to be decreased to a large extent (Prabhakar & Doble, 2009) In addition, understanding the metabolism and bioavailability of the interaction between these two compounds are vital. A part from that, these findings support a potential clinical application of combination treatment between polyphenols and antidiabetic agents in measurement of DM and its complications.

Cardioprotective effects in combined polyphenols treatment with synthetic drugs

The antioxidant properties of many polyphenols alone could have been proclaim to exhibit vasodilator, antithrombotic, anti-inflammatory, antiapoptotic, hypolipemic or antiatherogenic effects (Williams et al., 2004) that have been associated with decreased cardiovascular risk. Studies have shown that polyphenols contribute to vasodilator effects and can help to improve lipid profiles as well as mediates the oxidation of low-density lipoproteins. In addition, these polyphenols can also attenuate apoptotic process in vascular endothelium and contributes to anti-inflammatory effects. Hence, polyphenols can be considered good candidates for the prevention and treatment of cardiovascular diseases (Quinones, et al., 2013). Based on table 1, combination treatment between selected cardiovascular drug and polyphenols causes reduced atherosclerosis lesion and improved vascular re-reendothelialization *in vivo* (Chen et al., 2020; Li et al., 2019). These findings were supported by successful clinical trial incorporating combined polyphenols intake with cardiovascular drug therapy. Results of the clinical trial indicated that the action of combined compounds causes decrease of endothelial inflammation and mediation of vascular wall repair (Naruszewicz et al., 2007) Moreover, it was uncovered that the combination treatment also causes significant clinical improvement that includes decreased in pulse pressure variability, diastolic blood pressure and mean arterial pressure indicating relevant improvements in arterial stiffness (Curtis et al., 2013). Generally, the evidence listed provide researchers and clinicians with an outline to consider these combined approached as a readily available solution to helps reduced cardiovascular disease risk among high-risk patients.

Regenerative effects of combined therapeutic approaches and synthetic drugs with polyphenols

Regenerative medicine has been extensively explored to as an ideal solution for numerous existing diseases. The key factor in regenerative medicine is to replace or "regenerating" human cells, tissues or organs to restore or establish normal human function. The use of natural product and drug either by itself of synergistically combined, has been published in many researches as a drive component in regenerative medicine (Douglas et al., 2018). Current approaches in regenerative medicine is either via targeted drugs, cellular material, biomaterials as well as other therapeutic approaches that aims to mediate cellular regeneration and growth. Hence, in this review, the potential used of polyphenols combined with some of readily applied therapeutic approaches or drugs on cell regeneration to promotes healing were demonstrated. Yu *et al*, demonstrated the successful combination between bone marrow stromal cells (BMSCs), and green tea polyphenols on improving the condition of spinal cord injury in a rat model *in vivo* (Yu et al., 2015). Moreover, Raudhah *et al*, indicated successful combination between osteoporotic drug and polyphenols in bone regeneration *in vitro* (Raudhah et al., 2018). Additionally, the incorporation of material such as collagen scaffold combined with polyphenols was found to be successful for improving wound healing (Karri et al., 2016) and bone regeneration (Bhattarai et al., 2019) *in vivo*. Thus, these findings indicated various potential that can be explored to mitigate readily available drugs, biomaterial and synthetic material in order to promotes cell regeneration.

Conclusion

In summary, synergistic effects are achieved when two compounds increase each other's effectiveness by more than the sum of their single agent responses. We conclude that the use of polyphenols in combination of synthetic drugs or other therapeutic approaches against various diseases has significant preclinical effects; however, the evidence on clinical trial is still lacking and need to be uncover. The advantage of designing treatment regimens of synergistic combination in a particular disease setting provide the opportunity to lower the dosage of an individual agent, thereby reducing toxicity while maintaining the wanted effect on the target cells (Jia et al., 2009). Additionally, synergistic combination on the use of biomaterials was shown to enhanced its acceptance. Thus, provided numerous successful researches reported in this literature; it is strongly recommended that prioritizing the pharmacodynamics and pharmacokinetics concept in designing treatment combination should be considered in the later stages of clinical testing for optimization of successful clinically utilizable therapies.

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Availability of data and materials

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Authors' contribution

ARA, HH and NMZ drafted the manuscript. All authors read and approved the final manuscript. First author information ARA (Ph.D) is a lecturer in the Department of Biomedical Science 1, Faculty of Medicine and Health Science, Universiti Sains Islam Malaysia.

Conflict of Interest

The authors declare that they have no competing interests.

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REVIEW ARTICLE Open Access

Psychosocial and Socioeconomic Impacts of Atopic Dermatitis: A Comprehensive Review

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ABSTRACT

Atopic dermatitis or eczema is a chronic inflammatory skin disease caused by several factors, including environmental allergens, family history of atopy, barrier dysfunction, and immune dysregulation. Eczema has been widely recognized worldwide for its adverse clinical and psychological effects. Besides affecting the physical appearances of the patients, the bad perceptions of the surrounding people, including friends, families, and strangers, toward the patients can worsen their mental health status and their quality of life. The burden of eczema has long been recognized as a socioeconomic and psychosocial burden worldwide, thus initiating some approaches towards improving awareness and better treatment that can lead to significant improvement in the quality of life of eczema patients worldwide. Two of the Global Burden of Disease (GBD) studies, International Study of Asthma and Allergies in Childhood (ISAAC) and other eczema skin studies are included in this review to study the global burden of this skin disease using the Disability-Adjusted Life Years (DALY) to assess the overall burden of the disease and estimating the prevalence of eczema worldwide. Regardless of eczema's direct or indirect cost, those effects have significantly changed the patients' lives negatively; thus, it is an issue that needs to be addressed globally.

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Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by severe pruritus. AD is associated with external allergens and a family history of atopy (Silverberg et al., 2017a; Tham et al., 2019). Moreover, skin barrier dysfunction and exposure of sensitive skin to allergens can contribute to the severity of AD (Czarnowicki et al., 2017). AD usually begins in childhood, within the first 5 years of life, and the disease progression will be lowered after decades of presence in adults (Silverberg et al., 2017a).

Quality of life (QoL) is defined as a subjective measure of happiness associated with several preferences, such as financial security, job satisfaction, family life, health, and safety (Kagan, 2022). Health-related QoL (HRQOL) can be defined as a term related to the QoL in health aspects, generally considered to reflect the disease impact and disability, daily functioning treatment, and the perceived health impact on an individual's ability to live a fulfilling life (Saketkoo et al., 2021).

The term psychosocial is defined as the influence of social factors on an individual's mind or behaviour or to the interrelation between behavioural and social factors. It is also known as the interrelation of the mind and society in human development. The psychosocial impact can be either positive or negative towards the individual. Regarding AD, it concerns with the elevation of negative psychosocial impacts in the society and normally overlooked by the physicians in charge of the patients (Haraldstad et al., 2019).

Similarly, the severity of AD is associated with the socioeconomic burden of the parents or the AD patients themselves. The more severe the AD, the higher the financial resources needed to cover the treatment expenses. For instance, treatments using specialised routine skincare with strict regimens needs to be implemented, or else the AD symptoms could worsen (Wisuthsarewong et al., 2017).

Atopic Dermatitis as a Global Problem

AD is the most common chronic inflammatory disease that has affected up to a fifth of the population in developed countries. AD can be characterized by chronic, relapsing and highly pruritic dermatitis and has a characteristic age-dependent distribution (Vujnović & Domuz, 2017). Progression of atopic disorders from AD in infants to allergic rhinitis and asthma in children, adolescents and adults is describe as atopic march and may persists with increasing age (Porcaro et al., 2018).

Recent studies have revealed that AD is becoming more severe in terms of its prevalence and economic and psychosocial impacts on society worldwide (Kramer et al., 2017). It is known that AD affects approximately 20% of children and 3% of adults, and its prevalence is still increasing, particularly in low-income countries (Vaikili et al., 2019). Therefore, several studies have been conducted to study the impacts of AD globally.

The flexible design of GBD allows researchers to update the data and epidemiological studies regularly; thus, the tools are made available to be used globally, nationally, and locally to gradually understand the health trend further (Safiri et al., 2019). Two studies have used the data from GBD studies from 1990 to 2017 to analyse and understand the global burden of AD (Laughter et al., 2020; Al-Hemoud et al., 2018). DALY is used to assess the overall burden of diseases by using time-based measurement with combinations of years of life lost (YLLs) due to premature mortality and time lived in states of poor health and years of healthy life lost due to disability (YLD) (Laughter et al., 2020).

A study that reported the AD burden using prevalence rates and DALYs found that AD was deemed responsible for 0.36% of the total DALYs out of 359 diseases and injuries analysed by the GBD 2017. Moreover, among all of the other skin diseases (psoriasis, urticaria, scabies, and fungal skin diseases), AD represented the highest age-standardised DALYs in 2017. Both global DALY rates and global age-standardised prevalence rates remained stable from 1990 to 2017 (Al-Hemoud et al., 2018). Table 1 and Table 2 show the highest and lowest prevalence rate for the AD around the whole world.

A multinational, cross-sectional study was conducted in 2016 involving various countries with different prevalence rates of AD among the population of 18–65 years old. The prevalence rates for AD among adults were found to be 19,986 for the United State (US), Canada (10,004), France (9,964), Germany (9,971), Italy (9,897), Spain (9,924), UK (10,001) and 10,911 for Japan (Laughter et al., 2020).

In terms of measurement of the DALY, the higher rate of DALY indicates a higher rate of years lost due to the illness, disability, or premature death within the given population. For instance, DALY can be represented as a numerical value, such as one DALY equals one year lost due to the illness, disability, or premature death (Barbarot et al., 2018). Moreover, one DALY could also mean a one-year loss of healthy life, thus leading to the disease burden calculation by combining the mortality and morbidity, which allows comparison between different diseases (Dierick et al., 2020).

Table 1: Ten countries with the highest prevalence rate of AD per 100,000 (IHME, 2021)

No	Countries	Prevalence Rate per 100,000
1	Japan	5324.33
2	France	5087.17
3	Estonia	4786.64
4	Mongolia	4697.35
5	Uzbekistan	4680.62
6	Turkmenistan	4654.14
7	Georgia	4611.62
8	Italy	4276.3
9	United Kingdom (UK)	4250.15
10	Norway	4165.78

Table 2: Ten countries with the lowest prevalence rate of AD per 100,000 (IHME, 2021)

No	Countries	Prevalence Rate per 100,000
1	Rwanda	678.22
2	Egypt	853.93
3	Ghana	874.39
4	Kenya	914.12
5	Zambia	1036.29
6	Zimbabwe	1083.39
7	Togo	1099.44
8	Mali	1178.88
9	Qatar	1284.80
10	Saudi Arabia	1321.70

A study stated that the implementation of the ISAAC is among the best estimates of AD prevalence internationally due to the availability of the global map of AD, which allows for easy comparison of AD prevalence estimates between countries (Silverberg, 2017b). The ISAAC Phase Three study was conducted from 2001–2003 involving 234 collaborating centres, 98 countries, and 1,187496 children. Based on the ISAAC Phase Three study, an average of 60% of children within the age range of 6–7 years and 50.4% of children within the age range of 13–14 years had symptoms of eczema from 9 different countries with Northern and Eastern Europe having the highest percentage for both age group (66.7% and 58%) (Mallol et al., 2013).

Thereby, based on the studies conducted through the use of ISAAC and GBD, AD has already caught the whole world′s attention as its prevalence can be seen in most countries. Even though the surveys or studies have revealed AD as a burden as early as 2000, the present data still showed that AD is still overlooked as a serious skin disease that badly affects individuals with AD in society and the professionals from the medical field.

Psychosocial Impacts of AD

The psychosocial impact is the impact of a disease or other factors on a person′s mental health. It correlates with the QoL as negative psychosocial impacts cause a reduction in QoL. Some of the thoughts of males and females with AD, divided into six categories, including advice, suggestions, ways to cope with the disease, and their expression of dissatisfaction towards the people around them were recorded in previous studies (Birdi et al., 2020; Ghio et al., 2020; Bilyj, 2019). Their thoughts were recorded based on interviews from different studies, and some of them were posted on the websites as a guideline for the AD communities to assist them in managing AD.

AD Stigma

Physical disfigurement, psychological destruction, and social stigma often happened to patients with skin diseases (Zhang et al., 2019). Loneliness, social isolation, depressive symptoms, and decreased QoL were often associated with patients with skin diseases (Yew et al., 2020). In this case, those patients could have sufferred from psychosocial maladaptations of the skin diseases (Zhang et al., 2019).

Patients' maladaptive assumptions regarding their appearances and society's focus on the perfect body are some of the psychosocial impacts of skin disease (Zhang et al., 2019). In short, most of the AD individuals in those studies were trying to express the underestimation of AD based on society's thoughts on skin disease. Those thoughts lead to a conclusion of insufficient knowledge of the society towards AD.

Despite having negative thoughts and society's perceptions towards AD individuals, little is known about the differences in each person's understanding of the stigma between countries or societies (Topp et al., 2019). Likewise, the QoL of the AD patients were severely affected, with 85% of adults experienced interrupted sleep, 70% of them reported having social anxiety, 65% avoided exercise and sports, 52% avoided social activities and sexual intimacy, and 43% of them were depressed because of AD (Murray et al., 2020).

A meta-analysis and systematic review found that AD patients tend to have a significantly greater risk of developing suicidal thoughts and attempts than those without AD. (Sandhu et al., 2019). Based on the EuroQoL-Visual Analog Scale (EQ-VAS) (an indication of the patient to describe their overall health), AD adults had lower HRQoL than adults without AD. Furthermore, AD patients have a high-stress level, and the risk of suicide, depression and stress were approximately 1.7 times more prevalent in AD patients than those without AD (Kwak & Kim, 2017).

In terms of percentage, another finding reported a higher possibility of developing suicidal ideation in almost 44% of AD patients and a higher possibility (36%) of attempting suicide in AD patients than those patients without AD (Sandhu et al., 2019). More comprehensive efforts need to be implemented to reduce the social stigma experienced by those affected by the skin disease while promoting positive societal attitudes towards society concerning the diversity in appearances (Rumsey, 2018).

AD in Families

In the US, 7% AD adults had gone through divorced or separation. As proposed in the same study, separation and divorce could happen because of the negative life impacts of AD and comorbidities on social function and relationships (Hua & Silverberg, 2018). Data from a study indicated that separated or divorced individuals and unmarried individuals showed a higher prevalence of AD compared with the married AD individuals (Lee et al., 2017). Among 43.9% of mothers with AD who were raising their children alone, 18.3% were divorced, and 25.6% were widowed, which showed higher percentages of AD in these groups (Young & Keung, 2018).

Findings of a study found an increased AD prevalence with the possibility of increased severity was because of the stressors at home or neighbourhood (McKenzie & Silverberg, 2018). For both adults and children, stress is considered a very common trigger for AD. There are connections between stress and AD, resulting in an undesirable vicious cycle in which stress affecting the skin and AD symptoms which result in worsening the conditions (Bennington-Castro, 2019).

Maternal depression may lead to the development of atopic diseases, such as AD. For instance, the association between depression and AD can be observed in a study conducted among Korean children that showed a strong correlation between those two factors. The study found that the divorced mothers were significantly associated with maternal depression, eventually leading to the development of AD (Kim et al., 2018).

Mothers with maternal depressive symptoms due to lack of social support will eventually lead to low maternal sensitivity, resulting in a higher risk of their children developing AD (Letourneau et al., 2017). Additionally, a higher risk of childhood eczema in infancy can be observed among mothers with persistent prenatal depression. This event was likely due to maternal depressive symptoms at either early or late pregnancy (Wei et al., 2020).

Additionally, studies from Korea (Cho et al., 2010; Kanda et al., 2019) and UK (Kemmett & Tidman, 1991; Gratton et al., 2022) reported that 61% or 52% of female AD patients which experienced pregnancy had noticed AD deterioration. This is because of the effects from extremely high concentrations of estradiol and progesterone towards the Th2 activity and skin barrier (Kanda et al., 2019). During pregnancy, high level of estrogen caused the immune system's focus shifting from protecting the cells in the mother's body towards the fetus. Hence, causing the mother's body to be more susceptible towards AD (Gardner, 2022).

The impacts of AD children on their parents may cause their parents' QoL to be reduced significantly. Almost 43% of the parents out of 93 parents were reported to have moderate QoL, 23.66% of parents were affected severely, while the rest of them were affected at a low and normal level (Al-Hayyan et al., 2020). Furthermore, there was a possibility of the association between secondary depression or suicide ideation and sleep disturbances in mothers of AD children (Young & Keung, 2018).

Healthcare Professionals (HCP) and AD Patients' Relationship

Most of the time, AD patients seek treatments from healthcare professionals. Even though the physicians have suggested a few treatment options and regimens to the AD patients, the AD patients tend to not adhere to the treatment, resulting in a higher risk of unsuccessful treatments (Writers, 2017). This can be due to the patient-HCP relationship and the non-adherence can be avoided by increasing the regularity of appointments (Patel et al., 2017).

Nevertheless, the physicians might overlook the lack of information given to the patients. For instance, the physicians often do not address or quickly dismiss any discussion regarding complementary and alternative medicine (CAM). Therefore, a two-way discussion needs to be carried out between the doctor and the patient to signify that the patients' opinions are accepted in a good manner (Leow et al., 2018).

Most of the patients, who pay visits to the clinics, demand more information regarding their illness. In case of AD, the patients require more detailed information regarding the severity classes of the AD based on the symptoms. However, in-depth knowledge of the AD needs to be tailored as more knowledge received by patients results in generating unnecessary worry and could lead to negative impact (Leow et al., 2018).

Guidance comprised of several steps to provide supportive conversations between the healthcare professionals and the patients has been established. In summary, the steps in the guidance are to assess how AD affects the person's life, discuss the methods to manage the patient's AD on a daily basis, and maintain consistent control of their condition (Sanofi, 2019). Therefore, effective methods in AD treatment need to be developed and implemented towards AD patients to improve the patient-physician relationship.

Advantages of Having Reliable Platforms

Some of the advice were taken from the personal experiences of those affected by AD and posted at the National Eczema Association, which acts as the knowledge provider primarily for the eczema community. Adolescents and young adults require sufficient information regarding AD as they could not find any suitable information for their age. Luckily, those people from the same age groups were into blogging, and were able to help them in managing AD (Bilyj, 2019). Those experiences finally led to the first step towards better management of the AD.

Through online mediums, the virtual networking sites can help the AD patients in terms of facilitating online support communities and several platforms for them to interact with the medical professionals or experienced individuals in AD. Additionally, these sites serve as emerging platforms for the scientific journals to reach broader audiences and develop potential educational tools to assist them in coping better with their AD-related problems (Diaz et al., 2020).

Socioeconomic Burden

The services provided by the HCP, such as medical visits, days of hospital stay, emergency department visits, diagnostic and therapeutic requests, are considered as direct costs. Additionally, the transportation required for the appointment and the need for caregivers are parts of the direct cost (Murota & Katayama, 2018). Meanwhile, indirect costs include absenteeism, presenteeism, loss of employment, and social and psychological burden (Chung & Simpson, 2018).

AD patients need to plan their expenditures on a daily basis, due to the frequent application of moisturisers and other therapeutic products to control the AD symptoms and preventing its exacerbation (Adamson, 2017). By referring to the treatment outline, the treatment for AD starts with non-pharmacological treatment (education, emollient, avoidance of triggers, and quick baths with fragrance-free cleansers) and is followed by pharmacological treatment using the topical corticosteroids, phosphodiesterase-4 inhibitors, dupilumab, phototherapy, and immunosuppressants (Johnson et al., 2019).

Direct costs

A study conducted in Singapore stated that the AD infants and children with high impact on their HRQoL covered the most direct cost compared to those with moderate and low impacts, based on the average of total healthcare costs (Table 3). The HRQoL of the infants (<4 years old) were measured by the Infant's Dermatitis QoL Index (IDQOL), while the Children's Dermatology Life Quality Index (CDLQI) was used for the children (>4 years old) (Olsson et al., 2019). The infants and children with high impacts on the HRQoL and categorized in severe AD were accounted for 12.5% (64 out of 513), thus indicating the contribution of mild and moderate AD to higher impacts on the HRQoL. Thus, children in this category contributed significantly higher healthcare costs than the children with low impacts, regardless of the AD severity (Olsson et al., 2019).

Another study in Singapore which focused more on the severity of AD found that the overall annual total cost was 7,943 USD per child, summing up the

total cost of healthcare service utilisation, informal care, and out-of-pocket (OOP) expenses. The findings of the study revealed that informal care (46% of the total cost) came out as the highest total cost compared with the remaining costs (37% and 17%) (Table 3) (Olsson et al., 2020). Advanced treatments for AD, including dupilumab, systemic corticosteroids (SCS), systemic immunosuppressants (SIS), and phototherapy, were investigated and SCS was the most common advanced treatment followed by dupilumab, phototherapy, and SIS (Eichenfield et al., 2020). Data revealed a significant burden to the healthcare system with an annual cost of approximately more than 20,00 USD per patient. Although dupilumab showed the highest total cost compared to other treatments, the clinical efficiency of dupilumab might reduce the medical-related costs for dupilumab (Table 3) (Eichenfiled et al., 2020).

A study from Spain reported that the cost of the treatment on average for AD adults was 1,604.74 USD, with notable differences with the severe AD adults, which cost around 3,932.89 USD, four-fold higher than mild forms (865.32 USD). Around 75.5% of the total cost came from healthcare costs (Table 3), mostly contributed by both the drug prescription and specialist care, while the remaining 24.5% from the loss of productivity (Sicras-Mainar et al., 2019).

According to the Dermatitis Family Impact Questionnaire (DFIQ), the impact of the 75 childrens' AD towards their families was investigated and the result showed that the family expenditure of the AD children had the highest impact, including cost of treatment and clothes, on the main caregiver's life and housework (Siafaka et al., 2020).

A study in Ireland has recorded that most adults and parents spent up to 53.35 USD/year, and 10% of the respondents spent more than 213.40 USD/year on the alternative therapies for AD. One-quarter of those groups have spent up to 2,454.05 USD annually for the direct cost expenditures. Unfortunately, 52% of them cannot always afford the AD treatment, and 58% of the parents admitted that they have to cut some of their household spendings to afford their children's treatment (Murray et al., 2020).

A comparison of healthcare utilisations between controlled AD patients and uncontrolled AD patients in the Netherlands showed that the later groups used significantly more healthcare resources. The direct cost for uncontrolled AD in each category is higher than the controlled AD in which the healthcare resources accounted the highest (Table 3). Consequently, the mean total direct cost of uncontrolled AD was 7,461.39 USD per patient per year, while it was 4,695.78 USD for controlled AD (Ariens et al., 2019).

Out of 10,533 individuals with AD in the US, the annual direct medical costs were 11,660 USD per patient. Since AD is generally treated on an outpatient basis, the outpatient visits cost the highest, followed by prescription, ambulatory, inpatient, and emergency department costs. The study also found that more severe AD has higher direct costs for all categories included in this study than the less severe AD (Eckert et al., 2019). The OOP cost for both medical and non-medical consumptions that were not covered by health insurance differed based on the severity of AD in a study conducted in France. The approximate OOP costs for mild AD were 285.63 USD, 441.30 USD for moderate AD, and 753.07 USD for severe AD (Drucker et al., 2017). Additionally, this study stated the importance of social inequalities in the AD treatmtent as some of the patient treatments were covered by insurance (21.1%), while it was not the same case to other AD patients, and it further leads to a reduction in adherence to the treatment because of their incapabilities to continue with the cost of the treatments (Drucker et al., 2017).

A study that investigated the OOP costs for nine European countries (Czech Republic, Denmark, France, Germany, Italy, Netherlands, Spain, Sweden, and the UK) have found that besides 989.22 USD was spent per year on healthcare, the mean extra spending per month was 82.43 USD with emollients and moisturisers accounted for the highest monthly cost (Table 3) (Launois et al., 2019). All things considered, the direct cost of AD has been a huge burden not only towards the AD patients but also their parents. Hence, indicate the need of thorough understanding of this skin disease financially and support from the government itself towards the importance of aiding these families for them to adhere to the treatment and resolving their financial issues.

Table 3: Mean cost of different measurements of direct cost of Atopic Dermatitis

No	Categories	Cost (Mean)		References
1.	High Impact HRQoL Moderate impact HRQoL Low impact HRQoL	3,787 USD 2,548 USD 2,258 USD		Olsson et al., 2019
2.	Dupilumab (ACMC- 3620 USD + ACPC- 32,885 USD) SCS (ACMC- 12,066 USD + AC PC- 5858 USD) SIS (ACMC- 12,536 USD + A 12227 USD) Phototherapy (MC- 14,944 USD + PC- 2606 USD)	36,505 USD 17,924 USD 13,763 USD 17,550 USD		Eichenfield et al., 2020
3.	Severe AD Moderate AD Mild AD Informal care Out-of-pocket expenses Healthcare service utilizations	14,335 USD 7,935 USD 6,651 USD 3659.1 USD 2934.6 USD 1348.9 USD		Olsson et al., 2020
4.	Primary care Specialist care Total medication	754.35 USD 1575.93 USD 2799.76 USD		Sicras-Mainar et al., 2018
5.	OTC Treatments Prescription treatments Alternative treatments Doctor's fees	<26.67 USD - >16 USD <26.67 USD - >160.05 USD <26.67 USD - >160.05 USD <26.67 USD - >160.05 USD		Murray et al., 2020
6.	 Healthcare Resources Medication Diagnostic Tests Laboratory Tests Transportation and Parking	Controlled: 4695.78 USD 1630.35 USD 9.60 USD 264.61 USD 97.10 USD	Uncontrolled: 7461.39 USD 3569.05 USD 11.74 USD 294.49 USD 124.84 USD	Ariens et al., 2019
7.	 Inpatient costs Emergency department cost Ambulatory cost Outpatient cost Prescription cost	More severe: 3,063 USD 765 USD 3,234 USD 4,568 USD 3,492 USD	Less severe: 1,849.9 USD 425 USD 2,041 USD 3,029 USD 2,321 USD	Drucker et al., 2017
8.	Emollients Hygiene products Solar protection Dressing and Bandages Clothes Dietary supplements	808.77 USD 710.61 USD 413.99 USD 300.89 USD 186.72 USD 201.66 USD		Launois et al., 2019
9.	Emollients and Moisturisers Medications Doctors and Hospitals Bandages Travel expenses In-patient treatment	29.46 USD 18.93 USD 9.26 USD 7.60 USD 6.07 USD 2.07 USD		Zink et al., 2018

Indirect costs

Indirect cost can be defined as the productivity loss that affects the cost of the outcome of the productivity loss. From a societal perspective, a person is productive if this person is useful to the society for the work and goods produced by that person, the person involvement in unpaid activities, such as volunteer works, non-profit organisation, or even household duties. (Zink et al., 2018)

In indirect cost, there are two terms involved; presenteeism and absenteeism. Presenteeism can be defined by two definitions. The first definition is when the employee feels like it is better to stay at home rather than going to work (Fautrel et al., 2020). The other definition is when the staff is staying at work past the working hours or come to work earlier regularly; thus, they are working longer than within their contracted working hours (Fautrel et al., 2020). The definition of absenteeism is the frequent absence of employees from his or her work. Absenteeism is associated with habitual absence, excluding both authorised leaves and paid time off. In other words, absenteeism is any failure to report for or remain at work as scheduled, regardless of the reason (Price, 2021).

Presenteeism and absenteeism caused a much higher indirect cost towards the AD patients based on the European Union-5 (EU5) (Figure 1). The indirect cost for the EU5 countries (France, Germany, Italy, Spain, and the UK) based on the Dermatology Life Quality Index (DLQI) severity band are available in Figure 1 (Girolomoni et al, 2021). The study found a correlation between AD severity and work productivity loss; with more severe the AD, the higher the number of days lost at work. Moreover, the increasing severity of psychosocial comorbidities (sleep difficulties, anxiety, and depression) was associated with poorer HRQoL and work productivity loss. Thus, this study suggested that those comorbidities mainly affect the HRQoL and the indirect cost burden rather than the direct cost (Girolomoni et al, 2021).

From that Japan National Health and Wellness Survey (NHWS), the overall-work-impairment (OWI) in employed AD patients (30.61%) was rather higher than the employed non-AD controls (24.62%) (Arima et al., 2018). Furthermore, the severity of AD also affects the AD patients as more severe AD conditions lead to a higher rate of presenteeism (32.17% vs 26.10%), OWI (33.79% vs 28.08%), and activity impairment (35.14% vs 30.06%) (Arima et al., 2018).

An estimation of the productivity loss has been made and represented in OWI, as shown in Figure 1. The combination of direct medical cost, self-medication cost, OWI, and AI costs resulted in the nationwide disease burden in Japan, which was estimated to be 3036.9 billion Japanese Yen with a majority of the nationwide burden dominated by the OWI cost (69.7%) (Arima et al., 2018). In another study, 57% of AD patients had absenteeism for at least a day out of 5 days, 26% for at least 1 week (6–10 days), and 13% were absent from work for more than 11 days (Zink et al., 2018).

In Spain, the impairment of work productivity has risen the expenditure for AD patients. Approximately 1,989.71 USD has been accounted for mild to severe AD due to the impact of the productivity loss plus an amount of 108.62 USD was the cost they lose for a day when they were not working (Sicras-Mainar et al., 2019). Based on Figure 1, the more severe the AD, the higher the cost per day the patients will lose (Sicras-Mainar et al., 2019).

Around 10,774.93 USD per patient per year was the productivity loss for the AD patients in the Netherlands between January 2016 and September 2017 (Eckert et al., 2019). The work productivity and activity impairment (WPAI) of both controlled and uncontrolled AD were calculated based on OWI and activity impairment (Figure 1). The cost of productivity loss was higher than the total direct costs associated with the AD patients, which concludes that its impact on them was much bigger due to the impairment at work and daily activities associated with AD (Eckert et al., 2019).

The differences between employed subjects with AD and without AD showed a much higher rate of absenteeism (9.9% vs 3.6%), presenteeism (21.1% vs 16.1%), and OWI (25.6% vs 18.3%). Moreover, the annual indirect cost, taking into account the absenteeism, presenteeism, and OWI, was 2,400 USD higher for AD subjects than non-AD subjects (8,907 USD vs 6,517 USD). Regarding the impairment of the daily activities in all AD subjects, the mean percentage of AD subjects was much higher than non-AD subjects (33.6% vs 25.2%) (Murota et al., 2020).

The socioeconomic burden of AD in Denmark was associated with the increased use of social benefits, including paid sick leave and disability pensions which contemplate the negative impact on the work-life of AD adults and the financial burden of society. Paid sick leave was more prevalent in older populations for both mild/moderate and severe AD (Slagor et al., 2020). Both mild/moderate and severe AD for the younger groups had a more long-lasting and prevalent paid sick leave than the controls. Whereas only severe AD patients had more long-lasting and prevalent paid sick leave for the older

groups (Slagor et al., 2020).

Taking everything into acccount, the negative impact of AD towards the indirect cost result in a huge loss towards the AD patients. Even though there were some countries that offer paid sick leave and diasbility pensions, it does not reflect any positivity in terms of productivity for these patients. The most important thing for them is to improve their HRQoL in their employment aspect and to work without any worries of troubling the surrounding community.

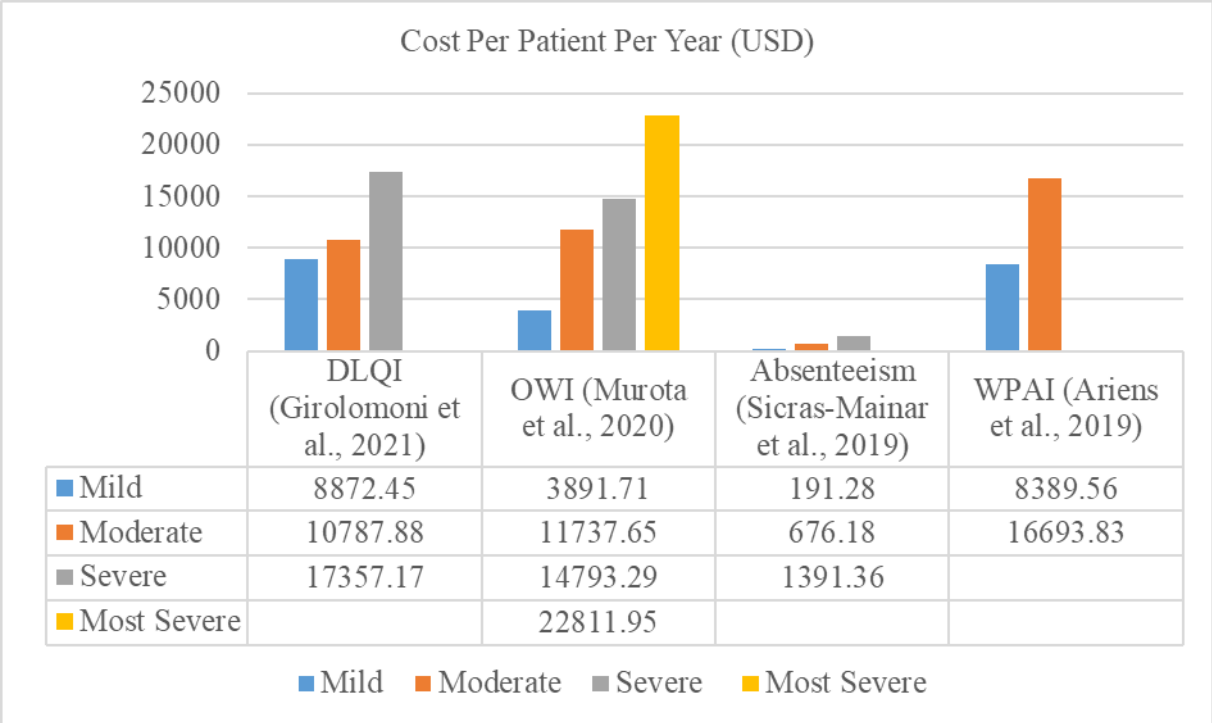

Figure 1: The cost per patient per year based on the severity of AD

Conclusion

In conclusion, it is best to consider AD as one of the skin diseases that required the attention of healthcare professionals and the society around them. AD cannot be underestimated as a normal skin disease as it can significantly affect the patient physically and mentally. The psychosocial impact and the economic burden of AD have brought a lot of attention from researchers around the world to solve the problems associated with AD as it affects the QoL of the patient. Further research studies in the burden of AD need to be conducted especially in regard to the number of participants or patients involved in the study and clinical identification or diagnoses of AD need to be proven by the healthcare professionals as a prove that the participants are truly affected by AD to strengthen the study's data. Better insights can be created for AD patients by spreading the knowledge to society and educating the whole world that equality is important in every aspect of life.

Conflict of Interest

The authors report there are no competing interests to declare.

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Journal of Pharmacy

REVIEW ARTICLE Open Access

Cosmeceutical benefits of stingless bee honey

Nuraqilah Zulkifli[1] and Hazrina Ab Hadi[1,2*]

ABSTRACT

Introduction: There were about 500 stingless bee (SB) species that had been reported in Afrotropical, Indo-Australian and Neotropical provinces with the highest diversity species originating in the Neotropical. In Malaysia, there are more than 38 species and around 33 species were reported in Peninsular Malaysia. Due to its high nutritional and medicinal values, recently, stingless bee honey (SBH) has been highly demanded by the food, pharmaceutical and cosmetic industries. Thus, this paper will describe the cosmeceutical potential of SBH as an antiacne, skin moisturizing and anti-hypertrophic scar agent. SBH can treat acne by reducing inflammation and irritation during acne formation due to its high flavonoid and phenolic content. Besides, SBH also possesses antibacterial activity due to its acidity, osmolarity and hydrogen peroxide content which is unfavourable for *P.acne* growth. It functions as a natural humectant due to its ability to attract water from the dermis and deeper epidermis to the epidermis and its high water-binding capacity. SBH also can reduce scar formation by improving the wound healing process. This is due to the both pro-inflammatory and anti-inflammatory properties in which it triggers the production of the pro-inflammatory cytokines to initiate the inflammation and inhibit the production of the pro-inflammatory cytokines when inflammation is in progress to avoid prolonged inflammation. Furthermore, SBH can stimulate skin reepithelialization and wound contraction. These will reduce the damaged tissue that needs to be repaired and eventually minimise the scarring area. In cosmeceutical, further research regarding the effectiveness of honey/ SBH in various cosmetic formulations had been investigated and the formulations developed were proven to be effective in treating the acne, moisturising the skin and minimising the scar.

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Introduction

Stingless bee (SB) is normally found in tropical and sub-tropical areas (Fletcher et al., 2020; Rattanawannee & Duangphakdee, 2019). There were 500 SB species that had been reported in Afrotropical, Indo-Australian and Neotropical provinces (Fletcher et al., 2020) with the highest diversity species originating in the Neotropical. In Malaysia, there are more than 38 species (Mustafa, Yaacob, & Sulaiman, 2018) and around 33 species were reported in Peninsular Malaysia (Ghazi, Zulqurnain, & Azmi, 2018). However, only four species are commercially nurtured which are *Heterotrigona itama, Geniotrigona thoracica, Tetragonula leviceps* and *Lepidotrigona terminate* (Mustafa et al., 2018). There are two tribes of SB which are Meliponina and Trigonina (Jalil, Kasmuri, & Hadi, 2017; Zulkhairi Amin et al., 2018) Melipona tribe is bigger compared to the Trigona tribe (Figure 1) (Fletcher et al., 2020; Jalil et al., 2017). It is called a stingless bee due to its non-functional vestigial sting. Since SB do not have a sting, thus they protect their nests by biting or putting plant resin on the enemies' skin, entering body cavities or hiding in the hair (Barbiéri & Francoy, 2020).

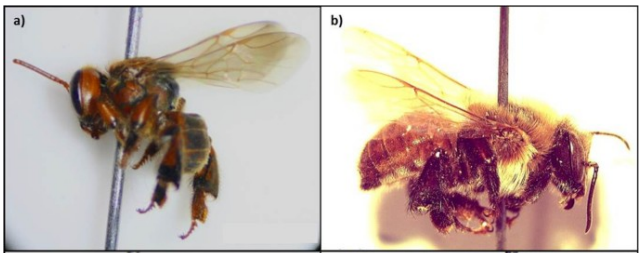

Figure 1 (a) Trigona genera (b) Melipona genera (Reyes-González, Camou-Guerrero, Reyes-Salas, Argueta, & Casas, 2014).

In Malaysia, the SB is known as *kelulut* (Mustafa et al., 2018). Nowadays, meliponiculture (stingless beekeeping) activities are very popular due to their easy management of the SB culture (Nordin, Sainik, Chowdhury, Saim, & Hj Idrus, 2018). As SB do not sting, therefore the extraction of honey, propolis and pollen are much easier compared to sting bee which needs proper safety equipment and training (Jalil et al., 2017; Nordin et al., 2018). Furthermore, the nesting behaviours of SB also make the meliponiculture activity easier since SB just construct their hive in the existing chamber or hollow area (Fletcher et al., 2020). Hence, the artificial hive can be used to control the colony and increase the honey production in meliponiculture activity (Jalil et al., 2017; Majid, Abu Bakar, Mian, Esa, & Kok Yeow, 2019; Nordin et al., 2018) Besides, the demand for stingless bee honey (SBH) has been increasing (Majid et al., 2019). This is because, over the last two decades, the food, pharmaceutical and cosmetic industries have become more interested in using SBH due to its highly nutritional and medicinal values (Ávila, Beux, Ribani, & Zambiazi, 2018).

SBH has been practised as a source in traditional treatment in many countries. The characteristics of SBH are amber-brown, sweet and sour taste, acidic and high moisture content (Rao, Krishnan, Salleh, & Gan, 2016; Zulkhairi Amin et al., 2018). It consists of carbohydrates, phenolic compounds, amino acids, organic acids, minerals, vitamins, enzymes and lipids (Fatima, Mohd Hilmi, Salwani, & Lavaniya, 2018). Its major constituent is fructose and glucose which are about 65% of sugars with a very minimal amount of hydroxymethylfurfural (Mustafa et al., 2018). However, the composition, bioactive compounds and physiochemical properties of the honey differ depending on the botanical source, geographical site, climatic condition, soil type, beekeepers' activities and storage treatments in commercial production (Mohammad, Mahmud-Ab-Rashid, & Zawawi, 2020). The amount of each constituent of SBH is listed in Table 1.

Table 1: The composition of SBH (Zulkhairi Amin et al., 2018).

Composition	Amount (%)
Total reducing sugars	54.90–87.00
Glucose	8.10–31.00
Fructose	31.11–40.20
Sucrose	0.31-1.26
Maltose	Not determined
Calcium	0.017
Potassium	0.07
Sodium	0.012
Magnesium	0.004
Ash	0.01–0.12

Minerals such as phosphorus, zinc, manganese, lead and iron are also found in the honey (Zulkhairi Amin et al., 2018). In *G. thoracica* and *H. itama* honey samples, there are four types of organic acids were indicated which are gluconic acid, lactic acid, acetic acid and citric acids (Shamsudin et al., 2019). The flavonoid and phenolic compounds of SBH are greater than in other honey types

(Mustafa et al., 2018) which makes it a potent antioxidant. As it has a high antioxidant capacity, thus SBH has the potential to treat numerous medical conditions such as cataracts and wounds. SBH also displays potential antimicrobial activity as a substitution treatment for inflammation and infection (Ávila et al., 2018).

STINGLESS BEE HONEY AS ANTI ACNE AGENT

Pathophysiology of Acne Vulgaris

One of the pathogenesis of acne vulgaris is increased sebum production. Sebum production is mediated by the androgen level in the pilosebaceous unit. The possible mechanism of high sebum production is either directly due to the high production of androgen hormones or the increased sensitivity of the sebaceous gland to normal androgen levels (Masterson, 2018). The level of androgen hormones including testosterone and 5α-dihydrotestosterone (DHT) will rise during puberty. This will lead to sebaceous gland hypertrophy, sebaceous lipogenesis and sebocyte differentiation. The sebocytes differentiation will possess abundant nuclear androgen receptors and peroxisome proliferator-activated receptors (PPARs) that make the sebaceous glands more hyperresponsive to androgens (Briganti, Flori, Mastrofrancesco, & Ottaviani, 2020). Besides, the high sebum in sebaceous follicles offers a nutrient source and anaerobic environment for *Propionibacterium acne* (Figure 2) to grow (Soleymani, Farzaei, Zargaran, Niknam, & Rahimi, 2020).

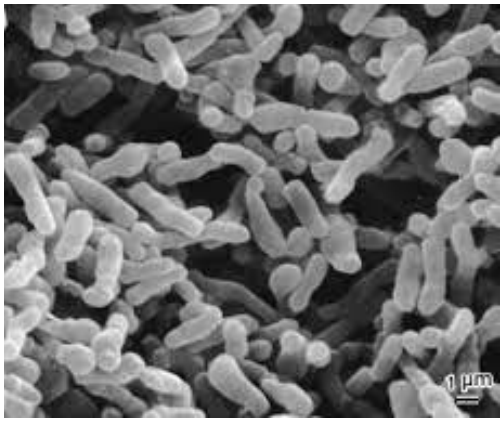

Figure 2: Scanning electron microscopic picture of P.acnes (Toyoda & Morohashi, 2001).

. The second pathophysiology of acne is the follicular hyperkeratinisation of the follicle. Many factors are believed to cause follicular hyperkeratinisation including reduced sebaceous linoleic acid concentration, increased androgen activity, inflammation and *P.acnes* biofilms. Reduced sebaceous linoleic acid concentration will enhance the cutaneous permeability to acne-causing inflammatory substances, thus disrupting the skin barrier function (da Cunha, Daza, Filho, da Veiga, & Fonseca, 2018). The proliferation and accumulation of keratinocyte cells at the follicle base will obstruct the pilosebaceous duct which leads to microcomedones formation. The microcomedone can be either a closed comedo (whitehead) in which the duct is almost entirely blocked or an opened comedo (blackhead) in which the duct is opened and exposed to the air (Figure 3). This opened comedo appears as a dark colour due to lipid and melanin oxidation (Brown, 2020).

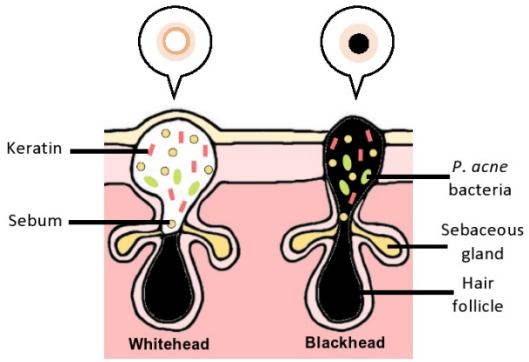

Figure 3: Illustration of whitehead and blackhead formation.

Furthermore, it is suggested that *Propionibacterium acne* does contribute to acne formation. Basically, *P.acnes* is one of the skin microbiota that acts as a first-line defence against external pathogens (Ramasamy, Barnard, Dawson, & Li, 2019). However, free fatty acids (FFA) produced by *P.acnes* triacylglycerol lipase activity are strong inducers for comedogenic, *P. acnes* biofilm formation and inflammation (Plewig, Melnik, & Chen, 2019). Lipase that is released by *P.acnes* will oxidise the squalene of the sebum and hydrolyse the triglycerides of the sebum into glycerol and FFA. Oxidised squalene can trigger the pro-inflammatory cytokines generation and PPARs activation. Meanwhile, glycerol can be the nutrient source for bacteria to grow and FFA promotes comedogenic, oxidative stress, inflammatory reaction and tissue destruction (da Cunha et al., 2018; Soleymani et al., 2020).

Lastly, inflammation is considered a significant element in acne pathogenesis. The expansion of microcomedone due to the buildup of keratin, sebum and bacteria will eventually cause the follicular wall to rupture and make the contents extrude into the dermis rather than above the skin surface. This will stimulate the inflammatory response and develop inflammatory acne lesions such as papules and pustules (Greydanus, Azmeh, Cabral, Dickson, & Patel, 2021). Inflammation is also

mediated by the action of *P.acnes* through both innate and adaptive immune responses. *P.acnes* actuates the Toll-like receptor-(TLR)-2 on neutrophils and monocytes, then leads to the generation of proinflammatory cytokines such as tumour necrosis (TNF-α), IL-12 and IL-8 (Soleymani et al., 2020). The summary of the pathophysiology of acne vulgaris is illustrated in Figure 4.

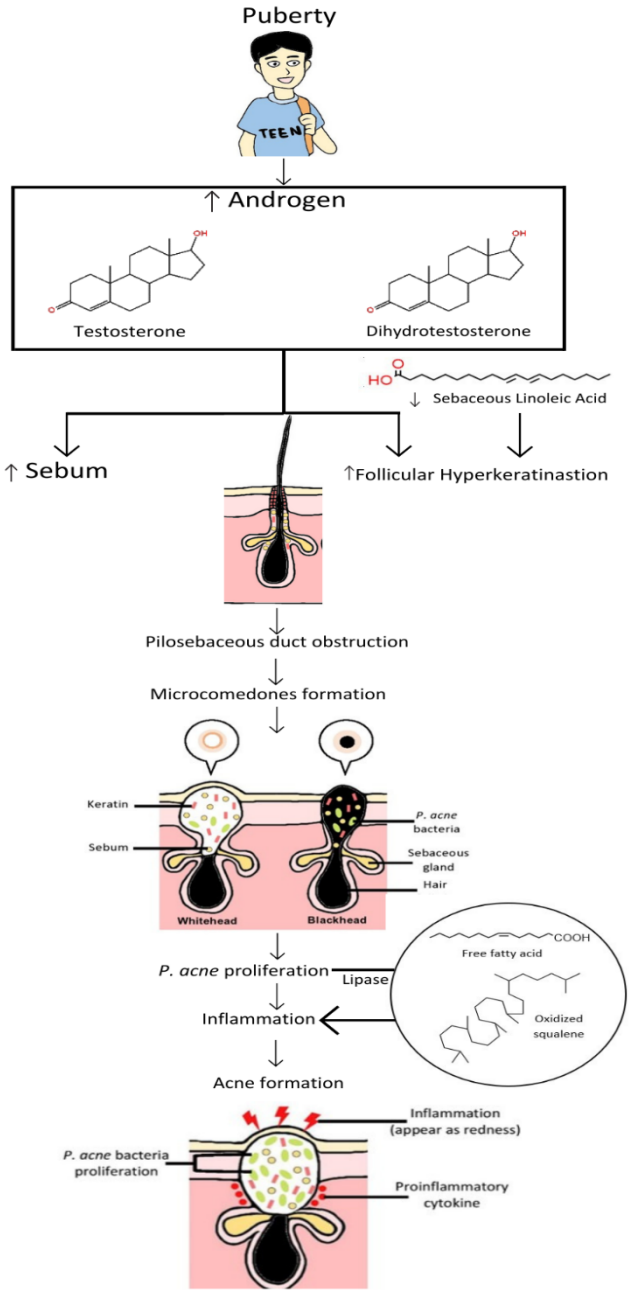

Figure 4: Summary of the pathophysiology of acne vulgaris.

Antioxidant and Anti-Inflammatory Activities of Stingless Bee Honey

As explained earlier, inflammation is one of the acne pathophysiologies. Oxidative stress that occurs due to ROS is known to cause many diseases either directly or indirectly. In acne, irritation during the infection is due to the free radicals formation specifically hydroxyl, superoxide and nitrous oxide. These free radicals are basically present in the sebum when the follicular walls of sebaceous glands become ruptured (Vora, Srivastava, & Modi, 2018). Antioxidative in honey produce anti-inflammatory properties which may decrease the inflammation during acne formation. Besides, studies show that the anti-inflammatory effects of honey can reduce the secretion of pro-inflammatory cytokines induced by *Propionibacterium acne* (Djakaria, Batubara, & Raffiudin, 2020). SBH possesses high antioxidative and anti-inflammatory properties since its polyphenolic compound is approximately ten times greater than other honey types (Mustafa et al., 2018). Research on the SB (*Trigona sp.*) in Malaysia indicated that the honey produced consists of a higher level of phenolic, flavonoids and total antioxidant activity compared with the honey produced by *Apis dorsata* (Ranneh et al., 2018).

Antimicrobial Activity of Stingless Bee Honey

The main mechanism of honey in treating acne is its antimicrobial property. Basically, the bacteria-killing effect of honey is attributed to its acidity, high osmolarity and hydrogen peroxide content (Albaridi, 2019). The acidic pH of honey will provide an unconducive environment for the bacteria, which in this case is *P.acnes* to grow as they favour neutral or slightly alkaline environments (pH 6.0 to 7.0) (Minden-Birkenmaier & Bowlin, 2018). Thus, the lower the pH, the higher ability of the honey to inhibit the growth of microorganisms (Fatima et al., 2018). SBH is considered the most acidic since it has the lowest value of pH (3.81 ± 0.02) when compared to *Tualang* honey (pH= 4.13 ± 0.02) and *Acacia* honey (pH= 4.20 ± 0.02) (Muhammad & Sarbon, 2021). Hence, this may deduce that SBH possesses potent antimicrobial activity since acidity contributes to its properties.

Moreover, it had been conjectured that reducing the water availability of the bacteria is a safe therapeutic approach for acne management (Eady, Layton, & Cove, 2013). Generally, honey is a supersaturated solution of sugar that has limited water content (Ab Hadi, Omar, & Awadh, 2016). Thus, this high sugar concentration of honey will induce a strong osmotic gradient on bacterial cells which

makes the water from bacterial cells flow out through the osmosis process. This makes the bacterial cells dehydrated and unable to grow and proliferate in hypertonic sugar solution (Almasaudi, 2020). In addition, certain types of sugars have an effect on antimicrobial activity. For example, glucose and maltose are substrates for the growth of bacteria and fungi in small concentrations (Mizzi et al., 2020). Hence, it can be concluded that the individual sugars making up the honey play role in the bactericidal activity of the honey.

Another factor that contributes to the antibacterial property of honey is the presence of hydrogen peroxide (Jalil et al., 2017). The formation of hydrogen peroxide content in honey is catalysed by the glucose oxidase through the oxidation process of the glucose ($Glucose \xrightarrow{Glucose\ oxidase} Gluconic\ acid + Hydrogen\ peroxide$) (Febriyenti, Lucida, Almahdy, Alfikriyah, & Hanif, 2019). This hydrogen peroxide will increase the cytokine production for the inflammatory response to kill the bacteria (Jalil et al., 2017). Studies showed that just a low concentration of hydrogen peroxide can effectively inhibit many types of bacteria (Febriyenti et al., 2019) since hydrogen peroxide itself is one of the reactive oxygen species (ROS). Thus, it possesses antibacterial action by destroying the bacterial cell structure. This destructive action basically will depend on the concentration of hydrogen peroxide in the honey (Ab Hadi et al., 2016). The summary of SBH properties and their effects in treating acne is tabulated in Table 2.

Nowadays, many cosmetic formulations that included honey in combination with other natural ingredients were studied for acne treatment. A tamarind soap with honey was developed and assessed for its antibacterial effect against *Staphylococcus aureus*. The result showed it able to inhibit the bacteria, thus it can be considered to have potential antibacterial properties. This soap also was tested on acne patients and it was effective in reducing the amount and size of the acne and able lightening the acne scars' colour and skin colour (de Jesus et al., 2020). In another study, a moisturising cream that contains SBH and propolis was also developed by Regional Apiculture Center (RAC) and tested on 15 volunteers. The volunteers that used it for their acne claimed that the cream able to prevent further acne progression (Mostoles, Rosario, Pasiona, & Buenaagua, 2021). The effect of natural products consisting of two different concentration of honey (44% and 54%) were also evaluated in acne patients. The acne severity proved a significant improvement in post-treatment than in the pre-treatment state in both groups. However, in the post-treatment, more patients were transferred to a mild acne state in the group that received the 54% honey concentration compared to the 44% honey concentration group (Helal, Mohamed, Farag, & Abdel Rashed, 2017). Therefore, this reveals that inclusion of honey in cosmetic formulation able to treat acne and higher honey concentration will treat acne better.

MOISTURIZING EFFECT OF STINGLESS BEE HONEY

Pathophysiology of Dry Skin

Dry skin is characterised by focal or generalised dry, rough, flaky or scaly skin. It is commonly accompanied by loss of skin elasticity, fissure and pruritus (Henning et al., 2021; Proksch, Berardesca, Misery, Engblom, & Bouwstra, 2020). The skin may also feel tense, sore or burning. Besides, dry damaged skin can be the entry for skin infections. Dry skin can be a symptom of itself or a symptom of other skin conditions including atopic dermatitis, ichthyosis, irritant contact dermatitis, psoriasis or asteatotic eczema (Mekić et al., 2019; Proksch et al., 2020). Besides, certain chronic diseases such as diabetes, hypothyroidism, HIV and renal insufficiency and also the use of some drugs (diuretics, statins or chemotherapeutic agents) can be accompanied by dry skin (Mekić et al., 2019).

The overall prevalence of dry skin is about 29% to 85% worldwide and it is the most common skin condition in middle-aged and elderly populations. Thus, it can be considered as a part of physiologic skin ageing (Mekić et al., 2019). This is because the lipid and water content are reduced in ageing skin which eventually impairs epidermal barrier function. Impaired epidermal barrier function will result in water loss (Chang et al., 2018). The onset of dry skin is mediated by intrinsic and extrinsic factors that cause modifications in the stratum corneum (SC) such as defective keratinisation and lipid cement abnormalities (Henning et al., 2021). This lipid cement is mainly composed of ceramides (~50%) followed by cholesterol (25%) and fatty acids (10-20%) (Moore & Rawlings, 2017). The ceramide content has been observed to have a strong

correlation with dry skin characteristics (Murphy et al., 2022). Many studies showed that ceramide content was reduced in dry skin

Table 2: Summary of SBH properties and their effects on treating acne.

Properties	Effective factors	Actions	References
Antioxidant and anti-inflammatory activities	Flavonoid and phenolic compounds	Reduce inflammation and irritation during acne formation	(Djakaria et al., 2020)
Antimicrobial activity	Acidity	Unconducive environment for bacteria growth	(Albaridi, 2019; Minden-Birkenmaier & Bowlin, 2018)
	Osmolarity	Dehydrate the bacterial cells	(Albaridi, 2019; Almasaudi, 2020)
	Hydrogen peroxide	Destruct the bacterial cells	(Albaridi, 2019; Jalil et al., 2017)

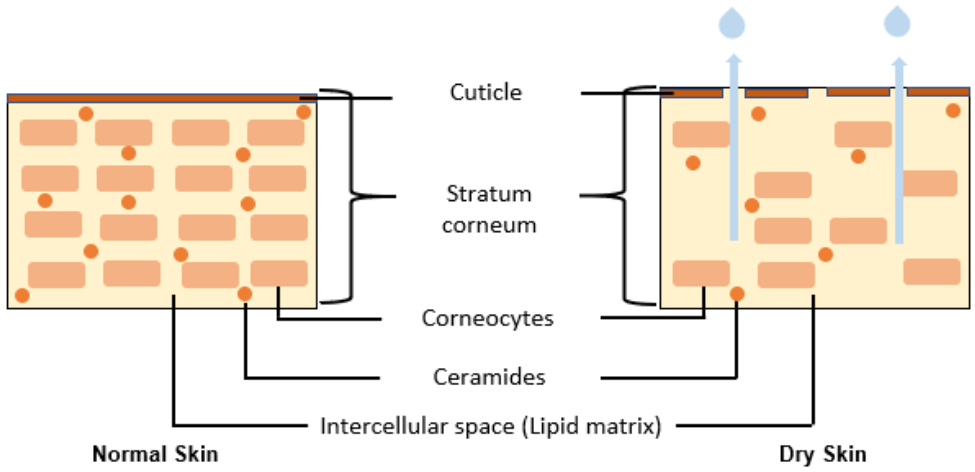

Figure 5: Illustration of the difference between normal and dry skins. The cuticle, corneocytes and ceramides number in the SC of dry skin are fragmented and depleted compared to normal skin. All these eventually lead to the loss of water.

(Moore & Rawlings, 2017). Some intrinsic factors are genetics, ageing and ethnicity meanwhile the extrinsic factors are sunlight exposure, low humidity, smoking, friction, bathing frequency and use of soaps (Henning et al., 2021; Proksch et al., 2020). A diagram illustrating the difference between normal and dry skin can be seen in Figure 5.

Role of Stingless Bee Honey as Humectant

Dry skin is normally been treated using humectants, occlusives or emollients to improve the SC's hydration and consequently repair the skin barrier (Murphy et al., 2022). Honey is a natural humectant due to its high moisture content (Ab Hadi et al., 2016; Eady et al., 2013). Humectants normally will possess emollient properties too (Purnamawati, Indrastuti, Danarti, & Saefudin, 2017). A humectant is basically a substance that acts like a sponge that draws water into the skin. It will attract water from the deeper epidermis and dermis and make the skin smoother by filling holes in the SC through swelling (Draelos, 2018). The presence of hydroxyl groups in honey

(Fletcher et al., 2020; Jalil et al., 2017) makes it has the water-binding ability and form hydrogen bonds with the water molecules. Hence, the hydrated skin condition can be preserved since the hydrogen bonds help to retain water in the SC by averting water loss through evaporation (Hadi, Razali, & Awadh, 2015).

Besides, the honey composition such as sugars, amino acids and lactic acid also exhibits a moisturizing effect (Fletcher et al., 2020; Jalil et al., 2017). SBH possesses high moisture content when compared to other types of honey. A study conducted in Malaysia showed that both SBH types (*Trigona thorasica* and *Trigona itama*) have high moisture content (28.3% to 33.7%) compared with the honey produced by *Apis dorsata* (24.8%) (Fatima et al., 2018). When compared to *Manuka* honey (*Apis mellifera*), the moisture content of SBH is also greater. The moisture content of honey by *Geneotrigona thorasica* and *Heterotrigona itama* range from 19.49% to 33.93% meanwhile *Apis mellifera* honey only consist of 14.74% water content (Shamsudin et al., 2019). Due to the great percentage of moisture content, thus SBH can be presumed to possess excellent moisturizing properties (Rao et al., 2016; Zulkhairi Amin et al., 2018). A diagram illustrating the mechanism of SBH preserves skin hydration can be seen in Figure 6.

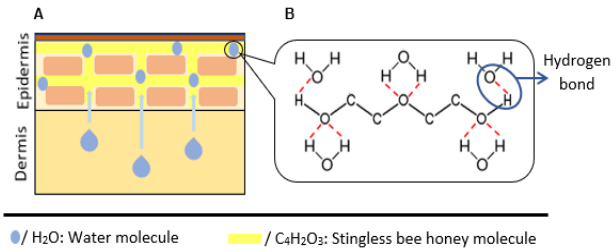

Figure 6: Illustration on how SBH preserves skin hydration by (A) attracting water from dermis to epidermis and (B) making a hydrogen bond with water molecules.

The effect on the skin of honey and bee products in cosmetic matrices had been studied. It showed that formulation consist of higher concentrations of honey hydrated the skin more effectively (Pavlačková, Egner, Slavík, Mokrejš, & Gál, 2020). More than 50% of volunteers rate the moisturizing effect of a bath soap consisting of SBH and propolis as acceptable (Mostoles et al., 2021). Furthermore, the moisture content of the lip balm formulation was enhanced with the presence of SBH. In this study, it shows that the higher the SBH concentration, the higher the moisture content of the lip balm. The lip moisture of all volunteers was also improved when applying the lip blam (Athirah, Yusof, Ajit, Sulaiman, & Naila, 2018). Hence, this indicates that the incorporation of SBH in cosmetic formulations enables to improve the skin hydration and also the moisture content of the formulation itself.

STINGLESS BEE HONEY REDUCES SCAR FORMATION

Formation of Scar

Scar formation is the result of the healing mechanism once the skin is injured. There are four corresponding stages in the wound healing process which are hemostasis, inflammation, proliferative and remodelling stages. Hemostasis and inflammation stages occur immediately after local aggression to restore the injury. Meanwhile, proliferative and remodelling stages involve the process of replacing the wound area with new skin. This new skin is formed by the deposition of collagen and the regeneration of new skin cells (Theoret, 2018). The summary of the wound healing process is illustrated in Figure 7.

Scar formation is a process that occurred in the remodelling phase. However, the extent of scarring will be depending on the severity of the trauma and the intensity of the inflammatory response. Although inflammation is one of the wound healing phases but once the inflammation fails to stop, the chronic inflammatory response will take over (Theoret, 2018). This will eventually impair the wound healing process and cause pathological scarring since permanent fibrotic scar tissue will accumulate at the site of injury. This fibrosis is characterised by the excessive accumulation of extracellular matrix (ECM) components including collagens, fibronectin and hyaluronic acid at the site of injury (Karppinen, Heljasvaara, Gullberg, Tasanen, & Pihlajaniemi, 2019; Theoret, 2018). This ECM will consequently form granulation tissue which is a secular connective tissue that contains macrophages, fibroblasts and capillaries (Karppinen et al., 2019).

Modulation of Pro-Inflammatory and Anti-Inflammatory Properties of Stingless Bee Honey

Honey possesses both pro-inflammatory and anti-inflammatory properties that may improve the wound healing process and eventually minimise scar formation. This is because honey will trigger the

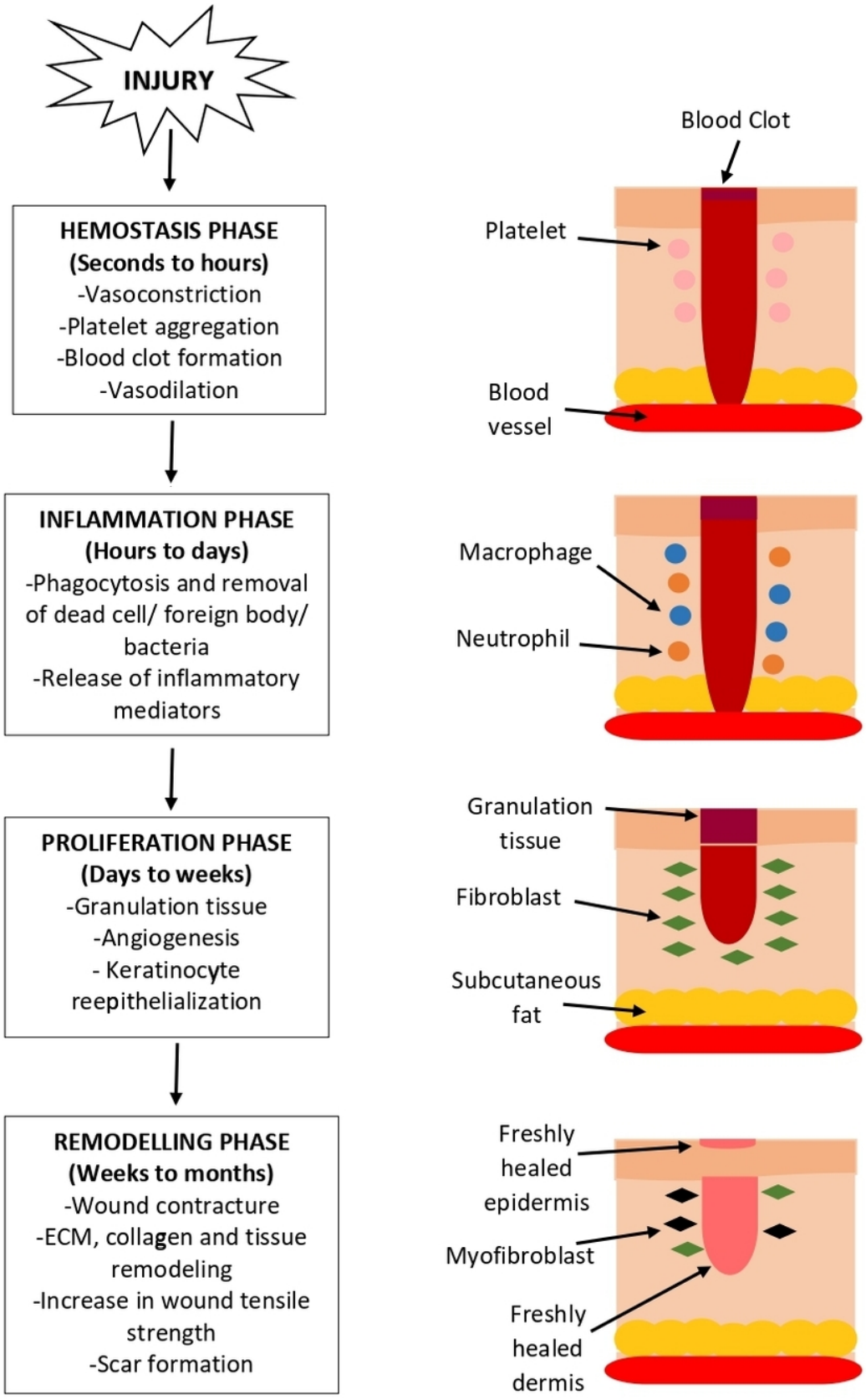

Figure 7: Illustration of the wound healing process.

release of inflammatory cytokines such as TNF-a IL-6, IL-1b, reactive oxygen species (ROS) and nitric oxide from the monocytes in the inflammatory phase. These cytokines play important roles in the initiation and magnification of inflammatory processes (Oryan & Alemzadeh, 2017; Oryan, Alemzadeh, & Moshiri, 2016). However, the prolonged inflammation phase can lead to tissue damage, impairs wound healing and eventually cause hypertrophic scarring due to hypergranulation and fibrosis. This is because the inflammatory mediators and cytokines are very cytotoxic and can destroy the essential components for the functional activities of all cells (Jalil et al., 2017). Hence, the use of honey can reduce tissue necrosis and hypertrophic scarring since honey possesses high anti-inflammatory properties.

The high anti-inflammatory properties of honey are attributed to the inhibition of leukocyte infiltration and reduction of matrix metalloproteinase-9 (MMP-9), cyclooxygenase-2 (COX-2), ROS and inducible nitric oxide synthase (iNOS) formation. This shows that honey can modulate both the pro-inflammatory and anti-inflammatory properties in which honey will trigger the production of the inflammatory cytokines when their concentrations are low. Meanwhile, the production of the inflammatory cytokines will be inhibited by the honey when inflammation is in progress (Oryan & Alemzadeh, 2017; Oryan et al., 2016) to avoid chronic wounds due to prolonged inflammation (Theoret, 2018). This anti-inflammatory property of SBH is contributed by the high flavonoid and phenolic compounds. Lastly, as mentioned before, SBH is a potent antioxidant. The antioxidant substances of SBH will remove and scavenge the ROS. It is important to prevent ROS formation as oxidative stress will increase tissue damage and prolong the wound repair process and form hypertrophic scar (Oryan et al., 2016).

Role of Stingless Bee Honey as Skin Reepithelialization and Wound Contraction Promoter

Honey which contains abundant sugars including SBH can stimulate skin reepithelialization by providing energy and a moist surrounding for the keratinocytes to migrate and proliferate to the wound surface in the proliferation phase (Oryan & Alemzadeh, 2017; Oryan et al., 2016). The high osmotic pressure of honey also facilitates skin reepithelialization acceleration by drawing out water from the tissue oedema and keeping the wound margin together. Besides, hydrogen peroxide content in honey also can promote epithelial cell growth (Nakajima et al., 2013). Wound contraction that occurred in the remodelling phase is basically a healing response that functions to decrease the damaged tissue size, so that lesser damaged tissue needs to be repaired and eventually minimise the scarring area. This response involves fibroblasts and myofibroblasts that are located adjacent to the wound (Lesperance, Francis, & Norton, 2006). As mentioned before, honey which is a source of energy promotes wound contraction by stimulating fibroblasts and myofibroblasts to deposit more collagen (Oryan & Alemzadeh, 2017; Oryan et al., 2016). The summary of the SBH effects on each wound healing phase in reducing scar formation is illustrated in Figure 8.

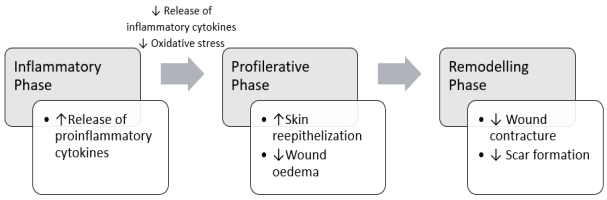

Figure 8: Summary of the SBH effects on each wound healing phase in reducing scar formation.

Recently, several formulations containing SBH had been developed and tested for wound healing. An *in vivo* healing efficacy test of PVA-natural biopolymer hydrogel incorporated with SBH on rabbit skin found that the SBH hydrogel exhibited an excellent wound healing property compared to no treatment and blank hydrogel. This is due to faster wound contraction and better wound closure was noticed in the SBH hydrogel groups (Abd Jalil, 2020). In another study, a nanofibrous composite membrane containing SBH and curcumin was developed for wound healing. The nanofibrous membrane-treated group showed a better healing rate (wound contraction and reduction) compared to the control group since significant collagen deposition and reepithelialization were observed (Samraj.S, Kirupha, Santhini, & Vadodaria, 2021). Besides, the volunteers of the RAC moisturising cream also mentioned that the skin irritations were easily reduced with little scar removal from the wounds (Mostoles et al., 2021). Hence, this shows that the addition of SBH in a formulation can promote wound healing and consequently reduce scar formation.

Conclusion

In conclusion, SBH may benefit the skin as it can treat acne, moisturise the skin and reduce the hypertrophic scar due to its constituents. SBH is a possible treatment for acne since it is highly antioxidant due to its high phenolic and flavonoid compounds. It also possesses anti-inflammatory properties that are contributed by the antioxidants which may decrease the inflammation during acne formation. Besides, the bacteria-killing effect of SBH is also attributed to its acidity, high osmolarity and hydrogen peroxide content. Moreover, honey's high moisture content is proven to give a hydrating effect on the skin due to the presence of hydrogen bonds in its chemical structure. This help to retain water in the SC of the skin by averting water loss through evaporation. Lastly, SBH honey can reduce hypertrophic scarring by modulation of both the pro-inflammatory and anti-inflammatory properties to avoid chronic wounds, stimulate skin reepithelialization and reduce wound contracture in the wound healing cascade. In cosmeceutical, further research regarding the effectiveness of honey/ SBH in various cosmetic formulations had been investigated and the formulations developed were proven to be effective in treating the acne, moisturising the skin and minimising the scar.

Conflict of Interest

None.

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ORIGINAL ARTICLE

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Determination of Zinc, Copper, Selenium, and Manganese in Human Milk using Acid Digestion by ICP-MS and its Application in Biological Trace Element Monitoring

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ABSTRACT

Introduction: Human milk contains essential trace elements which support healthy development of infants. Previous studies have reported various analytical methods using different instruments to measure trace elements in human milk. This study aimed to determine trace element concentration in human milk using a validated acid digestion method and its application in biomonitoring among postpartum mothers.

Method: Human milk samples were collected from three postpartum mothers and prepared using acid digestion method. All samples were analysed using inductively coupled plasma mass-spectrometry (ICP-MS) and all validation parameters were measured.

Results: Four trace elements which were zinc, copper, manganese and selenium were found to have good linearity ($r^2 > 0.99$), limit of detection in µg/L (0.06, 0.0001, 0.005, 0.00003, respectively) and limit of quantification in µg/L (0.18,0.0003, 0.02, 0.0001, respectively). Good accuracy (83.4 – 112.7%), inter-day, and intra-day repeatability were obtained. Method application on trace element monitoring over postpartum period of three participants showed the median concentration of zinc, copper and selenium in human milk gradually decreased with slight variation, whereas manganese remained stable. Positive significant correlations were observed for most of the elements ($r > 0.40$, $p < 0.001$) except for copper-manganese.

Conclusion: Acid digestion method is sensitive, accurate and precise to analyse and quantify zinc, copper, manganese and selenium concentrations in human milk simultaneously by ICP-MS. It can be applied to monitor trace elements concentration in human milk in clinical and public health settings.

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Introduction

Human milk has been reported to contain essential trace elements such as zinc, copper, iron, iodine, and selenium (Mohd-Taufek et al., 2016). The presence of these trace elements despite only in minute amount is very crucial to support infants' development and prevent trace element deficiencies. Determining the optimum trace elements concentration in human milk will ensure that infants get the most benefits and prevent deficiencies. Trace elements play vital roles in growth and metabolisms, and the concentration have been reported to vary in human milk of different study populations and at different lactational stage according to infant's requirement (Hunt & Nielsen, 2009). It is important to monitor trace elements concentration in human milk and identify reference range at different stages of lactation.

Previously, numerous analytical instruments have been reported to measure trace element analysis in human milk. Some of the reported techniques were using inductively coupled plasma–optical emission spectrometry (ICP-OES) (Durović, et al., 2017), flame atomic absorption spectrometry (FAAS) (Kim et al., 2012), inductively coupled plasma sector field mass spectrometry (ICP-SF-MS) (Ecsedi-Angyal et al., 2019) and graphite furnace atomic absorption spectroscopy (Ait lhaj et al., 2021). Currently, the inductively coupled plasma mass-spectrometry (ICP-MS) has been widely utilised to determine multi-element concentration in human milk due to its capability to detect many elements simultaneously at very low concentrations (Mohd-Taufek et al., 2016; Rovira et al., 2022; Tahboub et al., 2021).

Another advantage of ICP-MS is that it involves simple preparation of samples. For example, acid digestion and alkaline dissolution methods have been used to prepare human milk samples prior to analysis (Jagodic et al., 2020; Levi et al., 2018; Mohd-Taufek et al., 2016). Nitric acid and hydrochloric acid were the most often used acids in acid digestion method, whereas ammonium hydroxide and tetramethylammonium hydroxide (TMAH) were the most frequently used alkali in alkaline dissolution method (Wilschefski & Baxter, 2019). Nitric acid also has been reported to be the most optimum acid media for ICP-MS analysis due to its strong oxidising ability that is able to extract the trace element from the sample by forming soluble nitrate salts (Hu & Qi, 2014). Although there were studies which reported that the alkaline dissolution method was better than the acid digestion method, some elements were reported to be accurately analysed by the acid digestion method and there were also elements that produced the consistent results regardless of using alkaline dissolution or acid digestion method (Levi et al., 2018; Mohd-Taufek et al., 2016).

In human milk studies, there is no certified reference material for human milk available. Therefore, various certified standard reference materials in the form of milk powder such as NIST 1549, CRM 8534, and NIST 8435 and spiked samples have been used as the reference material to validate a developed analytical method (Alves Peixoto et al., 2019; Baranowska-Bosiacka et al., 2016; Jagodic et al., 2020; Mohd-Taufek et al., 2016; Pekou et al., 2022). Currently to our knowledge, there is no study available that has reported about a validated method to measure trace elements concentration in human milk in Malaysia probably due to limited access to analytical instruments and ethical issues about human milk samples. This study aimed to validate a simple acid digestion method to measure simultaneously the concentration of trace elements in human milk by ICP-MS and determine its application in biological monitoring among postpartum mothers.

Methodology

This study received International Islamic University Malaysia (IIUM) Research Ethics Committee (IREC) approval (ID No.: IREC 2021-053).

Study participants

A total of three postpartum women were recruited to provide milk samples. Participant information sheet and informed consent form were provided. The mothers were informed about the study and voluntarily provided human milk samples for analysis at their convenience. Data collection form was filled in by the participants for demographic information, medical and medication history, and their lifestyles. The participants provided expressed breast milk (EBM), which were collected in 2 mL syringes or the EBM plastic container, labelled with date of milk collection. All samples were frozen at -21ºC prior to analysis. Sample analyses were conducted at the Institute of Oceanography and Environment (INOS), University Malaysia Terengganu.

Sample Preparation

The frozen human milk samples were thawed at room temperature and manually shaken until the samples were completely homogenised. For the acid digestion method, 15.4 mL of 65% (v/v) nitric acid (Merck Suprapur) was diluted with deionised water up to 1 L to prepare 1% (v/v) nitric acid. Then, 1 mL of samples was then added to 9 mL of 1% (v/v) nitric acid in a 15 mL polypropylene conical tube (Corning Falcon) and was shaken manually until a homogenous solution was formed. The prepared sample were then analysed using ICP-MS.

Blanks Preparation

A total of 10 sets of blanks were prepared by adding 10 mL of 1% (v/v) nitric acid into a 15 mL polypropylene conical tube. The blanks were used to calculate the limit of detection (LOD) and limit of quantification (LOQ) for

method validation purpose.

Wash solutions

The Milli-Q water was used for wash purposes between samples analysis. Acid washout using 1% (v/v) nitric acid was done after analysing ten to twenty samples.

Standards Preparation

For the preparation of standards, a multi-element standard solution (PerkinElmer Pure VIII) with 100 g/mL of Al, B, Ba, Be, Bi, Ca, Cd, Co, Cr, Cu, Fe, Ga, K, Li, Mg, Mn, Na, Ni, Pb, Se, Sr, Te, Tl, and Zn (Merck Certipur) were used. To make a standard stock solution, 1 mL of multi-element standard solution was added to a polypropylene conical tube that contained 49 mL of 1% (v/v) nitric acid. For calibration purposes, six calibration standard solutions were prepared by mixing 1 mL of milk sample with 0.1 mL, 0.2 mL, 0.3 mL, 0.4 mL, 0.5 mL, and 0.6 mL of multi-element stock solution. All the standard solutions were then further diluted up to 10 mL with 1% (v/v) nitric acid to produce the following final concentrations: 0.02 µg/mL, 0.04 µg/mL, 0.06 µg/mL, 0.08 µg/mL, 0.1 µg/mL and 0.12 µg/mL for all elements.

Quality control

Skimmed milk powder ERM-BD150 was used as a certified reference material for quality control in this study. A total of 0.02 g of ERM-BD150 was weighed and diluted up to 10 mL with 1% (v/v) nitric acid in a polypropylene conical tube. The solution was manually shaken until all milk powder was fully dissolved. Three replicates of certified reference material were prepared daily for method validation purpose. The milk samples were analysed together with the blanks, reference materials, and spiked samples.

Samples Analysis

Sample analysis was conducted using Perkin-Elmer SCIEX ICP-MS model ELAN 9000 connected with DELL PC equipped with ELAN Instrument Control Session software (PerkinElmer Inc., Massachusetts, USA). The working condition for this instrument is presented in Table 1. During analysis, the samples were nebulised with a pneumatic nebuliser and then transported into the plasma for ion production using argon gas. Ions were brought into a quadrupole, where they were separated according to their mass/charge ratio (m/z), after passing through several focusing lenses and a reaction cell. On an electron multiplier detector (SimulScan™), specific isotopes of single-charged ions were identified, and the resulting electrical current was intensified to produce an intensity value in counts per second (cps). The system software was then used to translate the intensities for various isotopes in the tested samples into concentrations and compared them to those obtained from calibration standard solutions. Each sample underwent analysis for a total of one minute and forty seconds.

Table 1: ICP-MS working conditions (ELAN 9000, Perkin-Elmer SCIEX)

Nebulizer gas flow (L min-1)	0.94
RF power (W)	1100
Analog stage voltage (V)	-1700
Lens voltage (V)	6
Pulse stage voltage (V)	900
Ac rod offset (V)	-6
Discriminator threshold (V)	70
Scan mode	Peak hopping
Speed of peristaltic pump (rpm)	26
Detector Pulse Sweeps/Reading	50
Replicates	2
Sampler/Skimmer cones	Nickel
Dwell Time (ms)	2.5
Spray chamber	Ryton® Double-pass Scott-type spray chamber
Nebulizer	Gem-tip Cross-Flow pneumatic nebulizer

Linearity, LOD, LOQ, Accuracy and Repeatability

The validation parameters that were assessed and calculated included linearity, LOD, LOQ, accuracy and repeatability. To assess the linearity of the method, calibration graphs for each element were generated by the system software at final concentrations of 0.02 g/mL, 0.04 g/mL, 0.06 g/mL, 0.08 g/mL, 0.1 g/mL, and 0.12 g/mL. The linearity was assessed using the correlation coefficient value (r^2) derived from the calibration graph given by the system software. Ten series of blank solutions were employed to calculate LOD and LOQ. The ICH Harmonised Tripartite Guideline (2005) formula were used in this study to determine LOD and LOQ based on the slope and the standard deviation of the blank:

$$\mathrm{LOD} = \frac{3.3\ \sigma_{\mathrm{blank}}}{\mathrm{b}}$$

$$LOQ = \frac{10\ \sigma_{blank}}{b}$$

*σblank: standard deviation of 10 series of blanks
*b: slope of calibration curve

Accuracy which was reported in the form of recovery percentage was calculated using Microsoft Excel 2019 by comparing the result obtained from the analysis of ERM-BD150 to the certified reference value provided by the manufacturer.

Repeatability was assessed on the same day (intra-day) and three different days (inter-day). Inter-day repeatability was determined by analysing three ERM-BD150 samples and five human milk samples on three different days, whereas intra-day repeatability was determined by analysing three ERM-BD150 samples and five human milk samples on the same day. Repeatability was reported as %RSD and calculated using the following formula:

$$\%RSD = \frac{\text{Standard deviation}}{\text{mean}} \times 100\%$$

The inter-day repeatability was computed using the pooled relative standard deviation formula derived from the formula provided by Mc Naught & Wilkinson (2012). The inter-day repeatability was calculated as follows:

$$RSD_{pooled} = \sqrt{\frac{(n_1-1)RSD1^2 + (n_2-1)RSD2^2 + \cdots + (n_k-1)RSDk^2}{n_1 + n_2 + \cdots + n_k - k}}$$

k: different series of measurements
n: number of analysed samples

The acceptance range for accuracy and repeatability are 70-120% for recoveries with an RSD% ≤20% based on the 2017 Codex Alimentarius Commission.

Results

Calibration data obtained from this analysis were tabulated in Table 2 that showed good linearity (r^2 > 0.99) calibration curve for four trace elements which were zinc (Zn), copper (Cu), selenium (Se) and manganese (Mn). Zinc had the highest LOD value (0.06 µg/L), whereas Mn had the lowest (0.00003 µg/L).

The concentration of Zn, Cu, Se and Mn were also analysed in certified reference material ERM-BD150 in µg/L. The concentrations in µg/L were multiplied by the dilution ratio for the ERM-BD150 solution, then divided by 1000 to get milligrams per kilogram for the purpose of comparison with the manufacturer's certified value to measure the accuracy. The accuracy values for all four trace elements from the analysed ERM-BD150 were between 83.4% and 112.7%. All trace elements achieved satisfactory percentage recoveries within the acceptable range (Table 3). Except for the inter-day repeatability value of Se (18.37%), the intra-day and inter-day repeatability values for other elements in the ERM-BD150 samples were less than 10%.

Table 4 showed the repeatability values for intra-day and inter-day measurements for human milk samples. It was notable that the very low concentration of Mn and Se found in human milk demonstrated significant variability that lowered the method precision. Nevertheless, this method is considered precise as all repeatability values for all four trace elements were less than 20% which is considered acceptable by the guideline of 2017 Joint FAO/WHO Codex Alimentarius Commissions.

Application in biological trace element monitoring: Case studies

The validated method was then applied to analyse a total of 105 milk samples donated by three postpartum mothers. The data were reported as case studies in this research and demographics of the mothers were presented in Table 5.

Participant A was a 30-year old mother who delivered a male neonate at 41 weeks of gestation with a birth weight (BW) of 3.04 kilograms. She voluntarily donated a total of 64 breast milk samples, collected at different times of the day over six months, at her convenience. The infant was healthy and fully breastfed during the study period.

Participant B was a 27-year old mother who gave birth to a term male infant with a BW of 3.04 kilograms at 38 weeks of gestation. A total of 30 breast milk samples

Table 2: LOD, LOQ, slope and correlation coefficient for four trace elements measured in acid digestion methods.

Trace elements	LOD (µg/L)	LOQ (µg/L)	Slope	Correlation coefficient (r^2)
Zn	0.06	0.18	y= 937x - 5877	0.9976
Cu	0.0001	0.0003	y= 3747x - 21033	0.9992
Se	0.005	0.02	y= 203x - 967	0.9993
Mn	0.00003	0.0001	y= 10617x - 47720	0.9992

Table 3: Accuracy, inter-day (3 days), and intra-day repeatability of ERM-BD150.

ERM-BD150 (n=3)					
Trace elements	Certified value [mg/kg]	Observed value [mg/kg]	Accuracy [%]	Inter-day [%RSD]	Intra-day [%RSD]
Zn	44.8 ± 2.0	50.5 ± 3.7	112.7	4.79	4.39
Cu	1.08 ± 0.06	0.92 ± 0.04	85.2	3.52	2.56
Se	0.188 ± 0.014	0.188 ± 0.086	100	18.37	3.71
Mn	0.289 ± 0.018	0.241 ± 0.042	83.4	9.94	9.48

Table 4: Inter-day (3 days) and intra-day repeatability (n = 5) of human milk samples..

Trace elements	Inter-day [%RSD]	Intra-day [%RSD]
Zn	5.66	5.03
Cu	6.74	3.78
Se	14.10	16.24
Mn	18.10	12.09

Table 5: Demographics of participants (n = 3)

Demographic	A	B	C
Race	Malay	Malay	Malay
Body Mass Index	23.19	24.0	21.83
BW Pre-pregnancy (kg)	55	51.5	56
BW Pre-delivery (kg)	64	62	62
BW Post-delivery (kg)	55	54	58
No. of pregnancies	4	1	5
No. of deliveries	2	1	5
No. of miscarriages	2	0	0
Birth method	Caesarean	Normal	Normal
Dietary intake	RCBV	RCBV	RFCVFr
Medical history	No	No	Gestational diabetes, Asthma
Supplement	No	Yes (Se, Mn, Zn, Cu)	Yes (Zn, Fe)

BW: Body Weight; R: Rice; C: Chicken; B: Beef; F: Fish; V: Vegetable; Fr: Fruit

were collected from 12 months until 15 months postpartum at her convenience. At birth, the infant presented with high fever and jaundice 10 days after birth. He was fully breastfed only until the first six months and then continued with partial breastfeeding and solid food. There were no abnormalities in the growth and development of the infant except he was underweight and had mild eczema.

Participant C was a 27-year old mother who delivered a male infant with a BW of 3.08 kilograms at 37 weeks of gestation. She was able to donate a total of 11 human milk

samples that were expressed at different times of the day in the 21st month postpartum only, at her convenience. The infant showed normal growth and development with no other health problems except for jaundice after birth. He was fully breastfed only until the first six months and then continued with partial breastfeeding and solid food.

Table 6 showed the concentrations of Zn, Cu and Se were found to be the highest at one month postpartum compared to the later months in participant A. However, only Se and Cu gradually decreased wheras Zn showed some fluctuations. On the other hand, Mn exhibited no significant changes in concentration over the time period. The concentration of Zn and Cu also showed relatively higher concentration than Mn and Se. The correlations between trace elements in human milk also were examined with $p < 0.001$ was considered as significant. The Spearman's correlation coefficients and p-value between essential trace elements in human milk of participant A were: Zn-Se ($r = 0.607$, $p = <0.001$), Zn-Cu ($r = 0.615$, $p = <0.001$), Zn-Mn (0.471, $p = <0.001$), Cu-Se ($r=0.735$, $p = <0.001$), Mn-Se ($r=0.467$, $p = <0.001$). All trace elements showed significant positive correlations ($r > 0.40$, $p < 0.001$) except for Cu-Mn.

Table 7 showed the concentrations of Zn, Cu, Se and Mn in participant B at 12 to 15 months postpartum and participant C at 21 month postpartum. For participant B, the median Zn concentrations were slightly decreased from 430.5 µg/L at 12 months to 353 µg/L at 15 months postpartum. For Se, the median concentration was increased from 7.6 µg/L at 12 months to 10.9 µg/L at 13 months and remained around 10 µg/L at 14 and 15 months postpartum. There was a fluctuation in the levels of Cu during 12 to 15 months postpartum. The median concentration of Mn increased from 5.8 µg/L to 7.1 µg/L at 12-13 months and later declined to 4.5 µg/L and 4.7 µg/l at 14-15 months. For participant C, the median values of Zn and Mn were similar but Se and Cu seemed to be higher than participant B despite later lactation period.

Table 6: The concentrations of trace elements in the human milk of participant A over a period of 6 months postpartum (µg/L).

Trace Elements	Mean/ Median	Month postpartum					
		1	2	3	4	5	6
		N = 15	N = 8	N = 10	N = 15	N = 6	N = 10
Zn (µg/l)	Mean±SD	2698±1010	1211±304	1480±355	1296±773	967±221	1254±541
	Median	2640	1110	1495	1190	886	978
	(Range)	(1550-5870)	(907-1810)	(841-1960)	(352-3780)	(716-1250)	(713-2350)
Cu (µg/l)	Mean±SD	403 ± 84	180 ± 20	164 ± 26	116 ± 46	93 ± 29	77 ± 18
	Median (Range)	406 (265-621)	188 (147-200)	166 (114-215)	104 (61-223)	89 (61-143)	75 (50-111)
Se (µg/l)	Mean±SD	24.8 ± 7.2	11.9 ± 4.7	14.8 ± 4.5	12.3 ± 6.8	9.8 ± 4.0	11.3 ± 4.1
	Median (Range)	23.6 (14.6-43.1)	11.9 (2.7-18.3)	14.2 (9.4-22.9)	12.2 (4.4-32.0)	10.7 (4.4-15.0)	10.4 (5.9-19.7)
Mn (µg/l)	Mean±SD	4.7 ± 1.2	3.6 ± 1.1	4.7 ± 1.8	4.7 ± 3.1	3.3 ± 0.5	5.1 ± 3.0
	Median (Range)	4.4 (3.5-7.6)	3.6 (2.2-5.9)	4.3 (1.8-7.7)	3.8 (2.6-14.8)	3.2 (2.9-4.0)	3.5 (2.7-10.5)

Table 7: The concentration of trace elements in human milk of participants B and C at 12 months postpartum and above (µg/L).

Trace Elements	Mean/ Median	B				C
		12	13	14	15	21
		n = 16	n = 4	n = 6	n = 4	n = 11
Zn (µg/l)	Mean±SD	450±117	1178±1557	373±126	390±86	606±532
	Median (Range)	431 (243-642)	454 (292-3510)	354 (203-585)	353 (338-518)	450 (179-2150)
Cu (µg/l)	Mean±SD	8.4±2.3	12.8±5.4	10.1±1.7	9.8±2.0	15.3±4.3
	Median (Range)	7.6 (5.1-14.0)	10.9 (8.9-20.7)	10.3 (7.9-12.6)	10.2 (7.4-11.5)	16.4 (7.2-21.7)
Se (µg/l)	Mean±SD	76.7±23.9	84.2±22.2	82.4±31.2	105.3±40.3	155±33
	Median (Range)	72 (47-136)	84 (57-111)	72 (62-144)	106 (56-154)	166 (83-200)
Mn (µg/l)	Mean±SD	5.7±2.3	7.1±1.7	20.8±34.7	4.6±0.4	3.5±1.3
	Median (Range)	5.8 (3.2-11.2)	7.1 (5.0-9.1)	4.5 (3.8-90.8)	4.7 (4.2-5.0)	3.5 (1.1-6.3)

Discussion

We report a method using acid digestion to measure total elements of Zn, Cu, Se and Mn concentration in human milk. Previous studies that reported acid digestion method have used heat application during sample preparation (Alves Peixoto et al., 2019; Jagodic et al., 2020; Tahboub et al., 2021). Theoretically, by raising the temperature of the samples, the average kinetic energy will rise, thus increase the chances of acid and biological sample collisions and enhance trace element dissolution (Mohammed et al., 2017). Our method did not require heat application since human milk is a biological matrix that contains proteins such as mucins, caseins and whey proteins that can be affected by high temperature. An increase in temperature has been reported to increase the amount of protein adsorbed onto solid surfaces resulting in a lower trace element content in the pasteurized milk solution possibly due to protein precipitation (da Costa et al., 2003; Rabe et al., 2011). Our method was simple and produced good results for the required validation parameters to determine four trace elements simultaneously in 1 mL of human milk for biological monitoring purpose.

Homogenisation of milk samples before the digestion process is also a crucial step during sample preparation prior to the analysis. After being thawed, human milk is known to form two different layers, the fatty and aqueous layers. A study has reported that the concentration of iodine in the fatty layers was significantly different than those in the aqueous layers (Huynh et al., 2015). Therefore, sample homogenisation prior to mixing with nitric acid would ensure that all trace elements in the samples uniformly dispersed. Information about amounts of trace elements in two different layers of human milk have not been available to date. Further studies are needed to investigate the differences in the amount of trace elements in two distinct layers of human milk after thawing.

All validation parameters measured in this study produced good measurements which were within the acceptable range specified by the guideline, indicating that this method performed satisfactorily. The employed method in this study had a lower LOQ value for all four trace elements compared to other studies (Alves Peixoto et al., 2019; Mohd Taufek et al., 2016). The fact that the LOQ values of Zn, Cu, Se and Mn obtained using this method were also significantly lower than the anticipated range concentrations in human milk reported in other countries showed that this method may be used to determine the levels of trace elements in human milk over a wide variety of populations. This method did not utilise any internal standard. It has been demonstrated that the implementation of internal standards that had the mass numbers almost the same as tested trace elements could enhance the precision of the methods used (Vanhaecke et al., 1992). Although still within the acceptable range, Mn and Se in human milk samples had slightly lower precision in the present study. Therefore, the precision of this

method may be further optimised by utilising internal standards in the future.

For the purpose of method application in biological monitoring, we measured the concentration of Zn, Cu, Se and Mn in human milk collected at different postpartum period from three mothers as case studies. The median Zn concentration in participant A was high (2640 µg/L) at the first month postpartum and rapidly decreased (978 µg/L) at 6 months postpartum. This pattern was found to be similar to a previous report that highlighted a rapid decline of zinc concentration in term breast milk from 3000 µg/L at one month to 1200 µg/L at six months postpartum (Hunt & Nielsen, 2009). We found the mean±SD of Zn concentration at 1 month was 2698 ± 1010 µg/L and 1480 ± 355 µg/L at 3 months postpartum. A study by Motoyama et al. (2021) involving 78 Japanese mothers reported higher mean Zn concentration at 1-month and 3-months postpartum with levels of 3000 ± 1300 µg/L and 1680 ± 950 µg/L respectively. Similarly, another study by Dumrongwongsiri et al. (2015) also reported higher Zn levels with a range of 500-3200 µg/L at 4-6 months postpartum than the present case study (352-2350 µg/L). However, lower mean Zn values were found in term milk of 70 mothers in Spain of 1237.76 ± 949 µg/L at 1 month postpartum (Mandiá et al., 2021). In Table 7, the median levels of Zn for both participant B at month 12-15 and participant C at month 21 appeared to be relatively similar. Further monitoring on zinc concentrations in human milk over postpartum period may be needed to understand the relevance and factor behind its variation relative to the needs of infant's growth and development.

In our study, the median Cu values were 406 µg/L in the first month postpartum and 166 µg/L at 3 months, which were lower compared to the values reported by Motoyama et al. (2021) with values of 500 µg/L and 330 µg/L respectively. However, the mean Cu concentration in the first month postpartum in our case study was 403 ± 84 µg/L, which was higher than the reported value by Mandia et al. (2021) of 250.11 ± 163 µg/L. In Iran, a study also reported a higher mean Cu concentration of 1070 ± 1140 µg/L in the breast milk of 160 lactating mothers at 6 months postpartum (Sadeghi et al., 2020). A previous report also highlighted the decline of mean Cu levels from 250 µg/L in the first six months to 100-200 µg/L in seven to 12 months postpartum (Hunt & Nielsen, 2009). Currently, optimal levels of Cu in human milk have not yet been determined at different lactation stages.

The median Se concentration found in the current study was 23.6 µg/L in the first month then decreased to 14.15 µg/L at 3 months and 10.41 µg/L at 6 months postpartum. Another study also found similar level of Se at 1-month which was 22 (17-29) µg/L but higher level at 3 months postpartum, of 21 (16-25) µg/L (Motoyama et al., 2021). However, a previous study by Mandiá et al. (2021) reported lower mean Se values of 8.87 µg/L in the mature milk compared to the current study which was 24.82 µg/L. In a previous study involving 470 lactating women from Slovenia, the mean Se levels found in the human milk at 6 to 8 weeks after delivery was 12.6 µg/L (Snoj Tratnik et al., 2019). Based on the findings by Han et al. (2019), the adequate intake of Se for 0-3 months Chinese infants was 15.29 µg/day, which is almost the same with the levels reported in the current study (14.15 µg/L at 3 month). It is important to monitor selenium status in infants receiving different amounts of selenium from human milk to prevent deficiency or toxicity.

We also observed median Mn concentration that were relatively stable with 4.44 µg/L in the first month postpartum, 4.29 µg/L at three months and 3.54 µg/L at 6 months. These results were lower than those reported by a previous study by Motoyama et al. (2021) which found median Mn levels of 8 µg/L in the first month and 7 µg/L at 3 months postpartum. Similarly, Li et al. (2016) also reported higher Mn levels of 9.33 to 11.53 µg/L in the first 2 months and then declined at 4 to 6 months to a mean value of 7.69 µg/L. Our study suggests that Mn concentrations in human milk are relatively stable over the postpartum period. Human milk was reported to provide an adequate amount of Mn to prevent deficiency at about 3–10 µg/L (Horning et al., 2015). This study observed that trace elements concentration in human milk varied over postpartum period. The variations in the trace elements levels might be due to several factors such as lactation stages, geographical regions, dietary intakes or trace element supplements that were taken by some of the participants. Consistent with the previous reports (Li et al., 2016; Motoyama et al., 2021), we found positive significant correlations for most of the elements ($r > 0.40$, $p < 0.001$) except for Cu-Mn.

The limitation of our study includes the small population size due to challenges in recruiting participants. However, the milk samples provided by these participants were sufficient to validate the method and its application in biomonitoring of trace elements in human milk. Future studies with large sample size would be able to produce a thorough investigation on the factors that may influence the trace element concentrations in human milk. This study did not seek to evaluate the association of factors affecting trace elements in human milk. However, the findings from this study are important since this is the first study reported in Malaysia using a validated acid digestion method that is simple, accurate and sensitive to measure Zn, Cu, Se, and Mn. It is applicable and relevant for clinical and public health settings by using minimal amount of sample for biomonitoring to detect potential deficiency or toxicity relative to the needs of infants. Previous studies reporting data of Malaysian population have examined the concentration of a single trace element (Pb and Fe) in human milk in the past few decades (Huat et al., 1983; Loh & Sinnathuray, 1971). New data can be produced for Malaysian population using a simple and robust method reported in the present study.

Conclusion

Acid digestion method was simple, sensitive, accurate, precise, and robust to quantify zinc, copper, manganese and selenium simultaneously in human milk by ICP-MS. Human milk analysis is challenging due to high variability of trace elements contents between individuals and across postpartum period. There were significant correlations between concentration of Zn-Se, Zn-Cu, Zn-Mn, Cu-Se and Mn-Se. Future studies may utilise this method to determine reference range of trace elements concentration in human milk in larger study population.

Author Contribution

N.H. Mohd-Taufek, A.S. Mohmad Sabere & J. Bidai conceived and designed the experiment, conducted sample and data collection, data analysis, N.B. Amran, U.S. Mohamad Jamahari and A.R. Fata Nahas analysed the sample and data, all authors wrote the manuscript.

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Data Availability

The datasets analysed in the current study are available from the corresponding author upon reasonable request.

Competing Interest

N.H. Mohd-Taufek, A.S. Mohmad Sabere, J.A. Bidai, N.B. Amran, U.S. Mohamad Jamahari and A.R. Fata Nahas declare that there is no competing interest in this research.

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ORIGINAL ARTICLE

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Silver nanoparticle biogenically synthesised by Psychotria malayana Jack: Physicochemical, cytotoxic and antimicrobial characterisations

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ABSTRACT

Introduction: Silver nanoparticles are targeted for antimicrobial and cytotoxic properties to combat antimicrobial resistance and chemoresistance. Green synthesis of silver nanoparticle method is widely used because it is environmental-friendly using biological substances as reducing and stabilising agents. *Psychotria malayana* Jack is rich with a wide range of phytochemicals that able to synthesise silver nanoparticle. **Methods:** The leaves of P. malayana Jack was extracted with ethanol-water solvent via ultrasound assisted extraction and the extract was analysed using liquid chromatography- mass spectrometry (LC-MS). The extract was then added to silver nitrate solution for 24 hours. The formation of AgNPs-PM was analysed using UV-visible spectrophotometry, scanning electron microscopy, zeta particle size and zeta potential analysis. The synthesised AgNPs-PM were tested for their cytotoxicity on human colorectal adenocarcinoma cells (Caco-2) and human epithelial breast adenocarcinoma cells (MCF-7) using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colourimetric assay. For antibacterial activity, the nanoparticles were tested on Gram-negative Escherichia coli and Pseudomonas aeruginosa and Gram-positive Bacillus subtilis and Staphylococcus aureus using disc diffusion method. **Results:** AgNPs-PM were successfully synthesised using P. malayana Jack extract. LC-MS analysis showed the presence of flavonoids, amino acids and heterocyclic compounds . An attempt in cytotoxic activity test showed that at concentrations between 12.5 µg/ml to 400 µg/ml of AgNPs-PM, no cytotoxic activity was observed. Whereas, in antibacterial assay, 2 mg/ml AgNPs-PM tested on the bacterial strains showed weak inhibition on their growth. **Conclusion:** AgNPs-PM has been successfully synthesised and characterised. However, the AgNPs-PM possess low bioactivities of cytotoxic and antibacterial activities.

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Introduction

In the last few decades, research on nanotechnology is being widely adapted worldwide. Nanoparticles refer to particles within the size range of 1 nm to 100 nm and they have many potential applications in various areas such as biotechnology, medicine, pharmaceutics, industries, biology and agriculture (Song & Kim, 2009; Susanti, Haris, Taher, & Khotib, 2022). In medical study, nanoparticles assist in more rapid diagnosing of diseases and more efficient disease treatments because functional molecules can be attached selectively to the metallic nanoparticles such as silver, platinum and gold, which allow the transportation of the molecules to the target site under the influence of magnetic field (Ahmed *et al.*, 2022; Muhamad, Ab.Rahim, Wan Omar, & Nik Mohamed Kamal, 2022). In other field such as nanotechnology, metallic nanoparticles are being widely utilised due to their high reactivity and high surface area to volume ration (Muhamad *et al.*, 2022; Susanti *et al.*, 2022).

Silver nanoparticles (AgNPs) are gaining attention among other metallic nanoparticles as they have remarkable biological and physicochemical properties due to their distinct surface plasmon resonance (Muhamad *et al.*, 2022). This surface plasmon resonance refers to electronic oscillations of the conduction electrons on nanoparticle's surfaces due to the interaction with the electromagnetic radiation (Eze, Tola, Nwabor, & Jayeoye, 2019). In synthesising the metallic nanoparticles such as AgNPs, a few methods have been introduced: physical, chemical and biological (Susanti *et al.*, 2022). Physical method renders a few disadvantages which include small number of AgNPs yield, a long completion period of the whole process and the utilisation of high energy which can cause a release of excessive heat to the surroundings. With chemical method, the production yield may be high, but the production of the toxic by-products is a big downturn of this process (Muhamad *et al.*, 2022).

Due to disadvantages presented *via* physical and chemical methods, the synthesis of nanoparticles using biological method such as bacteria, fungi and plant mediated synthesis is now being widely adapted (Susanti *et al.*, 2022). From the economical factor, green synthesis is cost-effective as the biological component of the biological agents which include bacteria, fungi, yeast, plant, viruses and by-products of these agents can act as the reducing agents (Alarjani, Huessien, Rasheed, & Kalaiyarasi, 2022; Susanti *et al.*, 2022). They also function as capping agents which are crucial for the stability and biocompatibility of nanoparticles (Susanti *et al.*, 2022).

Figure 1: The leaves and fruit bunch of P. malayana Jack.

The mature plant possesses dark-green leaves with a fruit bunch (Figure 1). The phytochemicals presence in the leaves extract of *P. malayana* Jack, or also known as "*meroyan sakat*" or "*salung*" in Malaysia can be utilised as the reducing and stabilising agents for the synthesising process of AgNPs in this study. Plants from genus *Psychotria* are rich with alkaloids as their major compounds such as indole, quinoline, isoquinoline, monoterpene indole, flavonoids, cyclic peptides, terpenoids and coumarins (Calixto *et al.,* 2016). Among these phytochemicals, flavonoids are well known for its role as reducing and capping agent, as a replacement for the use of toxic chemical products (Ahmad *et al.*, 2022). In this study, the leaves extract of *P. malayana* Jack was utilised to produce AgNPs. Other advantages of using green synthesis method include environmentally friendly as toxic chemicals are not being used with no application of high pressure, temperature, and energy. It is also stated that the use of plant mediated synthesis in nanoparticles production is simple as it can be produced in a single step at a room temperature, easy to manage commercial-scale processes and highly stable in storage (Alarjani *et al.*, 2022; Susanti *et al.*, 2022).

Studies reported that the emergence of multidrug-resistant bacterial strains due to misuse of antibiotics has become a major concern to the human health all over the world (Murray *et al.*, 2022; Wang, Hu, & Shao, 2017). A predictive statistical models stated that the deaths associated with antimicrobial resistance in the year 2019 was estimated to be 4.95 million deaths (Murray *et al.*, 2022). It was proven in many previous literatures that silver ions and silver-modified inorganic materials such as AgNPs exhibit multiple antimicrobial activity. This includes their ability to interfere with the bacterial DNA activity and generation of free radicals which induce the reactive oxygen species leading to bacterial cell death (Ahmad *et al.*, 2022). These mechanisms are different to most antibiotics which commonly work by targeting the cell wall synthesis, translation machinery and DNA replication machinery. These unique properties of AgNPs make them a comparable choice to antibiotic in the treatment of microbial infections as antibiotic's resistance mechanisms are not applicable to them (Ahmad et al., 2022; Susanti et al., 2022).

Aside from that, reports state that AgNPs also demonstrate antitumourigenic activity on tumour models (Muhamad *et al.*, 2022). Based on the research, AgNPs which is concentration dependent can induce apoptosis or the programmed cell death in *in vitro* studies. Furthermore, AgNPs can also induce the alterations in the cell morphology, reduce cell viability and its metabolic activity and causing an increase in oxidative stress which lead to mitochondrial damage. This then leads to significant DNA damage and cell death. Thus, there is a potential usage of the AgNPs in cancer treatment (Zhang, Ma, Gu, Huang, & Zhang, 2020). Therefore, this study was done to investigate the antimicrobial activity and cytotoxicity of AgNPs-PM for their potential application in human use.

Methodology

1. Plant sample collection and extraction

Fresh leaves of *P. malayana* Jack, voucher specimen (PIIUM 0008-1) was collected at Kuantan, Pahang, Malaysia. The leaves were air dried at a controlled temperature drier (40 °C) for three days. The leaves were ground mechanically into fine powder using a mechanical grinder. 50g of the leaves powder were mixed with 500 ml ethanol-water (Ethanol 95%, GENE Chemicals) (80:20) in a 500 ml Erlenmeyer flask. The extraction was done via ultrasound-assisted extraction method at a temperature of 48 °C for 40 minutes using a probe sonicator (Qsonica Ultrasonic Sonicator Converter Model CL-334). The leaves extract was then filtered using filter papers (NICE Qualitative 102) and stored in a refrigerator at 4 °C (Bimakr, Ganjloo, Zarringhalami, & Ansarian, 2017).

2. Liquid Chromatography Mass Spectrometry Quadrupole of Flight (LC-MS-QTOF) Analysis

Sample preparation: P. malayana Jack leaves extract was dried via rotary evaporator (IKA HB 10 basic) at a speed of 130 rpm and a temperature of 50°C. The dried extract was reconstituted with methanol to a final concentration of 10 mg/ml, then it was diluted to a concentration of 1 mg/ml with methanol. Prior to analysis via LC-MS-QTOF (6520 Agilent Techologies, SA, USA), the extract was filtered using a 0.22 µm pore of PVDF membrane size syringe filter. LC-MS method: The chromatographic separation was operated using Agilent ZORBAX Eclipse Plus C18 Rapid Resolution HT (2.1 x 100 mm) 1.8 µm with (A) 0.1% formic acid in distilled water and (B) 0.1% formic acid in acetonitrile for positive mode. The gradient elution programme was 0.00 – 18.00 min, 5 – 95% (B); 18 – 23 minutes; 95% (B); 23.0 minutes; 5% (B). It was run for 30 minutes. Prior to new injection, re-equilibration of LC condition was conducted for 2 minutes. The sample injection volume and the flow rate of mobile phase was set at 2 µl and 0.25 ml/min, respectively. The mass spectrometer was operated in positive electrospray ionisation (ESI) mode with optimum gas temperature at 325°C, gas flow at 11 L/min and nebuliser at 35 psi. Data analysis: The chromatographic profiles were analysed using Agilent Mass Hunter Qualitative Analysis B.05.00 software (Agilent Technologies, Santa Clara, CA, USA) based on the accurate mass data identified and the predicted compounds were annotated using METLIN database (Al-Abd et al., 2015).

3. Preparation of silver nitrate ($AgNO_3$) solution

1 mM and 5 mM of $AgNO_3$ solutions were prepared by dissolving 0.0153g and 0.0764g of $AgNO_3$ powder (EMSURE, Macedonia) into 90 ml deionised water, respectively.

4. Synthesis of silver nitrate ($AgNO_3$) solution

10 ml of *P. malayana* Jack leaves extract was added slowly into a 90 ml $AgNO_3$ solution of two different concentrations (1 mM and 5 mM) under continuous stirring (100 rpm) using a magnetic stirrer to ensure the 1:9 ratio of the plant extract to $AgNO_3$ solution. The synthesis process was continued for 24 hours. After 24 hours, the synthesised solution was centrifuged at 7500 rpm for 15 minutes at 4 °C to purify it (Supra 22K, Korea). The resulting pellet was resuspended with a small amount of deionised water and was dried in an oven at 40 °C. The resulting powder of AgNPs-PM was left at room temperature for future use.

5. Characterization of silver nanoparticles (AgNPs-PM)

The characterisation of the synthesised AgNPs-PM was carried out by Ultraviolet and Visible spectrophotometer (UV-Vis), scanning electron microscopy (SEM), zetasizer and zeta potential analysis.

UV-Vis Spectroscopy analysis: The green synthesised AgNPs-PM were sampled at 1, 2, 4, 8 and 24 hours for analysis *via* UV-Visible double beam spectrophotometer (SHIMADZU UV-1800, Japan). Deionised water was used as a blank and reference solution. The spectrum was then recorded in the scanning range of 350 nm to 800 nm.

Morphological analysis: The image of the biosynthesised AgNPs-PM, the nanoparticles were analysed under Zeiss EVO-50X Scanning Electron Microscopy (SEM) instrument. Then, on a non-conduction carbon tape that functions as the stabiliser, AgNPs-PM powder was prepared by simply sprinkling 2 mg of the powder sample on the tape on the sample holder. After being fixed, SEM analysis was performed and the AgNPs-PM powder was analysed at room temperature.

Zetasizer and Zeta Potential analysis: The analysis sample was an aqueous solution of biosynthesised AgNPs-PM that was placed inside a Malvern Zetasizer instrument. The instrument then identified the electrical potential of ions surrounding a particle at its border as well as ions adsorbed in the diffuse layer at 25°C that was run 12 times.

6. Cytotoxic assay

Cell culture: Human colorectal adenocarcinoma cells (Caco-2) and human epithelial breast adenocarcinoma cells (MCF-7) were cultured at 37°C in a 5% CO_2 incubator (Thermo Scientific Heraeus BB15) in Eagle's minimal essential medium, EMEM (ATCC 30-2003, Manassas, VA) supplemented with 10% fetal bovine serum, FBS (Gibco, Brazil) and 1% (v/v) penicillin-streptomycin (Nacalai Tesque, INC., Kyoto, Japan). After the cells reached confluency at 80%, trypsinisation process was applied to detach and subculture the cells. The cells were then seeded into a 96-well plate at a density of 15,000 cells per well. Cell viability assay: The evaluation of the cytotoxic activity of the synthesised AgNPs was done via 3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colourimetric assay. The seeded cells were treated with various concentrations (400 µg/ml, 200 µg/ml, 100 µg/ml, 50 µg/ml, 25 µ/ml and 12.5 µg/ml) of AgNPs-PM and an anticancer drug Tamoxifen as positive control. The cells were incubated for 24 hours. After 24 hours, the cells were treated with 20 µg of MTT (5 mg/ml) and re-incubated for 30 minutes at 37°C. The formed crystals of formazan were dissolved using 200 µL of dimethyl sulfoxide, DMSO (EMPLURA, USA) and re-incubated for another 30 minutes at 37°C. Using a microplate reader, the difference in colour intensities (absorbance) was recorded at 630 nm.

7. Antimicrobial assay

Two Gram positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus* and two Gram negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa* were cultured in nutrient broth medium. Then, they were placed in the incubator for 18 hours at 37 ± 1°C. Disc diffusion assay was employed to screen for antimicrobial activity of AgNPs-PM. The grown microbes were sub-cultured on petri dishes and the discs were treated with 10 µl of 2 mg/ml AgNPs-PM, 10% *P. malayana* plant extract, 2 mg/ml $AgNO_3$ solutions and deionised water (negative control). Amoxicillin discs were used as the positive control. Then, the petri dishes were put in a CO_2 incubator (Binder) at 37°C for 24 hours. The zone of inhibition around the discs was measured in mm and was compared with the negative control.

8. Statistical study

Statistical analysis was done *via* Statistical Package for Social Sciences (SPSS). The tests were conducted in sets of three and the data were reported as mean ± standard deviation (SD) using one-way ANOVA test. The individual correlations were obtained *via* Duncan's technique. If $P < 0.05$, the value will be considered as significantly different (Anbumani *et al.*, 2022).

Results and Discussion

1. LC-MS-QTOF analysis of P. malayana Jack leaves extract

The preliminary compound identification was performed using LC-MS-QTOF analysis by comparing the m/z spectra belonging to each compound to the mass spectra database of METLIN (Figure 2). The analysis provided that there was a total of 80 compounds detected. Information on the chemical composition of the 10 major compounds from the analysis such as name, molecular formula, m/z, mass and classification of each compound were listed in Table 1. Among the compounds analysed were categorised as phospholipid, steroid, ketone, amino acids and flavonoids (flavans, falavanols and leucoanthocyanidin) (Table 1). Although, there were some unknown compounds as the data of the compounds are not available in the METLIN database.

The available literatures studying genus Psychotria reported that among chemical compounds found were indole alkaloids, cyclic peptides or cyclotides, quinoline and isoquinoline alkaloids, flavonoids, terpenoids, coumarins and tannins (Calixto *et al.*, 2016). This is in line with the findings from LC-MS analysis of *P. malayana* Jack (Figure 2 and Table 1). Many literatures also reported that among the phytochemicals that were responsible for synthesis of AgNPs were flavonoids, amino acids, tannins, polyphenols, sterols, heterocyclic compounds, triterpenoids, terpenoids, alkaloid, etc. due their ability to

act as the reducing, capping and stabilising agents (Ahmad *et al.*, 2022; Alarjani *et al.*, 2022; Nadaf *et al.*, 2022; Patil & Kim, 2017). Thus, attributable to the biological activity of *P. malayana* Jack, it can be utilised in the synthesis of AgNPs-PM.

2. Green synthesis of silver nanoparticles, AgNPs-PM

For the synthesis, 1 mM and 5 mM $AgNO_3$ solutions were added to the plant extract and changes were observed every 1, 2, 6, 8 and 24-hours (Figure 3A-3B). The changes in colour intensity when synthesising nanoparticles with 1 mM concentration were minimal, but darkest after 24 hours incubation. When the process was done with 5 mM, the colour of the solutions turned from green to dark brown after 1 hour incubation. As cited in many literatures, the formation of dark brown solution which is due to the excitation of AgNPs' surface plasmon resonance confirms the successful synthesis of AgNPs (Ahmad *et al.*, 2022). The yield of the AgNPs-PM was weighed after high-speed centrifugation process. The findings provided that 1 mM yielded the lowest and 5 mM the most (Figure 3C). The development of AgNPs-PM synthesised using 5 mM $AgNO_3$ solution was further observed using other characterisation methods.

2.1 Proposed mechanism for synthesis of AgNPs

A general mechanism for the formation of AgNPs include three main phases which are activation phase, growth phase and termination phase (Makarov *et al.*, 2014). During the activation phase, there will be reduction of metal ions and nucleation of the reduced metal atoms. Phytochemicals such as flavonoids in its keto form will convert into enol form. This will in turn release reactive hydrogen. However, due to the presence of two hydroxyl groups on the same carbon, the enol form is deemed as unstable, and thus, will convert back to its keto form. At this stage, the liberated reactive hydrogen causes the reduction of metal ions to metal atom, specifically Ag^+ to Ag^0, which then combine with each other forming small AgNPs. In addition to flavonoids, tannins also act as reducing agents (Ahmad *et al.*, 2022). In the growth phase, the small adjacent AgNPs coalesce spontaneously into larger particles. This process continues until the particles assume a stable shape and size. Finally, in the termination phase, AgNPs will acquire the most favourable conformation due to the influence of the phytochemicals that function as stabilising agents (Makarov *et al.*, 2014).

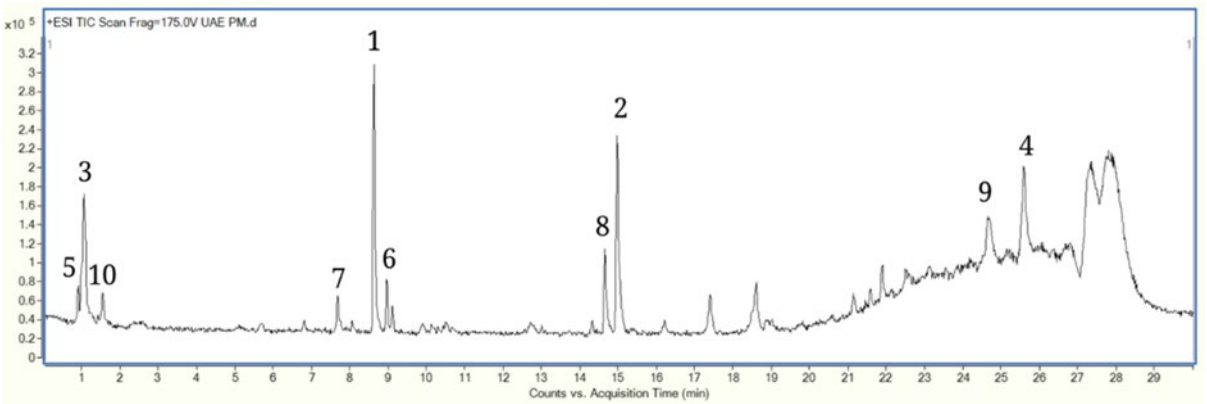
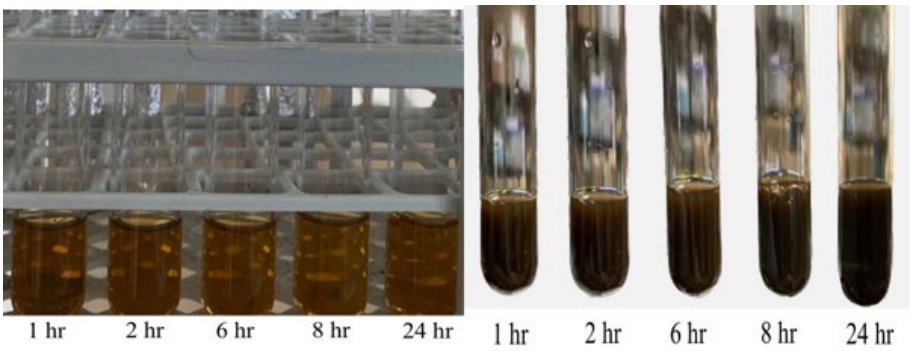


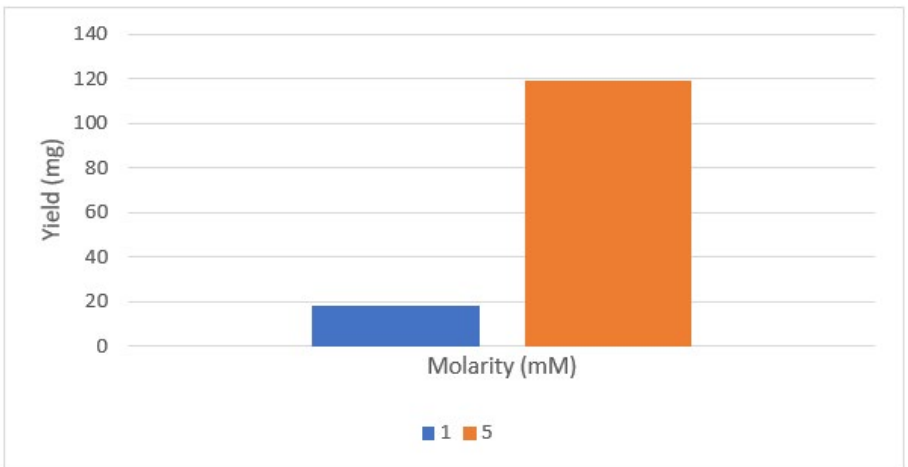
Figure 2: LC-MS analysis of *P. malayana* Jack. The sample was reconstituted with methanol to the final concentration of 10 mg/mL, then diluted to the concentration of 1 mg/mL with methanol. The sample was then filtered using a 0.22 µm pore size syringe filter before analysis. Chromatographic separation was performed at 40°C using Agilent ZORBAX Eclipse Plus C18 Rapid Resolution HT (2.1 x 100 mm) 1.8 µm with (A) 0.1% formic acid in dH20 and (B) 0.1% formic acid in acetonitrile for positive mode. Mass spectrometer was operated in positive electrospray ionisation (ESI) mode with optimum gas temperature at 325°C, gas flow at 11 L/min and nebuliser at 35 psi, respectively.

Table 1: 10 major compounds of *P. malayana* Jack analysed by LC-MS-QTOF analysis

No	Compound name	Molecular formula	m/z	Mass (g/mol)	Classification
1	LysoPE(0:0/18:4(6Z,9Z,12Z,15Z))	$C_{23}H_{40}NO_7P$	491.286	473.252	Lipid (Phospholipid)
2	Eplerenone	$C_{24}H_{30}O_6$	415.2116	414.2043	Lipid (Steroid)
3	4-(o-Carboxybenzamido) glutaramic acids	$C_{13}H_{14}N_2O_6$	295.0926	294.085	-
4	N-Cyclohexanecarbonyloentadecyl amine	$C_{22}H_{43}NO$	338.3421	337.3347	Ketone
5	2-Amino-3-methyl-1-butanol	$C_5H_{13}NO$	104.1069	103.0997	Amino acids
6	Unknown	-	533.3315	532.3242	-
7	Oritin-4beta-ol	$C_{15}H_{14}O_6$	291.0862	290.079	Flavonoids (Flavans, Flavanols and Leucoanthocyanidin)
8	Unknown	-	393.2862	375.2525	-
9	Unknown	-	568.4265	567.4192	-
10	Purine	$C_5H_4N_4$	121.0509	120.0473	Heterocyclic aromatic organic compound



(A) (B)



(C)

Figure 3: AgNPs-PM incubated at 1, 2, 6, 8 and 24hours incubation, respectively using (A) 1 mM $AgNO_3$ solution (B) 5 mM $AgNO_3$ solution (C) Yield of AgNPs-PM obtained from two different molarity of $AgNO_3$

3. Characterisation of silver nanoparticles, AgNPs-PM

3.1 UV-Visible spectrophotometer analysis

The formation of the synthesised AgNPs-PM was confirmed *via* UV-Visible spectroscopy analysis by measurement of surface plasmon resonance (Patra & Baek, 2014). UV-Vis absorption spectra of *P. malayana* Jack leaves extract showed the hypsochromic and hyperchromic shift of the UV-Visible spectra of the leaves extract, and AgNPs-PM at different incubation time (Figure 4). In this study, the collected absorption spectra within 350 nm to 800 nm showed maximum absorption at 412.0 nm (1 hr), 416.1 nm (2 hr), 422.2 nm (6 hr), 437.2 nm (8 hr) and 449.0 nm (24 hr). It was also observed that the absorption and intensity of the peak increased as incubation time increased. The broad peak in the range of 550 nm to 650 nm were attributed to the presence of flavonoids with aromatic benzene conjugated at C-2 and C-3. Another broad band that was centred at 520 nm suggested a conjugated benzene with electron donating group such as NH and OH, also suggesting flavonoids (Ahmad *et al.*, 2022). The data from LC-MS-QTOF analysis provided that one of the major phytoconstituents of *P. malayana* Jack extract was flavonoids. The observations on this analysis provided that flavonoids were the major constituent of *P. malayana* Jack that was responsible for the reduction and stabilisation of AgNPs-PM (Patil & Kim, 2017). The observation on the AgNPs-PM bands from this analysis provided that the hypochromic shift moved towards lower wavelength. These new maximum absorption peaks of AgNPs range from 410 nm to 450 nm represent the surface plasmon resonance of AgNPs-PM.

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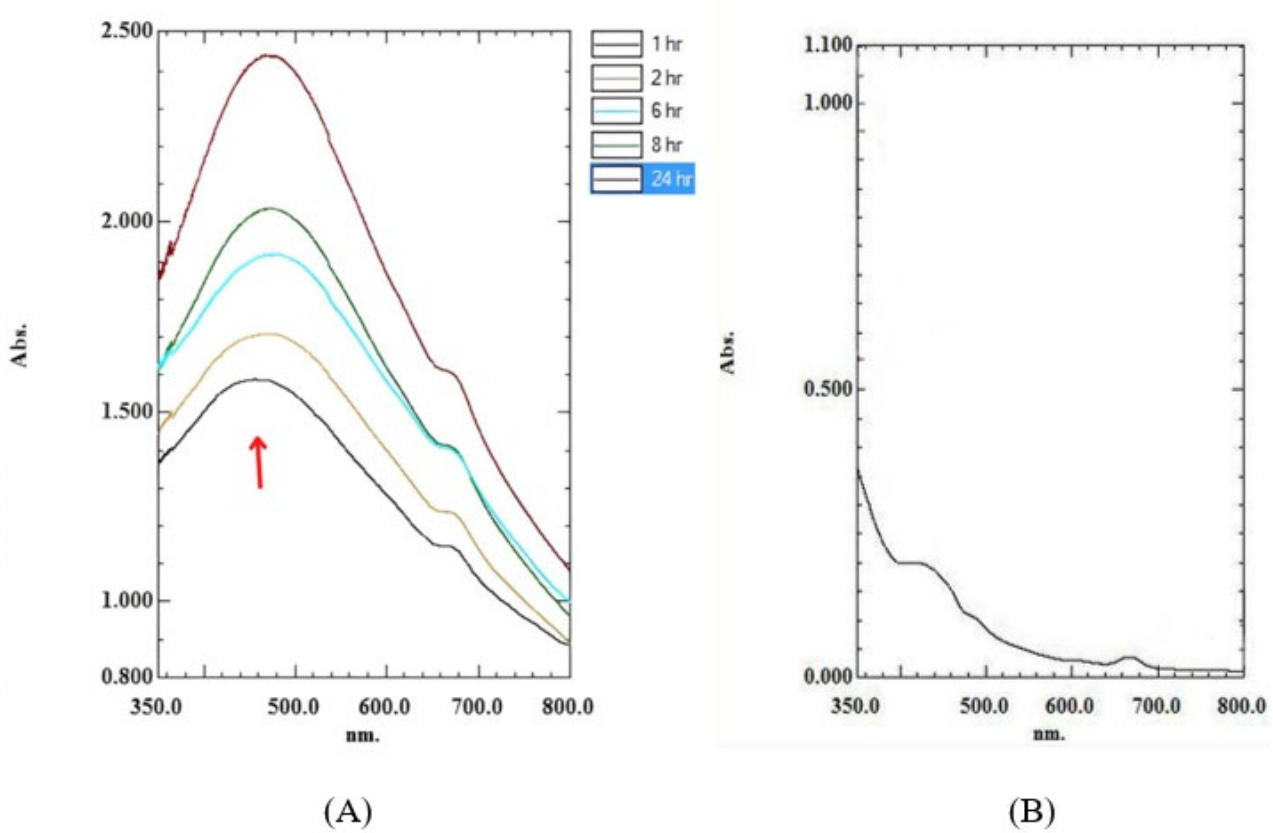

Figure 4: UV-Vis absorption spectra of (A) the green synthesized AgNPs-PM after incubation at 1, 2, 6, 8 and 24 hours (B) P. malayana Jack leaves extract measured between 350-800 nm. An arrow indicated a specific band for silver nanoparticles.

3.2. Scanning electron microscopy (SEM) analysis

The morphological characteristics of the synthesised AgNPs-PM such as size and shape were directly viewed using SEM *via* electron scanning. These characteristics were associated with the toxicity, drug and tissue targeting, drug release and the biological fate of AgNPs-PM (Patra & Baek, 2014). The SEM image indicated that the AgNPs-PM formed were mostly aggregated, which is attributed to the function of the phytochemicals of *P. malayana* Jack leaves extract (Figure 5). The formed AgNPs-PM were having a hexagonal cluster and the particle sizes of AgNPs-PM analysed ranged from 75 nm to 145 nm, which was magnified under 4000x. Some factors could have an influence in the formation of the size and shape of AgNPs-PM such as temperature of the reaction medium, time of the synthesising reaction, exposure to light and the storage conditions (Patra & Baek, 2014; Ye et al., 2022). Aggregation of nanoparticles may occur during the storage, in which they might shrink or grow, thus affecting their potential activities. However, the information provided by SEM about the size distribution and the true population average is limited (Patra & Baek, 2014).

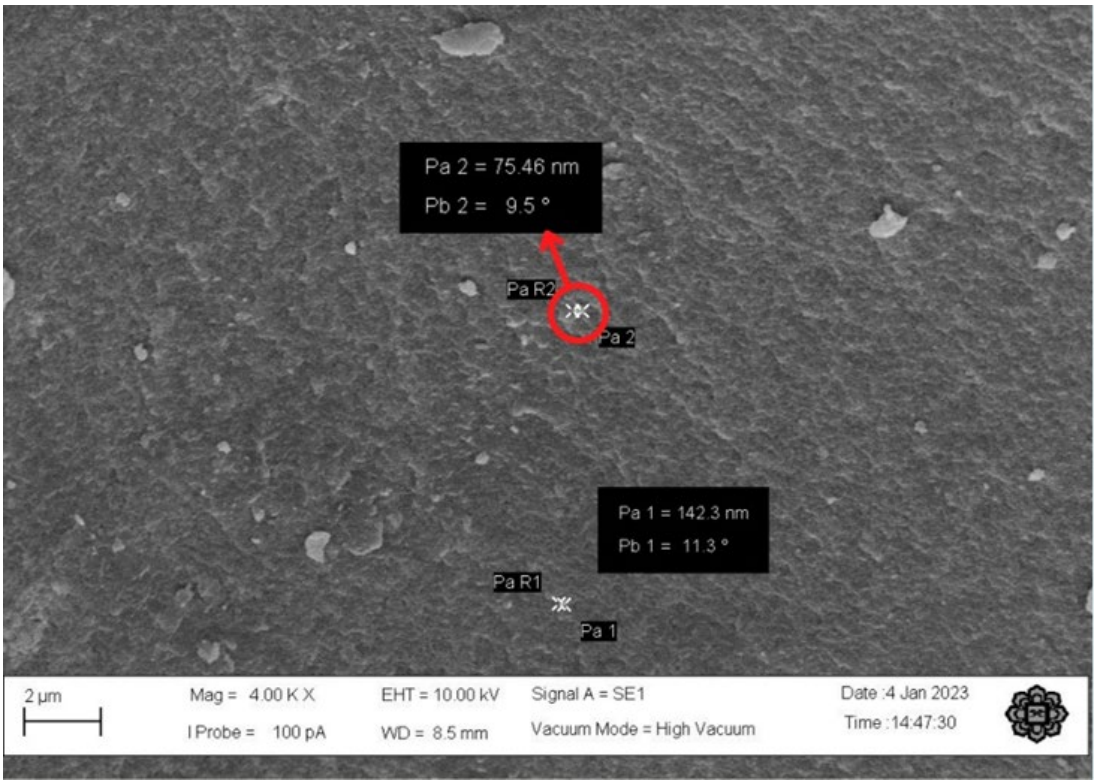

Figure 5: SEM analysis of AgNPs-PM. Characteristic morphology of silver nanoparticles produced in this study.

3.3 Zeta particle size and zeta potential analysis

Dynamic Light Scattering (DLS) analysis provided information on the particle hydrodynamic size, zeta potential and polydispersity index (PDI) of AgNPs-PM (Suriyakala *et al.*, 2022). In this study, the average size distribution of the synthesised AgNPs-PM as analysed *via* zetasizer nanomachine was 110.1 ± 64.66 nm (Table 2). Particle size and their morphology are the most important parameters in determining their properties. It had been proven in many studies that nanoparticles are more efficient in delivering drugs than microparticles due to nanoparticles having larger surface areas, thus more drug interactions can be observed (Patra & Baek, 2014). Many literatures accepted the descriptive size of nanoparticles to be between 1 nm to 100 nm (Susanti *et al.*, 2022). In pharmaceutical field, those particles having a diameter of 10 nm to 1000 nm are also regarded as nanoparticles (Mazayen, Ghoneim, Elbatanony, Basalious, & Bendas, 2022).

Zeta potential measurement can provide predictive data on the surface charge, which affects the storage stability of the colloidal dispersion (Patil & Kim, 2017; Suriyakala *et al.*, 2022). The maximum zeta potential value of the formed AgNPs-PM (Table 2) was approximately -117 ± 15.2 mV. This high negative value indicated that there were electrostatic repulsion between the synthesised AgNPs-PM, making them stable. The negative value also indicated that there were presence of negatively charged functional groups from the *P. malayana* Jack leaves extract (Suriyakala *et al.*, 2022).

In addition, zeta potential at ± 35 mV suggested a formation of stable particles (Buszewski *et al.*, 2018). Previous findings also stated that in order to avoid particle aggregation and ensuring its stability, either a high positive or a high negative value of zeta potential must be achieved (Patra & Baek, 2014).

Table 2. Average size distribution and zeta potential of AgNPs-PM analysed using zetasizer instrument.

Particle size analysis	
Particle size average (d.nm)	110.1 ± 64.66
PdI	0.287
Zeta potential analysis	
Zeta potential (mV)	-117 ± 15.2
Zeta deviation	18.2
Conductivity (mS/cm)	0.551

4. Cytotoxic Assay

Cancer is one of the most leading death-causing diseases and 1 out of 3 people has the possibility to get cancer (Alyami, Alyami, & Almeer, 2022; Zhang et al., 2020). Different types of cancer therapies being offered include chemotherapy, surgery, radiation, hormonal, targeted and immunotherapy. However, the therapies are challenging due to induction of enormous side effects, which include neuropathies, alopecia and gastrointestinal and skin disorders. In addition, high rate of recurrence and multi-drug resistance against common chemotherapeutic drugs are other factors that limit the therapeutic efficacy (Alyami *et al.*, 2022; Gavas, Quazi, & Karpiński, 2021). Thus, to avoid the systemic side effects and drug resistance, many researchers are focusing on the development of nanomaterials as an alternative formulation that can specifically target tumour cells (Zhang *et al.*, 2020).

Cytotoxic assay was used to measure the ability of tested compounds in killing cell lines (Shelembe, Mahlangeni, & Moodley, 2022). In this study, the cytotoxic effect of AgNPs-PM at different concentrations of 400 µg/ml, 200 µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml and 12.5 µg/ml was investigated against Caco-2 and MCF-7 cell lines using MTT cell viability assay. The formation of purple formazan crystals is dependent on NADPH and oxidoreductase enzymes of the cancer cells. Thus, the intensity of purple colour is directly proportional to the cell viability (Shelembe *et al.*, 2022). The absorbance of the dissolved formazan crystals was measured *via* microplate reader at 570 nm. The number of viable cells is proportional to the absorbance and percentage viability (%) was calculated using the following formula:

$$\% \text{ Cell viability} = \frac{A_{sample}}{A_{untreated}} \times 100 \qquad \text{(Eq. 1)}$$

Many studies from *in vitro* assays reported that AgNPs possess cytotoxic activity on several human cell lines, which include human peripheral blood mononuclear cells, human bronchial epithelial cells, red blood cells, liver cells (HepG2), human colorectal cells (Caco-2) and human epithelial breast cells (MCF-7) (Liao, Li, & Tjong, 2019; van der Zande *et al.*, 2016). In this study, the percentage viability of Caco-2 and MCF-7 cell lines was reduced after interaction against various concentrations of AgNPs-PM **(Figure 6)**. In Caco-2 cells, after incubation with 12.5 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml and 400 µg/ml, the cell viability calculated was 89.2%, 84.6%, 82.2%, 88.6%, 80.18% and 81.675, respectively. However, previous study reported that at a concentration of 5 µg/ml of AgNPs, 50% of cell will be inhibited after exposure for 48 hours (Zein, Alghoraibi, Soukkarieh, Salman, & Alahmad, 2020). In MCF-7 cells, the cell viability calculated against the increasing concentration of AgNPs-PM was 97.5%, 96.7%, 90.2%, 95.14%, 90.97% and 89.7%, respectively. Whereas the IC_{50} of AgNPs against MCF-7 in a study conducted by Fard, Tafvizi, and Torbati (2018) was found to be 9.85 µg/ml.

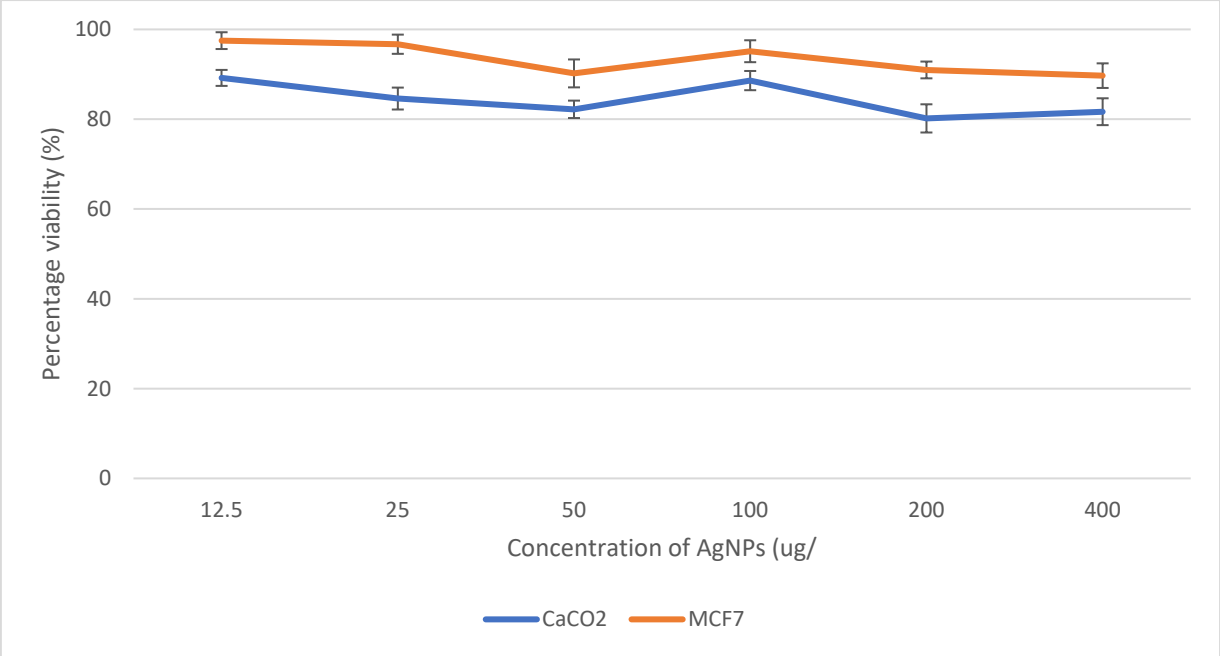

Figure 6: Percentage viability of AgNPs-PM on Caco-2 and MCF-7 against various concentrations ranging from 12.5 µg/ml to 400 µg/ml of AgNPs-PM

However, the findings from this study showed that the cytotoxicity of AgNPs-PM on Caco-2 cells and MCF-7 were both insignificant. It was also expected that the percentage viability of the two cell lines to decrease with increasing concentration of AgNPs-PM as AgNPs exhibit concentration-dependent cell death (Alyami *et al.*, 2022; Zhang *et al.*, 2020). However, in this study, the trend fluctuated. In addition to that, the standard errors of this assay were high, indicating that the results were not reliable, thus, the percent viability might not reflect a true value of the overall cytotoxic assay. This less reliable result might be due to variable density of cell in each well, unsuitable incubation time of cells in MTT, the wavelength at which the optical density was measured, and the type of culture media used (Ghasemi, Turnbull, Sebastian, & Kempson, 2021). In this assay, it was also observed that the percentage inhibition of AgNPs-PM against Caco-2 cells were more prominent than MCF-7 cells. This finding might be attributed to the higher sensitivity of Caco-2 cells to AgNPs-PM than MCF-7 cells (van der Zande *et al.*, 2016).

5. Antibacterial Assay

Many pathogenic bacteria are showing resistance to various antibiotics. To address this issue, new antibiotics are necessary (Ahmad *et al.*, 2022). AgNPs were proven to possess antimicrobial properties, making them suitable alternatives to antibiotics (Nguyen et al., 2021). This study investigated the activity of AgNPs-PM, $AgNO_3$ solutions and *P. malayana* Jack leaves extract on four bacterial strains *via* disc diffusion method. The tested bacteria were *B. subtilis, E. coli, P. aeruginosa* and *S. aureus*. In this study, the negative control discs were treated with deionised water, and the positive control discs were treated with antibiotic amoxicillin. For the sample discs, they were treated with 10% *P. malayana* plant extract, 2 mg/ml AgNPs-PM and 2 mg/ml $AgNO_3$ solutions

2 mg/ml AgNPs-PM and 2 mg/ml $AgNO_3$ solutions both showed antimicrobial activity on the microbial growth against all the tested bacterial strains (Figure 7). At a same concentration of 2 mg/ml, AgNPs-PM exhibited good inhibition on the growth of *P. aeruginosa,* followed by *S. aureus, B. subtilis* and *E. coli* at 3.00 ± 0.17 mm, 2.00 ± 0.28 mm, 1.75 ± 0.14 mm and 1.25 ± 0.26 mm, respectively. The activity of 2 mg/ml $AgNO_3$ solutions were almost comparable to AgNPs-PM, but with a larger zone of inhibition than AgNPs-PM. The zone of inhibition caused by $AgNO_3$ solutions was 2.00 ± 0.35 mm, 2.50 ± 0.41 mm, 4.00 ± 0.00 mm and 3.50 ± 0.22 mm on *P. aeruginosa,* followed by *S. aureus, B. subtilis* and *E. coli,* respectively. This is in line with the previous publications stating that silver ions and silver-modified materials like AgNPs possess multiple antimicrobial activity such as induction of reactive oxygen species (Ahmad *et al.*, 2022).

Since the activity of AgNPs-PM was both greatest on *P. aeruginosa* and least on *E. coli,* it is unknown

whether AgNPs work better on Gram-negative bacteria or Gram-positive bacteria. In addition, the true mechanism of how AgNPs exhibit antimicrobial activity is still unclear (Ahmad *et al.*, 2022). The proposed mechanisms are grouped into three main actions which are induction of oxidative stress, release of metal ion and non-oxidative mechanism. These actions can either act independently or simultaneously. This can lead to denaturation of the proteins and leaking of the cell contents (Goyal, Verma, Kharewal, Gahlaut, & Hooda, 2022).

However, when the bacterial strains were tested against the 10% *P. malayana* plant extract, no zone of inhibition was seen and measured, indicating that the extract does not possess antimicrobial activity. From this assay, it can be concluded that the antimicrobial activity of AgNPs-PM was caused by the synthesised AgNPs solely (Nguyen *et al.*, 2021). There is no significant difference of the antibacterial activity between the samples (AgNPs-PM and $AgNO_3$ solutions) and Amoxicillin against *B. subtilis* and *E. coli.* However, testing on *P. aeruginosa* and *S. aureus* showed that there is a large significant difference observed between the samples (AgNPs-PM and $AgNO_3$ solutions) and Amoxicillin.

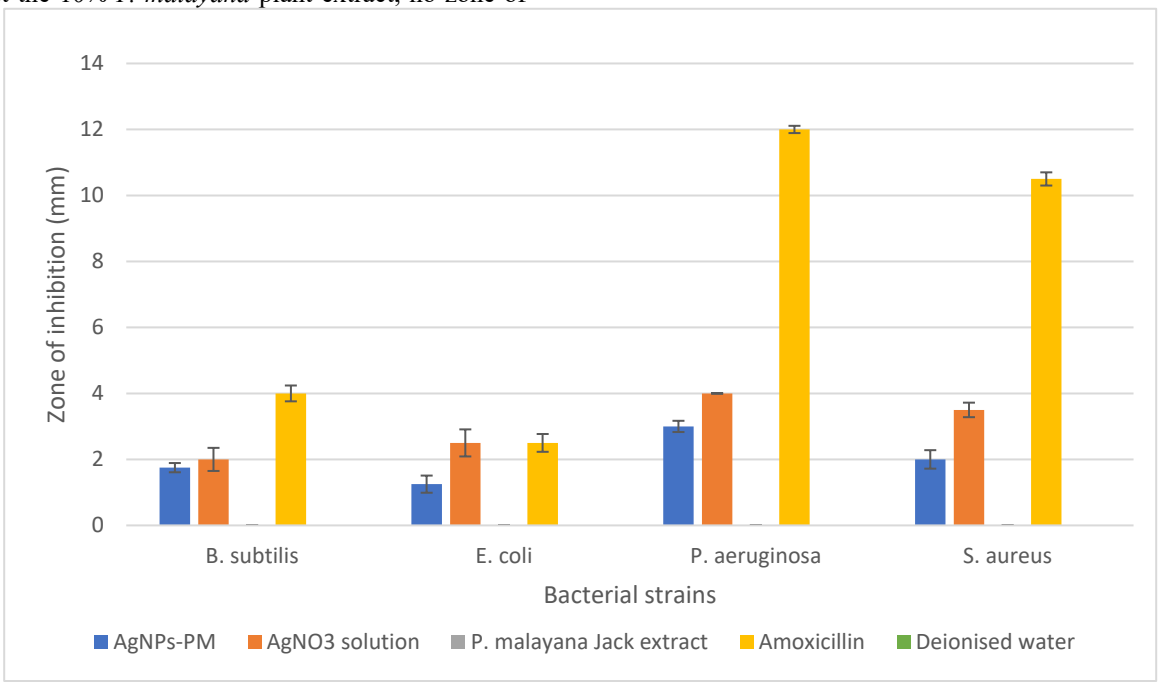

Figure 7: Zone of inhibition (mm) of four bacterial strains against AgNPs-PM, $AgNO_3$, *P. malayana* Jack extract. The test was conducted by disc diffusion method using amoxicillin disc as a positive control and deionised water as negative control.

Conclusion

The study was conducted by synthesising AgNPs-PM from *P. malayana* leaves extract *via* biological route which is known as green synthesis method. The major phytochemicals of *P. malayana* Jack as analysed by LC-MS-QTOF, namely flavonoids, amino acids and heterocyclic aromatic organic compound which were possible for the formation of AgNPs-PM as they act as the reducing and stabilising agents for the process. Characterisations were done on the synthesised AgNPs-PM to study their characteristics as the nature of the AgNPs play an important role in their application. The investigations proposed that the formed AgNPs-PM were having hexagonal cluster with the size of around 75 nm to 145 nm as viewed under electron microscope. Another analysis also confirmed that the size distribution of AgNPs-PM was 110.1 ± 66.64 nm with a zeta potential of approximately -117 ± 15.2 mV, indicating that stable AgNPs-PM were successfully synthesised using green synthesis method. Their cytotoxicity was also determined *via* MTT colourimetric assay and it was found out that at concentrations of 12.5 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml and 400 µg/ml of AgNPs-PM, the cell viability of both Caco-2 and MCF-7 cell lines were over 80%. This finding indicated that the percentage inhibition was low and insignificant. However, in this study, a few confounding factors may have an effect on the result, thus, making it unreliable. AgNPs-PM were also studied for antimicrobial activity against *B. subtilis, E. coli, P. aeruginosa* and *S. aureus* using disc diffusion method, and

it was observed that AgNPs-PM inhibited the growth of the four bacterial strains, with the highest inhibition on *P. aeruginosa* and the lowest inhibited was *E. coli.* Thus, the biogenic synthesis of AgNPs-PM might be a promising process for the production of other metallic nanoparticles which could have potential applications in various fields. However, the improvement on this study for cytotoxic and antimicrobial assays can be made by adjusting the size and formulation of nanoparticles.

Author contributions

MT and DS design the study. MT, DS, TK supervise the works. NA conduct the research and collect the data. NA wrote the manuscript. MT review the manuscript. All authors have read the manuscript.

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Conflict of Interest

The authors declare that there is no conflict of interest in the writing of this manuscript.

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