Faculty of Resource Science and Technology

In vitro and *In silico* Evaluation of Piperine Compound as a Potential Anticancer Agent in Inhibiting Growth of Nasopharyngeal Carcinoma Cells

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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ABSTRACT

Piperine is a popular secondary metabolite derived from the fruits of black pepper plant. Prior researches have shown that piperine exhibited anticancer effects against numerous carcinoma cell lines. To date, there have not been any studies conducted to evaluate the anticancer effect of piperine against nasopharyngeal carcinoma (NPC) cell lines. Hence, this study aimed to investigate the cytotoxicity and selectivity potential of piperine on NPC cells and to study the molecular interaction of piperine and a panel of proteins via molecular docking technique. A total of five cell lines (NP69, TW01, HK1, TW04 and C666-1) were used in this study. The cytotoxicity assay revealed that piperine inhibited the proliferation of normal nasopharyngeal epithelial (NPE) and NPC cells. With reference to the reported IC_{50} value, SI value for all four NPC cell lines was recorded to be less than 1. This demonstrated that piperine treatment did not show selective toxicity for NPC cells. In addition, the *in vitro* results suggested that the caspase-3 activity of the NPE and NPC cell lines were effectively increased upon piperine treatment at 20 g/mL and 40 µg/mL. Conversely, in the 60 µg/mL treatment group, the lowest caspase-3 activity was reported for all cell lines. This suggested a possibility for piperine to act through a different signalling pathway or cell death mechanisms in order to suppress cell proliferation. The AutoDock Vina software was used for the molecular docking simulation Generally, a negative binding value was recorded for all the protein-ligand complexes. This indicated a spontaneous binding between piperine and the relevant proteins. Additionally, a lower binding affinity was reported in several Toll like receptor (TLR) proteins which were TLR2, TLR6 and TLR8. This showed a higher potency for piperine to regulate cellular responses via the TLRs signalling pathway. In summary, the *in vitro* results showed that piperine suppressed the growth of NPC cells by the activation of

apoptosis whereas the *in silico* data provided an additional information on the molecular interaction between piperine and the corresponding proteins.

Keywords: Piperine, apoptosis, AutoDock Vina, TLR proteins, nasopharyngeal carcinoma

Penilaian in vitro dan in silico mengenai Antikanser dan Aktiviti berkaitan Apoptosis Sebatian Piperine ke atas Saluran Sel Karsinoma Nasofaring

ABSTRAK

Kompaun piperine ialah metabolit sekunder yang popular yang diperoleh daripada buah-buahan tumbuhan lada hitam. Penyelidikan terdahulu telah menunjukkan bahawa piperine mempamerkan kesan antikanser terhadap banyak sel karsinoma. Sehingga kini, belum ada kajian yang dijalankan untuk menilai kesan antikanser piperine terhadap saluran sel karsinoma nasofaring (NPC). Oleh itu, kajian ini bertujuan untuk menyiasat potensi sitotoksisiti dan selektiviti piperine pada sel NPC dan untuk mengkaji interaksi molekul piperine dan panel protein melalui teknik dok molekul. Sebanyak lima garisan sel (NP69, TW01, HK1, TW04 dan C666-1) telah digunakan dalam kajian ini. Ujian sitotoksisiti mendedahkan bahawa piperine menghalang percambahan sel epitelium nasofaring (NPE) dan NPC normal. Dengan merujuk kepada nilai IC50 yang dilaporkan, nilai SI bagi keempat-empat garisan sel NPC direkodkan kurang daripada 1. Ini menunjukkan bahawa rawatan piperine tidak menunjukkan ketoksikan terpilih untuk sel NPC. Di samping itu, keputusan in vitro mencadangkan bahawa aktiviti caspase-3 bagi garisan sel NPE dan NPC telah meningkat dengan berkesan apabila rawatan piperine pada 20 g/mL dan 40 µg/mL. Sebaliknya, dalam kumpulan rawatan 60 µg/mL, aktiviti caspase-3 terendah telah dilaporkan untuk semua garisan sel. Ini mencadangkan kemungkinan piperine bertindak melalui laluan isyarat yang berbeza atau mekanisme kematian sel untuk menyekat percambahan sel. Perisian AutoDock Vina digunakan untuk simulasi dok molekul Secara amnya, nilai pengikatan negatif direkodkan untuk semua kompleks protein-ligan. Ini menunjukkan pengikatan spontan antara piperine dan protein yang berkaitan. Selain itu, pertalian pengikatan yang lebih rendah telah dilaporkan dalam beberapa protein reseptor

seperti Tol (TLR) iaitu TLR2, TLR6 dan TLR8. Ini menunjukkan potensi yang lebih tinggi untuk piperine untuk mengawal selia tindak balas selular melalui laluan isyarat TLR. Ringkasnya, keputusan in vitro menunjukkan bahawa sebatian piperine menindas pertumbuhan sel NPC dengan pengaktifan apoptosis manakala data in silico memberikan maklumat tambahan mengenai interaksi molekul antara piperine dan protein yang sepadan.

***Kata kunci**: Piperine, apoptosis, AutoDock Vina, protein TLR, karsinoma nasofaring*

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LIST OF ABBREVIATIONS

Å	Armstrong
ANOVA	Analysis of Variance
APAF-1	Apoptotic protease activating factor-1
Bad	Bcl-2-associated death promoter
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma-2
Bcl-xL	B-cell lymphoma-extra large
BFGS	Broyden-Fletcher-Goldfarb-Shanno
Bid	BH3 Interacting Domain Death Agonist
Bim	Bcl-2 Interacting Mediator of cell death
CASTp	Computed Atlas of Surface Topography of proteins
CO_2	Carbon dioxide
D-PBS	Dulbecco- Phosphate Buffer Saline
DAMP	Damage Associated Molcular Patterns
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
EBV	EpsteinBarr Virus
FADD	Fas-associated death domain
FasL	Fas ligand
FBS	Fetal bovine serum
HLA	Human leukocyte antigen
HPV	Human papilloma virus

HSD	Honestly significantly difference
hTERT	Human telomerase reverse transcriptase
IAP	Inhibitor of apoptosis
IC_{50}	Half maximal inhibitory concentration
IgA	Immunoglobulin A
IL-6	Interleukin-6
IRAK	Interleukin-1 receptor-associated kinase
IRF3	Interferon regulatory factor 3
JNK	c-Jun N-terminal kinase
KSFM	Keratinocyte serum free media
LPS	Lipopolysaccharides
MAPK	Mitogen-activated protein kinases
MOMP	Mitochondrial outer membrane permealisation
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carbomethoyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
MyD88	Myeloid differentiation factor 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NPC	Nasopharyngeal carcinoma
NPE	Nasopharyngeal epithelial
NF-kB	Nuclear factor kappa-light-chain enhancer of activated B cells
PAMP	Pathogen-associated molecular pattern
PARP	Poly-ADP ribose polymerase
PDB	Protein data bank
PDBQT	Protein Data Bank, Partial Charge (Q) & Atom Type (T)

PRRs	Pattern recognition receptors
PS	Penicillin-streptomycin
RCSB	Research Collaboratory for Structural Bioinformatics
RIP1	Receptor interacting protein kinase 1
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SDF	Structured Data File
SI	Selectivity Index
SMAC	Second mitochondria-derived activator of caspase
TLR	Toll-like receptor
TME	Tumour microenvironment
TNF-α	Tumour necrosis factor alpha
TNFR-1	Tumour necrosis factor receptor 1
TRADD	TNFR-1-associated death domain protein
TRAF6	TNF receptor associated factor 6
TRAIL	TNF-realted apoptosis-inducing ligand
TRAM	Translocating chain-associating membrane
TRIF	TIR domain-containing adaptor-inducing interferon-ß
TRPV1	Transient receptor potential vanilloid subtype 1
WHO	World Health Organisation
WST-8	Water soluble tetrazolium-8

CHAPTER 1

INTRODUCTION

Nasopharyngeal Carcinoma (NPC) was one of the top five cancer affecting Malaysians in the year 2020 (The Globocan, 2021). NPC is a unique malignancy that mainly affects the Cantonese from China and the Bidayuhs from East Malaysia (Yeo et al., 2018; Zhan et al. 2019). Men are thought to be two to three times more susceptible to NPC than women (Malaysia Health Technology Assessment Section, 2016). All these suggests that NPC is a geographical, racial and gender dependent disease. Besides that, genetic heredity, dietary habits, the rate of exposure to nicotine, the presence of Epstein-Barr virus (EBV) and Human Leukocyte Antigen (HLA) were some common risk factors for NPC (Okekpa et al., 2019). An increased exposure to these risk factors and a lack of access to health care services have led to a rise in the prevalence rate of NPC (Salehiniya et al., 2018; Chen et al., 2021).

Currently, the treatments that are available to delay cancer progression and relieve cancer symptoms include chemotherapy, radiotherapy and surgery. Until now, chemotherapy and radiotherapy have been the standard treatment used for NPC patients. Patients in the early stages of NPC were often treated with radiotherapy alone (Chan et al., 2010). However, as the malignant tumour progresses to the final stage, concurrent or neoadjuvant chemoradiotherapy will be included in the treatment. The chemotherapy drug, cisplatin remained as the first-line regimen for NPC patients (Jiromaru et al., 2022). While effective, cisplatin treatment would usually associate with side effects such as nausea, vomiting and myelosuppression (Haider et al., 2020). Late prognosis and the development of drug resistance were some reasons for the failure of NPC therapy (Stanta & Bonin, 2018; Perri et al., 2019). Chemotherapy resistance remained as a major barrier to effective

treatment and often happened because of the heterogeneity nature of cancer cells (Dagogo-Jack & Shaw, 2018). In addition to the side effects, there are also limited options of the chemotherapy drug that are available for patients who experiences cancer recurrence and metastasis. Hence, the discovery of the new compound with selective, anticancer potential is needed for effective treatment.

Abnormal growth and apoptosis evasion remained as two main features of cancer cells (Hanahan & Weinberg, 2011, Hanahan, 2022). Thus, effective treatment targeting the cell proliferation and apoptosis mechanism of cancer cells will inhibit cancer progression. As natural compounds showed excellent therapeutic activity and reduced cytotoxicity, this makes natural products and its derivatives to be potential anticancer agents for NPC therapy. (Asma et al., 2022). Since long ago, black pepper has been widely used all over the world for its culinary and medicinal purposes (Gorgani et al., 2017). Piperine, found in the fruits of *Piper nigrum.* possess antioxidant, anticarcinogenic, anti-inflammatory, and antimicrobial properties and was commonly used in traditional medicine to treat health problems (Shityakov et al., 2019). Data from several studies suggested that piperine showed toxicity to various cancerous cell lines by increasing the level of reactive oxygen species (ROS), causing DNA fragmentation thus inducing cell death events (Siddiqui et al., 2017; Jafri et al., 2019). The damaged DNA will be repaired before being synthesized or apoptosis reaction will take place if the damage persists (George et al., 2019). As a result, the growth of cancerous cells was inhibited. Additionally, studies had also showed that piperine inhibited the cell growth through apoptosis via the regulation of different proteins such as Bcl-2, Bcl-xL, Bax, p53, caspase-1, caspase-3 and caspase-9 (Lin et al., 2014; Si et al., 2018; Chen et al., 2020; Jafri et al., 2019).

Although prior studies revealed that piperine possess anticancer potential against other carcinoma cells, there has not been any research reported on how piperine alone affects the proliferation of NPC cells. Therefore, this remained as an understudied issue. As prior studies demonstrated that piperine exhibited cytotoxic and selective properties against cancerous cells that were of epithelial origin, piperine was expected to show a similar effect on the studied NPC cell lines. Moreover, apoptosis event was expected to occur in piperine-treated NPC cells.

With that, this study aimed to evaluate the effectiveness of piperine in inhibiting proliferation of NPC cells through biological and computational methods. In this study, a normal nasopharyngeal epithelial (NPE) cell line, NP69 was also included to investigate whether piperine would show selective cytotoxicity towards the NPC cells. Moreover, cisplatin that served as positive control was included in the *in vitro* research. In other words, the objectives of this research were:

1. To determine the anti-proliferative and cytotoxic effects of piperine on NPC cells via the WST-8 assay.

2. To detect potential apoptotic activity in NPC cells after piperine treatment by quantifying the activity of caspase-3 protein.

3. To determine the physiological mechanisms behind piperine's impact on NPC carcinogenesis via molecular docking.

CHAPTER 2

LITERATURE REVIEW

2.1 Nasopharyngeal Carcinoma (NPC)

Nasopharyngeal carcinoma (NPC), also known as nasopharynx cancer is a unique malignancy originates from the epithelium of the nasopharynx (Shah & Nagalli, 2022). Furthermore, NPC is also a type of squamous cell carcinoma that is located within the fossa of Rosenmüller which is a cone-shaped extension of the nasopharynx (Prasad, 1979; Wei, 2001; Chua et al., 2016). With reference to Figure 2.1, the nasopharynx is an anatomical component that travels through oropharynx to connect the nasal cavities to the larynx and trachea (Mankowski & Bordoni, 2023).

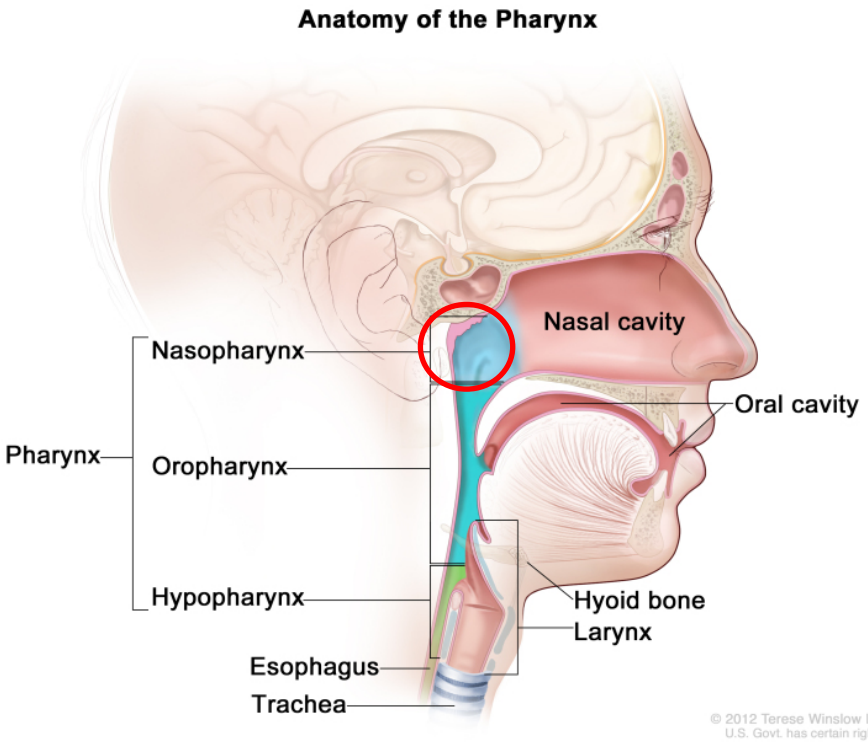

Figure 2.1: Illustration for the anatomy of human pharynx in which location of fosa of Rosenmüller is marked using a red circle, taken from National Cancer Institute (2022).

2.1.1 NPC classification system

The World Health Organisation (WHO) has categorized NPC cells into three different subtypes which are keratinizing squamous cell carcinoma (Type I), non-keratinizing squamous cell carcinoma (Type II) and basaloid squamous cell carcinoma (type III) based on its histopathological characteristics (Sinha & Gajra, 2022). The non-keratinising NPC subtype is further divided into non-keratinising differentiated carcinoma (Type IIa) and non-keratinising undifferentiated carcinoma cells (Type IIb) (Stepan et al., 2021; Stelow & Wenig, 2017). The non-keratinising differentiated squamous cell carcinoma (Type IIa) is an EBV negative cell line and is often associated with local and regional cancer development, along with a lower sensitivity towards chemotherapy and radiotherapy (Jicman Stan et al., 2022). On the other hand, undifferentiated, non-keratinising squamous cell carcinoma, Type IIb is often linked with presence of EBV and will share some unique traits such as distant metastasis and a higher sensitivity towards chemotherapy and radiotherapy. Of all NPC subtypes, Type IIb is found commonly in NPC patients from the endemic region (Rueda Domínguez et al., 2022).

The NPC classification system plays a significant role in the prognosis of NPC and the selection of the appropriate course of treatment. However, the data provided by WHO were insufficient. Therefore, Wang et al. (2016) proposed another classification system that consisted of additional information on the NPC morphological traits and suggested that their proposed NPC classification system would serve as a better prognostic tool. Besides that, a research carried out by Ding et al. (2021) demonstrated that each NPC subtype would respond differently towards drug and radiation therapy, mainly because each of these NPC subtype would retain its distinctive molecular features that lead to the activation of a specific signalling pathway. Thus, this resulted in the activation of different proteins. All these

suggested the importance of classifying NPC based on its tumour subtype and thus allowing clinicians to select a suitable mode of treatment. This would help in predicting the treatment response for the NPC patient (Peng et al., 2012).

2.1.2 Cell lines used in NPC research

Representative cell lines are valuable preclinical models used in the discovery of novel therapies and to understand the pathological properties and development of NPC. According to Yip et al. (2018), there were a total of 30 NPC cell lines established so far. The properties of these NPC cell lines were summarised and shown in Table 2.1. While most of the recorded NPC cell lines in Table 2.1 were derived from human biopsies collected from the primary tumour site, there were a few cell lines such as NPC-BM1 and CNE-3 that were derived from the metastasis tumour site. Apart from human biopsies, there were also a few NPC cell lines that were derived from xenograft models. For example, C666-1 and SUNE-1 cell lines were derived from Xeno666 and SUNT1 respectively. Besides that, a few NPC sublines namely NPC-BM29, NPC-BM00, 5-8F and 6-10B) were also established from the existing NPC cell lines.

In most of the NPC cell lines, EBV episomes seemed to disappear upon long term propagation where underlying reasons remained unclear (Yip et al., 2018). As the presence of EBV is strongly associated with development of Type IIb (undifferentiated) NPC, the establishment of representative NPC cell lines that harbour EBV episomes is crucial in understanding the role of EBV in NPC development and pathogenesis (Yip et al., 2018). Among all NPC cell lines that were listed in Table 2.1, there were only a few cell lines namely C666-1, C17 and NPC43 that retained EBV episomes after continuous propagation (Cheung et al., 1999; Lin et al., 2018; Yip et al., 2018).